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INATIVAÇÃO FOTODINÂMICA MEDIADA PELOS CORANTES XANTENOS ROSA BENGALA E EOSINA CONTRA PATÓGENOS DE ORIGEM ALIMENTAR *Salmonella* Typhimurium E *Staphylococcus aureus*

ADRIELE RODRIGUES DOS SANTOS

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Tese apresentada ao Programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá para a obtenção do grau de Doutora em Ciência de Alimentos.

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Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

Paula A. Z. Campanerut-Sá

Profa. Dra. Paula Aline Zanetti Campanerut-Sá

Prof. Dr. Evandro Bona

Prof. Dr. Alex Fiori da Silva

Camila Fabiano de Freitas

Profa. Dra. Camila Fabiano de Freitas

Profa. Dra. Jane Martha Graton Mikcha
Orientadora

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BIOGRAFIA

Adriele Rodrigues dos Santos nasceu em 1986, na cidade de Campo Mourão – PR. Graduou-se em Tecnologia de Alimentos pela Universidade Tecnológica Federal do Paraná, *campus* Campo Mourão, no ano de 2008 e, especializou-se Vigilância Sanitária de Alimentos pela Universidade Tecnológica Federal do Paraná em 2009. Concluiu, em março de 2014, o mestrado em Ciências de Alimentos, pela Universidade Estadual de Maringá. Trabalhou em Laticínios durante quatro anos e, trabalhou como professora contratada pela Universidade Tecnológica Federal do Paraná, *campus* Campo Mourão, durante dois anos. Atualmente é técnica de laboratório de alimentos da Universidade Tecnológica Federal do Paraná - *campus* Campo Mourão. Tem experiência nas áreas de microbiologia de alimentos, controle de qualidade na indústria de alimentos e tecnologia de leites e derivados.

Dedico

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Santos A. R., Batista A. F. P., Gomes A. T. P. C., Neves M. G. P. M. S., Faustino M. A. F., Almeida A., Hioka N. and Mikcha J. M. G.. The Remarkable Effect of Potassium Iodide in Eosin and Rose Bengal Photodynamic Action against *Salmonella* Typhimurium and *Staphylococcus aureus*. *Antibiotics* **2019**, 8(4), 211; https://doi.org/10.3390/antibiotics804021.

GENERAL ABSTRACT

INTRODUCTION. Foodborne diseases are a growing public health problem and are an important cause of morbidity and mortality worldwide. Between 1998 – 2016, in the United States, *Salmonella* spp. was responsible for 2.585 outbreaks of foodborne disease with 8.021 hospitalizations and *Staphylococcus aureus* for 671 outbreaks with 526 hospitalizations. In Brazil, between 2009 – 2018, among the identified agents of foodborne disease, 11.3 % was *Salmonella* spp. and 9.4 % was *S. aureus*, making them two important foodborne pathogens. Preventing outbreaks of foodborne disease requires the control of microorganisms in the food production chain. In this context, an efficient tool for inactivating microorganisms is antimicrobial photodynamic therapy (aPDT), which is a promising and low-price technology effective against a several types of foodborne bacteria. In a PDT, the inactivation of microorganisms is caused by oxidative stress induced by the interaction of a light-excited photosensitizer (PS) in the presence of molecular oxygen. The outcome from the action between PS and visible light is irreversible damage to various molecular constituents of the cells (lipids, proteins, enzymes, and DNA). This technique presents several advantages when compared with the antimicrobials methods, showing to be efficient independently of the antimicrobial resistance profile and to prevent further development of resistance even after several cycles of treatment. Xanthene dyes have been considered good PSs to induce bacterial photoinactivation due to their low price, high molar absorptivity, and high singlet oxygen quantum yield (Φ_{Δ}). The xanthene dyes rose bengal (RB) and eosin Y (EOS) have already proven to be effective against bacteria, however, these dyes showed to be more effective against gram-positive bacteria. This limitation can be overcomed using different organic salts such as potassium iodide (KI). Some xanthene dyes are approved for use in drug, cosmetic, and medical applications, and as food additives, while the safety of KI has been reported by Food and Drug Administration (FDA). Several light sources with different wavelengths are available, including the light-emitting diodes (LED). They are becoming a promising alternative to aPDT because of greater flexibility in terms of irradiation time, a wide range of the visible electromagnetic spectrum, including green light (490–570 nm region), and its low price. The mathematical models, as response surface methodology (RSM), can be used to predict the best conditions for inactivating microorganisms by aPDT. These models are valuable tools for applying this technology in the food industry.

AIMS. The aim of this work was to apply RSM to investigate the antimicrobial photodynamic effect of RB alone against *Salmonella enteric* serotype Typhimurium (ATCC 14028) and *Staphylococcus aureus* (ATCC 25923), as well the combination of the xanthene dyes RB and EOS with the inorganic salt KI against the same bacteria.

MATERIAL AND METHODS. A stock solution of RB and EOS at 1.0 mM and KI at 5 M, prepared in phospahate buffer saline (PBS) pH 7.2 and, a green LED homemade device prototype (10 mW/cm^2; 530 ± 40 nm) were used in this work. For the photoinactivation assays 500 µL of bacterial suspension (*S.* Typhimurium and *S. aureus*) at 10^7 CFU/mL with different concentrations of RB or EOS with or without KI were used. Simultaneously, four control groups were included: a positive control; light control (LC); KI control (KIC) and; PS control. After a dark incubation, the samples and the controls LC and KIC were exposed to the green LED light for diferent irradiation times. Finally, samples were serially diluted in 0.85% saline solution, plated in duplicate onto Tryptic Soy Agar and the CFU/mL was counted. In the first paper it mathematic models were used to investigated the interaction of RB concentration and illumination time in the photoinactivation of *S.* Typhimurium and *S. aureus*. So, for the statistical experimental design, it was used a sequence of designed experiments to model the combined effect of each factor (PS concentration and iradiation time) on the response through mathematical quadratic fitting of the experimental results. A second-order polynomial empirical model describing the relationship between PS concentration and illumination time was developed. It was also perfomed the the PS uptake, as well as the scanning electron microscopy (SEM), transmission electron microscopy (TEM) and flow cytometry. In the second paper it was investigated the use of RB or EOS combined with KI. For this, the same photoinactivation protocol, described above, was used. The photostability and aPDT resistance development assays were also performed.

RESULTS AND DISCUSSION. The derived model used in the first paper predicted the combined influences of PS concentration and illumination time on *S. aureus* and *S.* Typhimurium counts, in accordance with predictions and experimental observations (R^2 = 0.8483 and p = 0.0013 for *S. aureus*; R^2 = 0.9191 and p = 0.0001 for *S.* Typhimurium). Total inhibition of *S. aureus* and *S.* Typhimurium was observed when applying a light dose of 0.125 J/cm^2 and 152.0 J/cm^2, respectively.The study demonstrates that RB concentrations and illumination times used in the statistical experimental design were generally low, proving the effectiveness of aPDT mediated by RB and green LED light against the microorganisms tested.

The application of the RSM showed that the concentration of PS and illumination time are important, but it is necessary to evaluate them separately for each microrganisms to reflect their true influence on the response. *S. aureus* was more susceptible to RB concentration and, the photoinhibitory action increased in a concentration-dependent manner. The illumination time has more influence than PS concentration in the aPDT of *S.* Typhimurium. Bacteria exposed to aPDT lost membrane integrity, as showed by SEM, TEM and flow cytometry. This loss represents significant damage for cells, once multiple functions are linked to the plasma membrane and, a damage in the membrane may lead to cellular content leakage and can cause cell death. To improve the aPDT effect of the xanthene dyes, in the second paper, it was tested the combination of EOS or RB with KI. All PS and KI concentrations tested were able to efficiently inactivate *S.*Typhimurium and *S. aureus*. This combined approach allows a reduction in the PS concentration up to 1000 times, even against one of the most common foodborne pathogens, *S.*Typhimurium, a gram-negative bacterium. Besides, our results showed that there was no significant increase ($p < 0.05$) in resistance of *S.* Typhimurium and *S. aureus* to photosensitization after 10 consecutive sessions of 10 min of irradiation with EOS or RB with KI.

CONCLUSIONS. The use of xanthene dyes in aPDT was able to efficiently inactivate *S.*Typhimurium and *S. aureus*. Appling the RSM in aPDT treatment the photoinhibitory activity could be predict and a new way to use mathematic modeling tools together with aPDT could be provided. So, this approach could be further optimized and applied in food industries. Besides, the addition of KI can strongly potentiate the aPDT mediated by the xanthene derivatives EOS and RB. It was also confirmed that *S.* Typhimurium and *S. aureus* did not develop resistance when submitted to consecutive cycles of aPDT protocol. Therefore, the effective inactivation of both bacteria with low PS concentrations and the low price of the xanthene dyes show that this technology has potential to be easily transposed to the food industry.

KEY WORDS: Inorganic salts; photodynamic inactivation; *Staphylococcus aureus*; *Salmonella* Typhimurium; response surface methodology; xanthene dyes.

RESUMO GERAL

INTRODUÇÃO. As doenças transmitidas por alimentos são um crescente problema de saúde pública, além de ser uma importante causa de mortalidade em todo o mundo. Entre 1998 - 2016, nos Estados Unidos, *Salmonella* spp. foi responsável por 2585 surtos de doenças transmitidas por alimentos com 8021 hospitalizações e *S. aureus* por 671 surtos com 526 hospitalizações. No Brasil, entre 2009 e 2018, entre os agentes identificados de doenças transmitidas por alimentos, 11.3% eram *Salmonella* spp. e 9.4% eram *S. aureus*, tornando-os dois importantes patógenos de origem alimentar. Prevenir surtos de doenças transmitidas por alimentos requer o controle de microbiano em toda a cadeia de produção de alimentos. Nesse contexto, uma ferramenta eficiente para inativar microrganismos é a terapia fotodinâmica antimicrobiana (TFDa), que é uma tecnologia promissora, de baixo custo e eficaz contra vários tipos de bactérias transmitidas por alimentos. Na TFDa, a inativação microbiana é causada por estresse oxidativo induzido pela interação do fotossensibilizador (FS) excitado pela luz na presença de oxigênio molecular. O resultado é um dano irreversível a vários constituintes moleculares das células (lipídios, proteínas, enzimas e DNA). Essa técnica apresenta várias vantagens quando comparada ao uso de métodos antimicrobianos tradicionais, mostrando ser eficiente independentemente do perfil de resistência antimicrobiana e de impedir o desenvolvimento de resistência, mesmo após vários ciclos de tratamento. Os corantes xantenos têm sido considerados bons FSs para induzir a fotoinativação bacteriana devido ao seu baixo preço, alta capacidade de absorção molar e alto rendimento quântico de oxigênio singlete ($\Phi\Delta$). Os corantes xantenos rosa bengala (RB) e eosina Y (EOS) já provaram ser eficazes contra bactérias, no entanto, esses corantes mostraram-se mais eficazes contra bactérias gram-positivas. Essa limitação pode ser superada pelo uso de diferentes sais orgânicos, como o iodeto de potássio (KI). Alguns corantes xantenos são aprovados para uso em aplicações farmacêuticas, cosméticas e médicas e como aditivos alimentares, enquanto a segurança do KI foi relatada pela *Food and Drug Administration* (FDA). Várias fontes de luz com espectros diferentes estão disponíveis, incluindo os diodos emissores de luz (LED) que estão se tornando uma alternativa promissora para TFDa devido a maior flexibilidade em termos de tempo de irradiação, uma ampla gama de espectro eletromagnético visível, incluindo luz verde (490 –570 nm) e seu baixo preço. Os modelos matemáticos, como metodologia da superfície de resposta (MSR), podem ser usados para prever as melhores condições para a inativação de microrganismos pela TFDa. Esses modelos são ferramentas valiosas para a aplicação dessa tecnologia na indústria de alimentos.

OBJETIVO. O objetivo deste trabalho foi aplicar a MSR para investigar o efeito fotodinâmico antimicrobiano do RB isoladamente contra *Salmonella enterica* sorotipo Typhimurium (ATCC 14028) e *Staphylococcus aureus* (ATCC 25923), bem como a combinação de RB e EOS com o sal inorgânico KI contra as mesmas bactérias.

MATERIAIS E MÉTODOS. Uma solução estoque de RB e EOS a 1,0 mM e KI a 5 M, preparadas em PBS pH 7.2 e um protótipo caseiro de dispositivo de LED verde (10 mW / cm^2; 530) foram utilizados neste trabalho. Para os ensaios de fotoinativação foram utilizados 500 µL da suspensão bacteriana a 10^7 UFC / mL com diferentes concentrações de RB ou EOS com ou sem KI. Simultaneamente, quatro grupos controle também foram avaliados: controle positivo; controle de luz (CL); controle de KI (CKI) e; controle do FS. Após incubação no escuro, as amostras e os controles CL e CKI foram expostos ao LED verde por diferentes tempos de irradiação. Finalmente, as amostras foram diluídas em solução salina a 0,85%, semeadas em duplicata em Ágar Tryptic Soy e as UFC / mL foram contadas. No primeiro artigo foram utilizados modelos matemáticos para investigar a interação da concentração de RB e do tempo de irradiação na fotoinativação de *S.* Typhimurium e *S. aureus.* Assim, para o delineamento experimental estatístico, foi utilizada uma sequência de experimentos projetados para modelar o efeito combinado de cada fator (concentração de FS e tempo de irradiação) na resposta, por meio do ajuste quadrático matemático dos resultados. Um modelo empírico polinomial de segunda ordem que descreve a relação entre a concentração de FS e o tempo de iluminação foi desenvolvido. Também foram realizados os ensaios da captação do FS, microscopia eletrônica de varredura (MEV), microscopia eletrônica de transmissão (MET) e citometria de fluxo. No segundo artigo, investigou-se o uso combinado de RB ou EOS com KI. Para isso, foi utilizado o mesmo protocolo de fotoinativação, descrito acima. Os ensaios de fotoestabilidade e resistência TFDa também foram realizados.

RESULTADOS E DISCUSSÃO. O modelo derivado utilizado no primeiro trabalho previu, as influências combinadas da concentração de FS e do tempo de iluminação nas contagens de *S. aureus* e *S.* Typhimurium, em conformidade com previsões e observações experimentais (R^2 = 0,8483 e p = 0,0013 para *S. aureus* R^2 = 0,9191 e p = 0,0001 para *S.* Typhimurium). Foi observada inibição total das células de *S. aureus* e *S.* Typhimurium ao aplicar uma dose de luz de 0,125 J/cm^2 e 152,0 J/cm^2, respectivamente. O estudo demonstrou que as concentrações de RB e os tempos de iluminação utilizados no delineamento experimental estatístico para *S.*

aureus e *S.* Typhimurium foram geralmente baixos, comprovando a eficácia do TFDa mediado por RB e luz verde LED contra esses microorganismos. *S. aureus* foi mais suscetível à concentração de RB e a ação foto inibidora aumentou de maneira dependente da concentração de FS. Enquanto *S.* Typhimurium foi mais suscetível ao tempo de iluminação do que à concentração do FS. As bactérias expostas a TFDa perderam a integridade da membrana, como demonstrado por MEV, MET e citometria de fluxo. Essa perda representa um dano significativo para as células, uma vez que várias funções estão ligadas à membrana plasmática e um dano na membrana pode levar ao vazamento do conteúdo celular, causando a morte celular. Para melhorar a ação da TFDa dos corantes xantenos, no segundo trabalho foram testadas as combinações de EOS ou RB com KI. Todas as concentrações de FS e KI testadas foram capazes de inativar eficientemente *S.* Typhimurium e *S. aureus*. Essa abordagem combinada permitiu uma redução na concentração de FS de até 1000 vezes, mesmo contra um dos patogenos de origem alimentar mais comum, *S.* Typhimurium, uma bactéria gram-negativa. Além disso, os resultados mostraram que não houve aumento significativo (p <0,05) na resistência de *S.* Typhimurium e *S. aureus* à fotossensibilização após 10 sessões consecutivas de 10 min de irradiação com a combinação de EOS ou RB com KI.

CONCLUSÕES: O uso dos corantes de xantenos em TFDa foi capaz de inativar eficientemente *S.* Typhimurium e *S. aureus*. Aplicando a MSR no tratamento da TFDa, a atividade foto inibidora pode ser prevista e uma nova maneira de usar ferramentas de modelagem matemática juntamente com aPDT pode ser fornecida. Portanto, essa abordagem pode ser, futuramente, otimizada e aplicada nas indústrias de alimentos. Além disso, a adição de KI potencializou fortemente a TFDa mediada por EOS e RB. Também foi confirmado que S. Typhimurium e *S. aureus* não desenvolveram mecanismos de resistência quando submetidos a ciclos consecutivos do protocolo da TFDa. Portanto, a inativação efetiva de ambas as bactérias com baixas concentrações de FS e o baixo preço dos corantes xantenos mostram que essa tecnologia tem potencial para ser facilmente transposta para a indústria de alimentos.

PALAVRAS CHAVE. Corantes xantenos; inativação fotodinâmica; metodologia de superfície de resposta; *Staphylococcus aureus*; sais inorgânicos; *Salmonella* Typhimurium.

Response Surface Methodology can be used to predict photoinactivation of foodborne pathogens using Rose Bengal excited by 530 nm LED.

Photoinactivation of pathogens by RB using RSM

Adriele Rodrigues dos Santos[a*], Alex Fiori da Silva[b], Camila Fabiano de Freitas[c], Marcos Vieira da Silva[d], Evandro Bona[e], Celso Vataru Nakamura[f], Noboru Hioka[g], Jane Martha Graton Mikcha[h].

[a]Programa de Pós-Graduação em Ciência de Alimentos, Universidade Estadual de Maringá, Maringá, PR, Brasil. adrielesantos@hotmail.com.

[b]Programa de Pós-Graduação em Ciência da Saúde, Universidade Estadual de Maringá, Maringá, PR, Brasil. alex_skiba@hotmail.com.

[c]Programa de Pós-Graduação em Química, Universidade Estadual de Maringá, Maringá, PR, Brasil. camila.freitas1989@hotmail.com

[d]Instituto Federal de Educação, Ciência e Tecnologia Farroupilha, Alegrete, Rio Grande do Sul, Brasil. marcosvs.83@gmail.com

[e]Departamento de Alimentos, Universidade Tecnológica Federal do Paraná – campus Campo Mourão, Campo Mourão, PR, Brasil. ebona@utfpr.edu.br

[f]Departamento de Ciências Básicas da Saúde, Universidade Estadual de Maringá, Maringá, PR, Brasil. cvnakamura@uem.br

[g]Departamento de Química, Universidade Estadual de Maringá, Maringá, PR, Brasil. nhioka@uem.br

[h]Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, PR, Brasil. jmgmikcha@uem.br

*Corresponding Author: Laboratório de microbiologia de alimentos, Departamento de análises clínicas e biomedicina, Universidade Estadual de Maringá, Avenida Colombo, 5790, zona 07, Maringá, 87020-900, PR, Brasil. e-mail: adrielesantos@hotmail.com.

https://orcid.org/0000-0003-3061-6669

Abstract: In this work the photodynamic bactericidal effect of Rose Bengal, combined with green LED light, against *S. aureus* and *S.* Typhimurium, was investigated. The interaction of RB concentration and illumination time was evaluated using a response surface methodology, and a second-order polynomial empirical model was adjusted to the experimental data. The derived model predicted the combined influences of these factors on *S. aureus* and *S.* Typhimurium counts, in accordance with predictions and experimental observations (R^2 = 0.8483 and P = 0.0013 for *S. aureus*; R^2 = 0.9191 and P = 0.0001 for *S.* Typhimurium). Total inhibition of *S. aureus* and *S.* Typhimurium was observed when applying a light dose of 0.125 J cm^{-2} and 152.0 J cm^{-2}, respectively. The treatments also showed loss of membrane integrity, morphological changes and internal cell structural alterations. In sum, the polynomial model developed could provide accurate information on the combined influences of RB and green LED light in aPDT treatment and, that this combination was able to inactivate *S. aureus* and *S.* Typhimurium.

Practical applications: Foodborne diseases are a great public health trouble around the globe and the ingestion of contaminated food represent a substantial risk for millions of people, as well as associated with serious economic consequences for society. However, the commom food preservation techniques (chemical preservation; salting, drying, acidification and heat treatment) is related to undesired changes in the nutritional and sensory characteristics of food products. The improvement of new non-thermal technologies that inactivate foodborne pathogens and maintain the cost-effective relation, not causing damage to the environment and microbial resistance is necessary. This study provides an alternative method for foodborne pathogens inactivation the Antimicrobial Photodynamic Therapy (aPDT). Appling the response surface methodology in aPDT treatment the photo inhibitory activity could be predict

and a new way to use mathematic modeling tools together with aPDT could be provide and this approach could be further optimized and applied in food industries.

Keywords. *Salmonella* Typhimurium; *Staphylococcus aureus;* RSM; Xanthene dyes; photodynamic inactivation.

1. INTRODUCTION

Foodborne diseases consist of a broad spectrum of diseases caused by the ingestion of foodstuffs contaminated at any stage in the process (Miller & Cawthorne, 2017). According to the Centers for Disease Control and Prevention (CDC, 2019) between 1998–2016, in the United States, *Salmonella* spp. was responsible for 2,585 outbreaks of foodborne disease with 8,021 hospitalizations and *S. aureus* for 671 outbreaks with 526 hospitalizations. In Brazil, between 2009 – 2018, among the identified agents of foodborne disease, 11.3 % was *Salmonella* spp. and 9.4 % was *S. aureus* (Ministério da Saúde, 2019), making them two important foodborne pathogens.

Antimicrobial Photodynamic Therapy (aPDT) is an emerging technology that has been shown to be promising and effective against a wide range of foodborne bacteria (Bonin et al., 2018; Hu et al., 2018; Penha et al. 2017; Silva et al., 2018; Tao et al., 2019; Yassunaka et al., 2015). In aPDT, a visible light of a suitable wavelength is used to irradiated a photosensitizer (PS) that, in presence of molecular oxygen, generates reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), superoxide radical (O_2^-) and singlet oxygen ($^1O_2$), promoting the inactivation of microorganisms (Bartolomeu et al., 2017; Marciel et al., 2018). These cytotoxic species cause oxidative stress and irreversible damage to various biological components of the cell wall (lipids, proteins, enzymes, and DNA) (Martins et al., 2018).

Among the substances that could be used as a PS, the anionic water-soluble xanthene dye Rose Bengal (RB) has significant potential in aPDT, cause is an inexpensive PS and its photodynamic mechanism operate largely converting oxygen molecules (O_2) into singlet oxygen ($^1O_2$) upon irradiation with green light (Fadel & Kassab, 2011; Pérez-Laguna et al., 2018; Wen et al., 2017).

Several light sources with different spectra are available, including the light-emitting diodes (LED) that are becoming a promising alternative to aPDT because of greater flexibility in terms of irradiation time, a wide range of the visible electromagnetic spectrum, including green light (490–570 nm region) and its low price that could facilitate the study of new compounds for aPDT (Costa et al., 2011; Freire et al., 2014; Peloi et al., 2008).

Statistical techniques that can predict the best conditions for inactivating microorganisms by aPDT are valuable tools for applying this technology. One of these techniques is response surface methodology (RSM). It can be used to reduce the number of tests even when studying the effects of several factors at different levels and their influence on each other with a minimum number of tests (Rauf MA, Marzouki N, Körbahti BK. 2008; Zhang et al., 2013).

Most of the studies that investigate aPDT mediated by RB use the dye with a modified structure, encapsulated, or added to other compounds (Anju et al., 2018; Gu et al., 2010; Vieira et al., 2018; Wen et al., 2017). In addition, they use fluorescent and halogen lamps instead of a green LED as a source of light (Anju et al., 2018; Cahan R, Schwartz R, Langzam Y & Nitzan Y., 2011; Decraene V, Pratten J & Wilson M. 2006; Gu et al., 2010; Vieira et al., 2018; Wen et al., 2017). To our knowledge, there are no reports of the use of aPDT mediated by RB, combined with green LEDs, against *S. aureus* and *Salmonella* spp. using predictive mathematical models. Hence, the aim of the present study is to evaluate the effects of aPDT using RB excited by 530 nm LED against *Salmonella* Typhimurium and *Staphylococcus aureus*, via response surface methodology.

2. MATERIALS AND METHODS

2.1.Bacterial strains and culture conditions

Salmonella enterica serotype Typhimurium (ATCC 14028) and *Staphylococcus aureus* (ATCC 25923) were used in this study. The strains were stored at −20 °C in Brain and Heart Infusion Broth (Difco, Becton Dickinson, Sparks, MD, USA) that contained 20 % (vol/vol) glycerol and, prior to use, they were grown overnight at 37 °C in 5 ml of Tryptic Soy Broth (Difco). Then the microorganisms were harvested by centrifugation at 5000 x *g* for 4 min, washed three times with 0.85% saline solution. Afterward, the inoculums were adjusted to approximately 1 x 10^8 colony-forming units (CFU) ml^{-1} using a spectrophotometer at 580 nm (%T 25–30). This standardized suspension was diluted in 0.85% saline solution to approximately 1 x 10^7 CFU ml^{-1} for use in the experiments (Yassunaka et al., 2015).

2.2.Photosensitizers and LED light source

A stock solution of RB (Sigma Aldrich, Darmstadt, Germany) at 1 x 10^{-3} µM was prepared in PBS pH 7.2, filter sterilized at a 0.22 µm mixed cellulose esters membrane filter, standardized in a spectrophotometer (UV-Vis Beckman Coulter DU *800) and kept in the dark under refrigeration until use (Bonin et al., 2018).

The green LED homemade device prototype (Figure S1 – supplementary material) has 252 LEDs appropriately arranged on a plate of 13 cm length x 8 cm width, with a distance from the microplate surface of 3.5 cm. The prototype has a fluency rate of 10 mW cm^{-2} and a wavelength of 530 ± 40 nm. The spectral emission of the LEDs system was obtained using a spectrofluorimeter (Varian Cary Eclipse, San Diego, CA, USA). The absolute irradiance of the LEDs was evaluated with a Spectroradiometer USB2000+RAD (Ocean Optics,Winter Park, FL, USA).

Light doses (D_{Abs}) and LED beam array of RB at the respective concentrations and irradiation time were calculated as described by Gerola et al. (2012) and Silva et al. (2018) (equations 1 and 2), considering the irradiated area of 1.9 cm^2 and the irradiance of 10 mW cm^{-2}.

$$D_{Abs} = \frac{t}{A} \cdot \int_{\lambda 1}^{\lambda 2} P_{Abs}\, d\lambda \qquad (1)$$

A is the irradiated area, t is the exposure time, and P_{Abs} was calculated according to the following equation:

$$P_{Abs} = X_{Abs} P_{LED\ Emitted} \qquad (2)$$

X_{Abs} is the absorbed light fraction by the PS, P_{Abs} is the absorbed irradiance by the PS, and $P_{LED\ emitted}$ is the irradiance emitted by LED.

2.3. Determination of photoinhibitory activity of rose bengal and green LED light using a Statistical Experimental Design

A Rotational Central Composite Design generated by the software Statistica® 8.0 (StatSoft Incorporation, Tulsa, OK, USA, 2007) was used to establish the conditions for the photoinhibitory activity of RB against *S.* Typhimurium and *S. aureus* (Table 1). The PS concentrations and illumination times used in the statistical experimental design were pre-determined in preliminary studies. Twelve experiments, including three replicates of the central point, were conducted to evaluate the effects of two independent variables: X_1, the concentrations of RB (10 to 25 nM, to *S. aureus* and, 10 to 75 µM, to *S.* Typhimurium) and X_2, the illumination time (5 to 15 min for both microorganisms).

Table 1. Experimental design with coded and real values of the two independent variables (PS concentration and illumination time) and the light dose values calculated according to Gerola et al. (2012) and Silva et al. (2018) evaluated for their influence on *S.* Typhimurium and *S. aureus* cell viability.

Experiments	**Coded values**		***S. aureus*** **Real values**				***S.* Typhimurium** **Real values**			
	X_1	**X_2**	**Concentration (nM)**	**Time (min)**	**Light Doses (J cm^{-2})**	**Cell viability (Log CFU ml^{-1})†**	**Concentration (µM)**	**Time (min)**	**Light Doses (J cm^{-2})**	**Cell viability (Log CFU ml^{-1}) †**
Control (PS-L-)‡	---	---	0	0	0	5.49 ±0.05	0	0	0	7.34 ±0.03
1	-1.00000	-1.00000	12.00	5	0.029	3.99 ±0.23	11.80	6.5	26.900	6.73 ±0.17
2	-1.00000	1.00000	12.00	15	0.086	2.10 ±1.13	11.80	13.5	55.900	3.21 ±0.33
3	1.00000	-1.00000	24.00	5	0.057	2.98 ±0.37	64.20	6.5	73.200	4.11 ±0.32
4	1.00000	1.00000	24.00	15	0.172	1.40 ±0.82	64.20	13.5	152.000	Bql§
5	-1.41421	0.00000	9.50	10	0.046	4.11 ±0.28	9.47	10	35.100	7.26 ±0.16
6	1.41421	0.00000	26.50	10	0.125	Bql§	75.05	10	120.000	1.77 ±0.99
7	0.00000	-1.41421	18.00	3	0.027	4.09 ±0.22	38.00	5.05	44.000	5.73 ±0.15
8	0.00000	1.41421	18.00	17	0.155	1.77 ±0.99	38.00	14.95	130.200	0.92 ±0.69
9	0.00000	0.00000	18.00	10	0.091	3.12 ±0.81	38.00	10	87.100	1.40 ±0.93
10	0.00000	0.00000	18.00	10	0.091	3.43 ±0.17	38.00	10	87.100	2.00 ±0.24
11	0.00000	0.00000	18.00	10	0.091	2.56 ±1.11	38.00	10	87.100	3.69 ±0.25
12	0.00000	0.00000	18.00	10	0.091	3.30 ±0.18	38.00	10	87.100	2.24 ±0.97

†Values are mean followed by standard deviation

‡Positive control, containing only the inoculum in PBS and without illumination.

§Bql - below the quantification limit of 2 log CFU mL^{-1}.

In a 24-well microplate, an aliquot of 25 µL of bacterial suspension at 1 x 10^7 CFU mL^{-1} was homogenized with 475 µl of RB at different concentrations and kept in the dark for 10 min. After incubation, the microplate was illuminated with a green LED light (PS+L+) up to the maximum time of 15 min. Three controls were also evaluated: PS control, containing the inoculum and PS without illumination (PS+L-); light control, containing only the inoculum in PBS under illumination (PS-L+), and; the positive control, containing only the inoculum in PBS and without illumination (PS-L-).

After photoinactivation treatment, the samples were diluted in 0.85 % saline solution, 10 µL droplets of each dilution were plated on Trypticase Soy Agar (Difco) plates in order to determine the number of culturable cells (CFU mL^{-1}). The plates were incubated at 37 °C for 24 h and the CFU were counted (Bonin et al., 2018).

2.4.Statistical analysis

A second-order polynomial model was used for fitting the experimental data and its coefficients were obtained by multiple linear regression (Equation 3).

$$\hat{Y} = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad (3)$$

Where $\hat{Y}$ is the predicted response; b_0 is the regression coefficient for the intercept; b_1 and b_2 are the regression coefficients representing the linear effect terms; b_{11} and b_{22} are the quadratic effect terms; b_{12} is the interaction effect terms; and X_1 and X_2 are the independent variables in coded values. A t-test was performed for analyzing the regression coefficients and excluding those that were not significant ($p > 0.05$) to the mathematical models. The models were considered significant for the prediction of the responses via the determination of the coefficients of determination (R^2), the adjusted coefficients of determination (R^2adj), and the

ANOVA ($p < 0.05$). The mean square error (MSE) was also used as residual analysis of the model. The software Statistica® 13 (TIBCO Software Inc, 2017) was used for the statistical analysis described above and for generating the response surface plots.

2.5.Rose bengal uptake

Bacterial suspensions, prepared according Manoil D, Filieri A, Schrenzel J & Bouillaguet S. (2016) with modifications, in the presence of different concentrations of RB (Table 1), were incubated at room temperature in the dark for 10 min. Control and treated samples were harvested by centrifugation at 9.500 x g for 5 min and washed thrice with 0.85% saline solution. To extract RB incorporated into cells, the bacterial pellet was dissolved in 99.9 % DMSO, sonicated (10 min at 35 kHz, Sonorex, Bandelin electronics, Berlin, DE). The samples were transferred into a quartz cuvette and measured fluorescence emission in a spectrofluorimeter (Varian Cary-Eclipse). Lysed bacteria in DMSO, without RB, were used as blanks. A calibration curve of fluorescence emission of RB versus concentration, in DMSO, was used to determine the concentration of RB in samples. Experiments were performed in triplicate and repeated three times.

2.6.Scanning electron microscopy

Sample preparation was performed according Bonin et al. (2018). Control and treated bacteria were fixed with 2.5 % glutaraldehyde (Sigma-Aldrich, Louis, MO, USA) in 0.1 M cacodylate (SEM, Hatfield, PA, USA) at 4 °C for 2 h. Then the samples were centrifuged, washed thrice in the same buffer, and placed in coverslips containing poly-L-lysine for one hour. The coverslips were washed with cacodylate buffer and dehydrated using increasing concentrations of ethanol (50, 70, 80, 90, and 100 %). They were submitted to CO_2 critical-

point-dried and covered with gold. The samples were observed in a QUANTA 250 scanning electron microscope (FEI, Amsterdam, Netherlands).

2.7.Transmission electron microscopy

Sample preparation was performed according to Jiang et al. (2013) with modifications. After photodynamic treatment, control and treated bacteria were fixed with 2 % glutaraldehyde in 0.1 M cacodylate for one day. After fixation, the samples were centrifuged, washed thrice with cacodylate buffer, and post fixed with 1 % osmium tetroxide for one hour. Then, the cells were dehydrated in graded acetone and embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA), so the ultrathin sections (80 to 100 nm) were prepared, stained with uranyl acetate and lead citrate. The observation of the ultrastructure of the samples was in an electron transmission microscope (JEM-1400; Jeol, USA).

2.8.Flow cytometry

Flow cytometry was performed according Bonin et al. (2018) using the LIVE/DEAD BacLight Bacterial Viability and Counting Kit (L34856), (Molecular PROBES, Oregan, USA). Treated bacteria, positive control (PS-L-) and negative control were centrifuged at 10.000 x g for 3 min and resuspended in 1 mL of 0.85 % saline solution. Subsequently, were added to the samples 1.5 μL of propidium iodide (PI) and 1.5 μL of SYTO 9, then the samples were kept in the dark for 15 min. The stained samples were analyzed on a flow cytometer (BD Fascs Canto II).

3. RESULTS

3.1.Light doses

In this study, the absorbed potency (P_{Abs}) and the light doses (D_{Abs}) were calculated as previously described (Gerola et al., 2012; Silva et al., 2018). It was necessary to explaining the exact fraction of the LED power (P_{Emit}) that was absorbed by the different concentrations of the PS in varying illumination times, as shown in Table 1. It is also possible to observe that the intensities of the RB spectral profiles are directly proportional to the dye concentrations (Fig. 1) and, consequently, the light doses increase as the PS concentrations are higher.

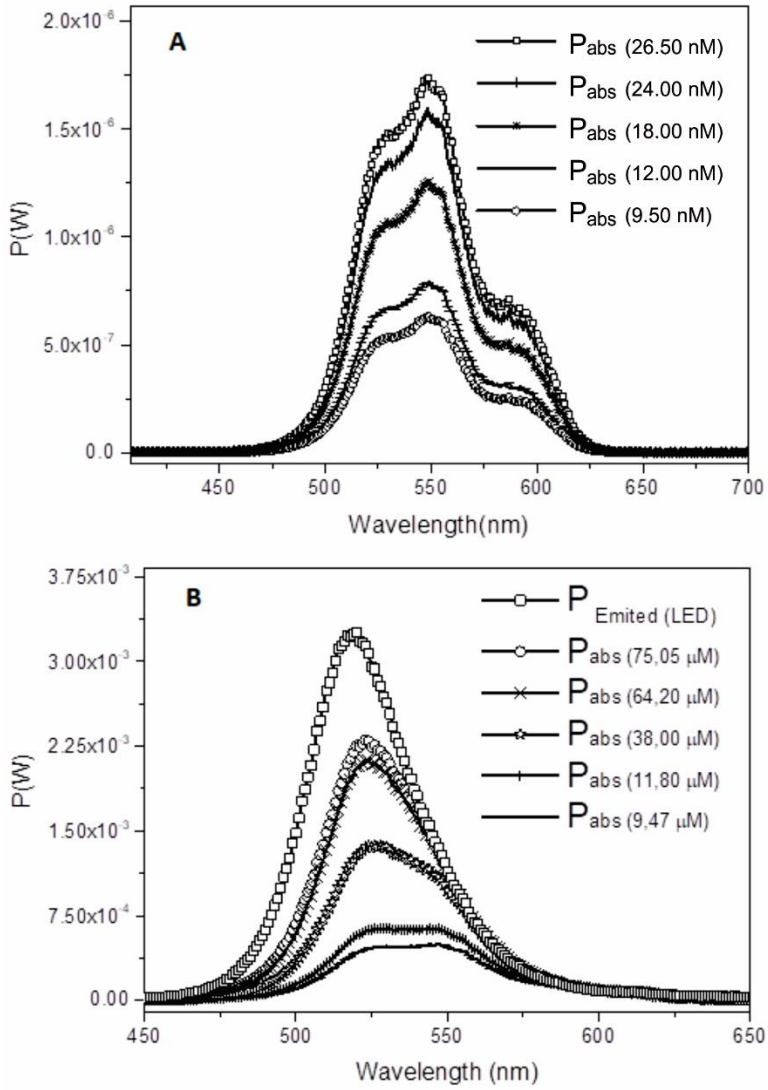

Fig. 1 - Spectra of light emitted by LED ($P_{LED\ Emitted}$) and power absorbed by RB (P_{Abs}) in different concentrations for *S. aureus* (A), (26.50 nM; 24.00 nM; 18.00 nM; 12.00 nM; 9.50 nM) and *S.* Typhimurium (B), (75.05 µM; 64.20 µM; 38.00 µM; 11.80 µM; 9.47 µM). Potency (Watt); wavelength (nm).

The complete inhibition of *S.* Typhimurium was observed at 64.20 µM and an illumination time of 13.5 min (152 J cm^{-2}), as shown in Table 1. However, with a small increase in the illumination time (14.95 min.) and just over half of the PS concentration (38 µM), it was possible to observe more than 6 log CFU mL^{-1} of reduction in the bacterial viability. Maintaining the PS concentration (38 µM) and decreasing the illumination time to ten minutes, we still obtained approximately 4 log CFU mL^{-1} of reduction in the viability of *S.* Typhimurium.

Table 2. Regression coefficients of the full mathematical model to predict the photoinhibitory effects of RB and green LED light against *S.* Typhimurium and *S. aureus.*

	Staphylococcus aureus		***Salmonella* Typhimurium**	
Coefficient†	**Regression coefficient**	***P*-value**	**Regression coefficient**	***P*-value**
b_0	3.0578	0.0001	2.3316	0.0010
b_1	- 0.9405	0.0066	- 1.6994	0.0009
b_{11}	- 0.4927	0.1057	0.9873	0.0193
b_2	- 0.8455	0.0107	- 1.8039	0.0006
b_{22}	- 0.0555	0.8374	0.3951	0.2516
b_{12}	0.0781	0.8194	- 0.1481	0.7200

†b_0 – intercept; b_1 – linear coefficient of PS concentration; b_2 – linear coefficient of illumination time; b_{11} – quadratic coefficient of PS concentration; b_{22} – quadratic coefficient of illumination time; b_{12} - interaction coefficient between PS concentration and illumination time.

In the mathematical models, only significant coefficients ($p < 0.05$) were considered (Table 2). Although the quadratic coefficient of PS concentration (b_{11}) was not significant, it was considered in the composition of the mathematical model for *S. aureus*, since the model evaluation were better with it, that is, by keeping this coefficient the adjusted R2 increases and the residual variance decreases.

The analysis of variance (ANOVA), showed at table S1, demonstrates that the models are significant for both microorganisms (*S. aureus*, $p = 0.0013$; *S.* Typhimurium $p = 0.0001$) and did not show lack of fit (*S. aureus*, $p = 0.196$; *S.* Typhimurium $p = 0.8161$). The R^2_{adj} value (0.7879 for *S. aureus* and 0.8887 for *S.* Typhimurium) being the measure of the goodness of fit of the model, indicates that 78.79 % (for *S. aureus)* and 88.87 % (for *S.* Typhimurium) of the total variation is explained by the model. The MSE was 0.2181 for *S. aureus* and 0.4009 for *S.* Typhimurium. Considering the R^2_{adj}, MSE, the significance of prediction, lack of fit of these models, and the natural variability related to the microbiological experimentation (Doria Filho, 2001), the models were considered adequate for predicting the photo inhibitory activity of RB with green LED light.

In Table 2, it is possible to observe that the models showed no significant interactions between PS concentration and illumination time (b_{12}) ($p > 0.05$), for both *S. aureus* and *S.* Typhimurium. The regression coefficients of the mathematical models showed that the quadratic effect for the variable illumination time (b_{22}) was not significant either ($p > 0.05$), for both bacteria.

For the *S. aureus* model, the ANOVA, table S2, showed that the variables PS concentration and illumination time exhibited linear negative effects (Table 2 and Fig 2A); in other words, as PS concentration or illumination time increases, there is a tendency to decrease the bacterial counts. However, the linear effect of the PS concentration was the most significant, demonstrating that PS concentration has a greater influence than illumination time in reducing

S. aureus counts. Even the quadratic effect of the PS concentration variable was not significant but was considered in the composition of the *S. aureus* mathematical model and indicated that the PS concentration has a low photo inhibitory effect until approximately 6.00 nM (Fig 2A). However, the photo inhibitory effect was pronounced when PS concentration was higher than 10.00 nM (Fig 2A).

For the *S.* Typhimurium model, the ANOVA (Table S2 – supplementary material) showed that the linear negative effect of illumination time was the most significant, followed by the linear negative effect of PS concentration, similar to *S. aureus*. These results also demonstrate that the time of exposure to light has higher influence than the concentration of RB in *S.* Typhimurium photoinactivation. The less significant effect was the quadratic positive of PS concentration, which indicated that the increase of PS concentration promotes a decrease in the bacterial count; however, from approximately 65 μM (Fig 2B), this term begins to show a decrease in its inhibitory effect.

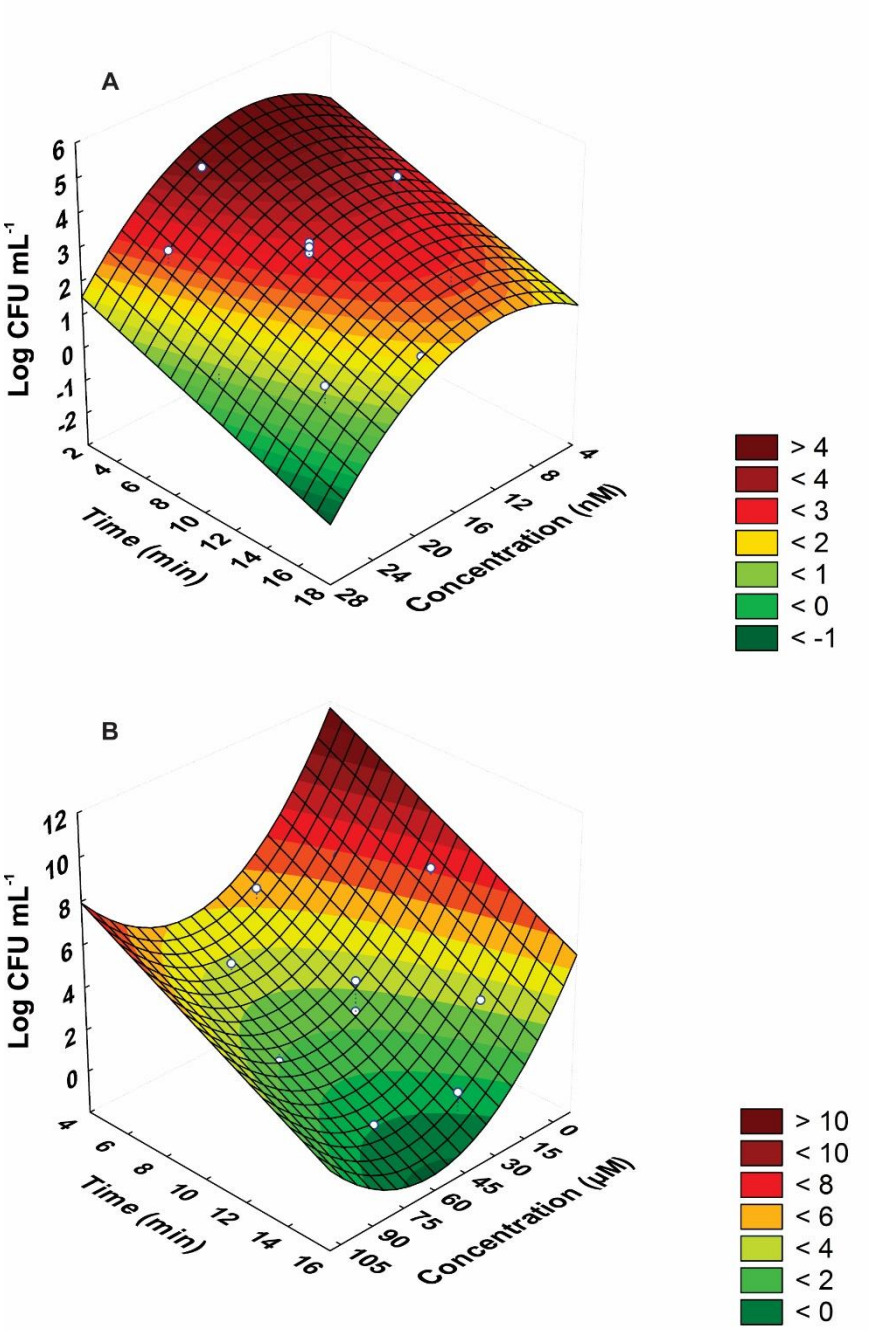

Fig. 2 – Response surface describing interactive influence of the two independent factors tested: PS concentration and illumination time for *S. aureus* (A) and *S.* Typhimurium (B) counts (> 4 log CFU ml^{-1}; < 4 log CFU ml^{-1}; < 3 log CFU ml^{-1}; < 2 log CFU ml^{-1}; < 1 log CFU ml^{-1}; < 0 log CFU ml^{-1}).

3.2.Rose bengal uptake

Figure 3 shows that both *S. aureus* and *S.* Typhimurium immobilized the photosensitizer after 10 min incubation. When the concentration of RB was increased up to 18 nM for *S. aureus*

and 38 μM for *S.* Typhimurium, it was possible to observe an increase in the PS uptake in a dependent manner. However, when RB concentration was increased, above those already mentioned, the uptake showed decay for both strains.

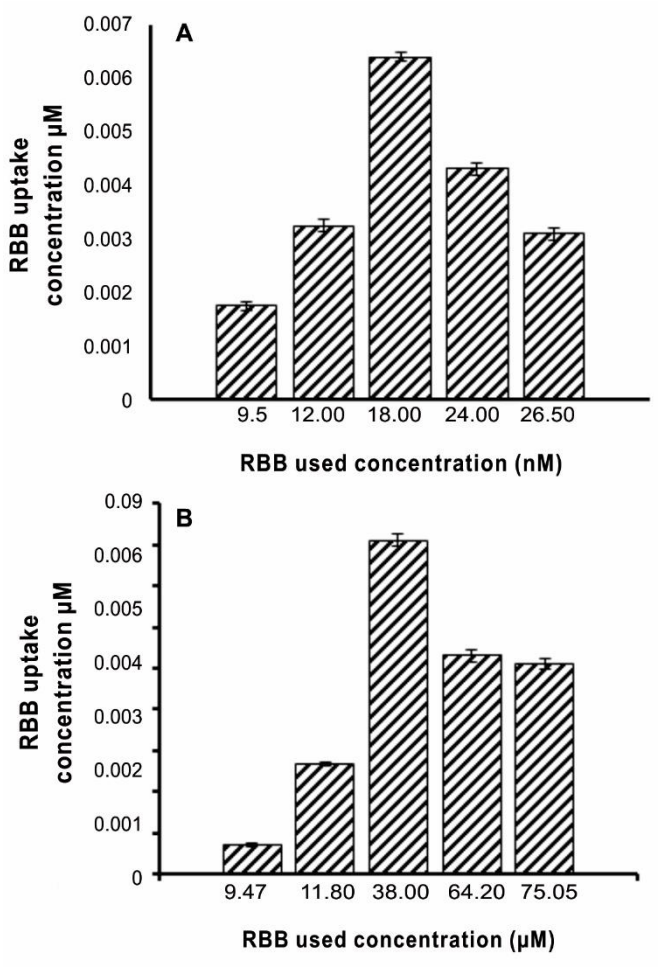

Fig. 3 – Rose Bengal uptake by *S. aureus* (A) and *S.* Typhimurium (B): concentration of RB extracted after 10 min incubation.

3.3.Scanning electron microscopy

The morphological alterations of *S. aureus* (Fig. 4A) and *S.* Typhimurium (Fig. 4B) induced by RB at 24.00 nM and 64.20 μM illuminated for 5 and 6.5 min, respectively, were evaluated by SEM. Positive control (PS-L-) was with its characteristic morphology and had a uniform and smooth cell surface (Fig 4 – A1 and B1), while the photoinactivated cells (Fig 4 –

A2, A3, B2 and B3) showed their cell membrane and morphology with distortions, appearing wrinkled and withered.

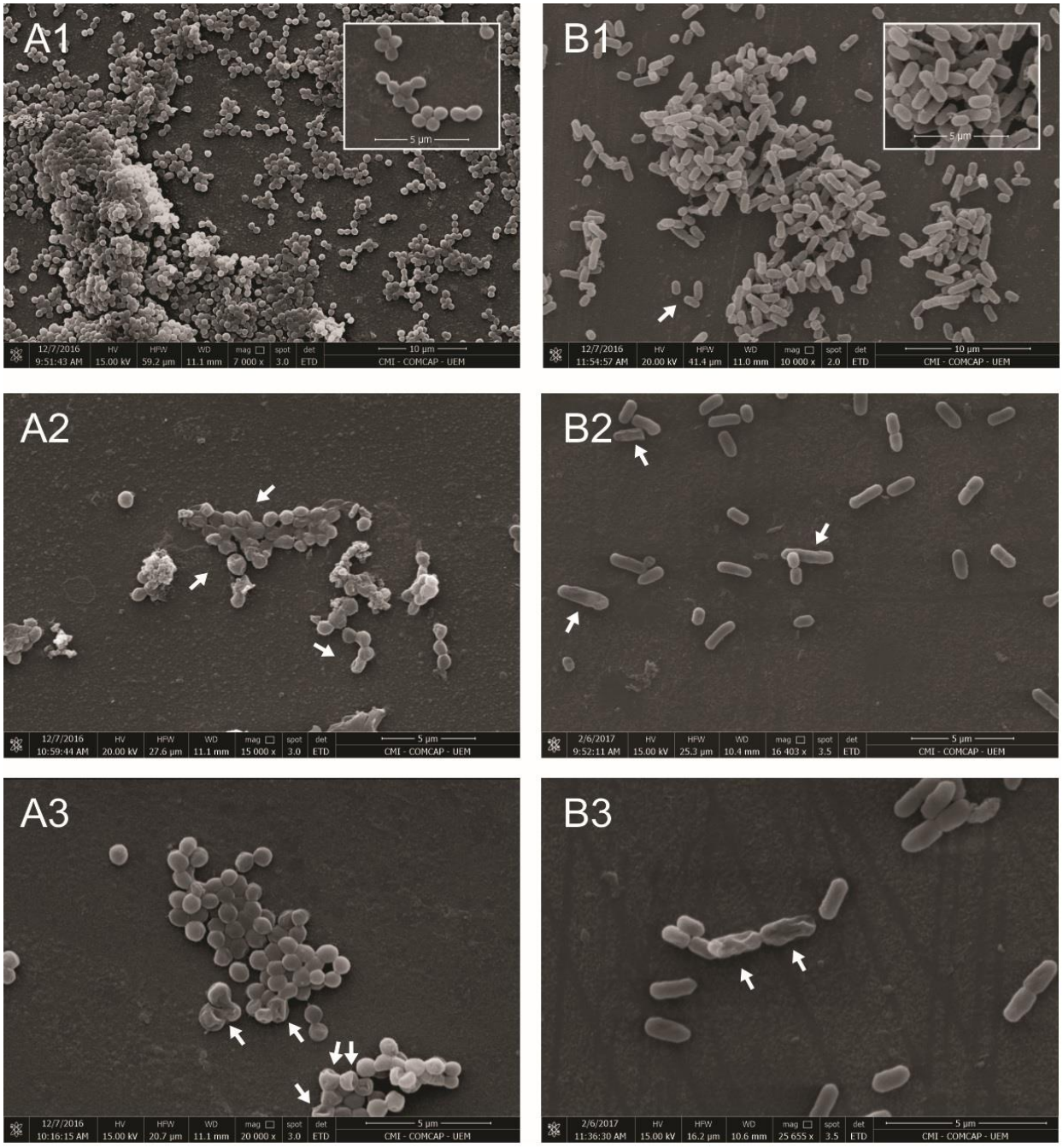

Fig. 4 – Scanning electron microscopy of *S. aureus* (A), positive control (PS-L-) (A1 - magnification 7.000x), bacteria treated with 24.00 nM of RB and 5 min of illumination (A2 - magnification 15.000x and A3 - magnification 20.000x) and; *S.* Typhimurium (B), positive control (PS-L-) (B1 - magnification 10.000x), bacteria treated with 64.20 µM of RB and 6.5 min of illumination (B2 - magnification 16.403x and B3 - magnification 25.655x).

3.4.Transmission electron microscopy

The ultrastructural morphology of *S. aureus* (Fig. 5A) and *S.* Typhimurium (Fig. 5B) induced by RB at 24.00 μM and 64.20 μM illuminated for 5 and 6.5 min, respectively, were observed using TEM. The *S. aureus* control cells presented normal and spherical morphology, with dense and homogeneous cytoplasm and intact cell walls and membranes (Fig. 5 – A1). In contrast, treated cells appeared to have undergone lysis, with damaged cell walls and membranes, resulting in leakage of cellular contents with translucent cytoplasm (Fig. 5 – A2). The *S.* Typhimurium cells in the control group had a densely stained cytoplasm and were in a shape of a rod (Fig. 5 - B1). In contrast, the cells in the treatment group showed that the cytoplasm was damaged and leaking, with loss of structural integrity of the membrane and cell wall also being observed, as evident in Fig. 5 – B2.

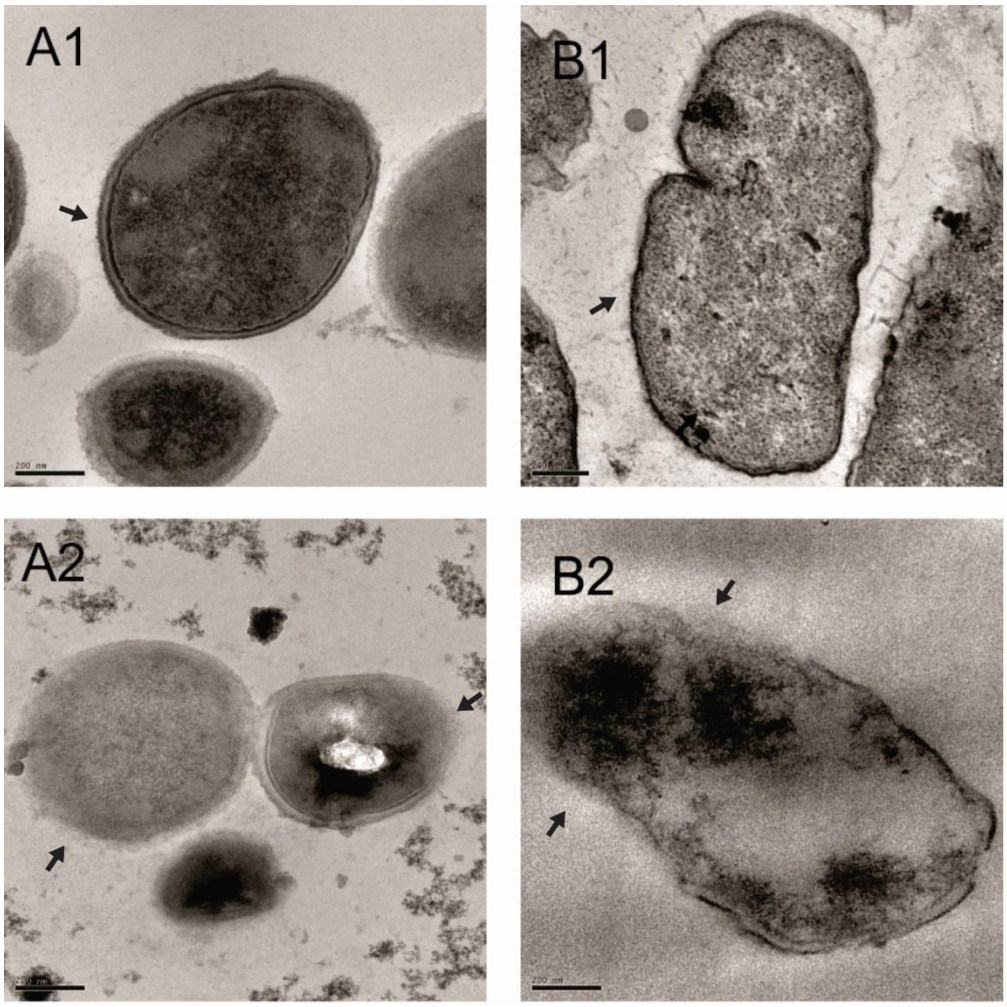

Fig. 5 – Transmission electron microscopy of *S. aureus* (A), positive control (PS-L-) (A1), bacteria treated with 24.00 nM of RB and 5 min of illumination (A2) and; *S.* Typhimurium (B), positive control (PS-L-) (B1), bacteria treated with 64.20 µM of RB and 6.5 min of illumination (B2). Bar represent 200 nm.

3.5.Flow cytometry

The detection of different subpopulations based on the membrane integrity of *S. aureus* and *S.* Typhimurium are shown in Figure 6. The negative control (bacteria death with isopropyl alcohol) exhibited, for *S. aureus*, 95.8 % of dead cells and 1.77 % of injured cells (Fig. 6 – A1), while *S.* Typhimurium showed 94.0 % of dead cells and 4.67 % of injured cells (Fig. 6 – B1). The positive control (PS-L-) showed that 96.2 % of the *S. aureus* cells showed up positive for SYTO 9 with a strong green fluorescence, indicating the integrity of the membrane. Already, 2.60 % of the *S. aureus* cells showed double-staining, indicating that the cells were injured (Fig. 6 – A2). For *S.* Typhimurium, the positive control (PS-L-) showed 91.7 % of live cells, 4.45 % of injured cells, and 2.19 % of dead cells (Fig 6 - B2).

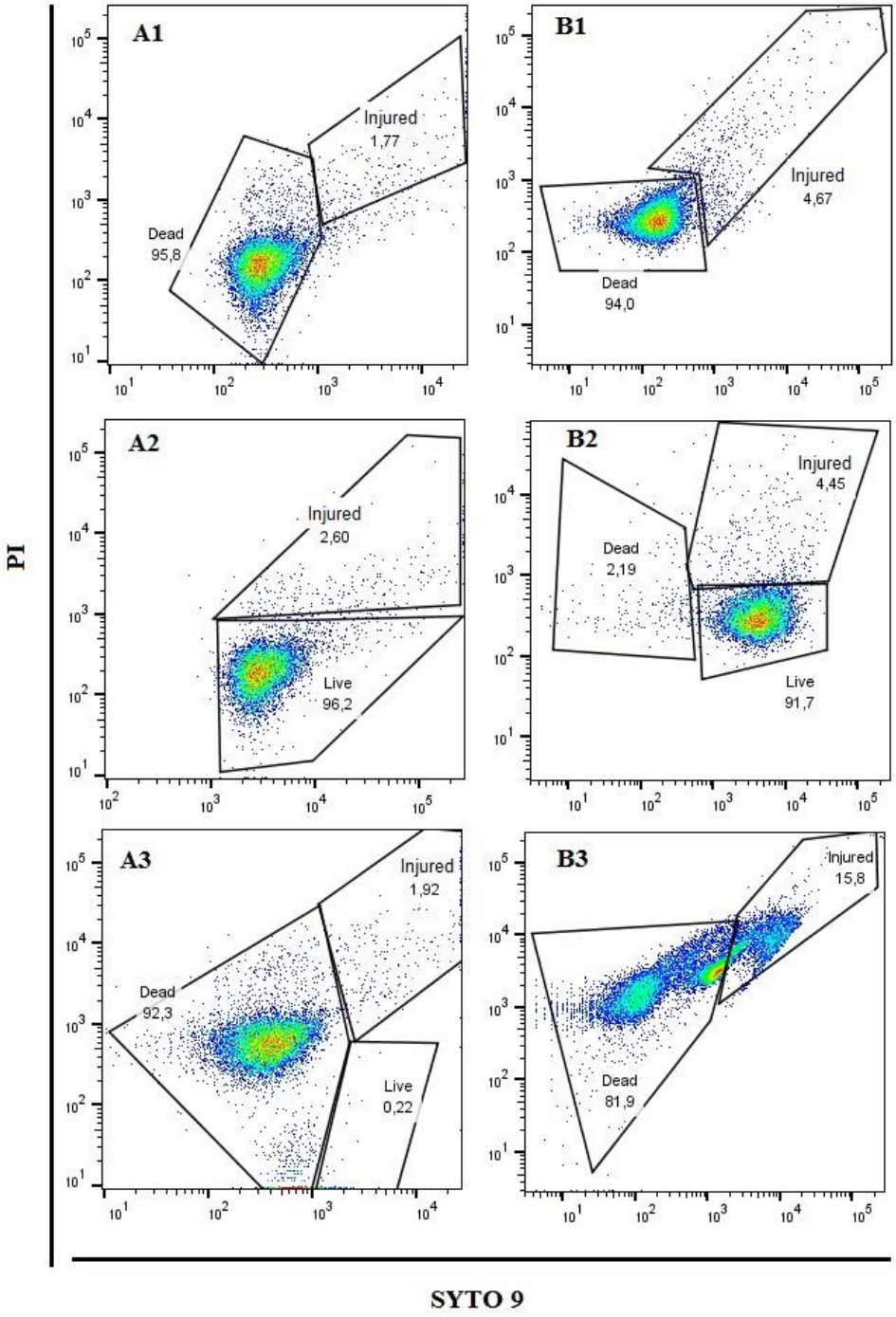

Fig. 6 - Flow cytometric analysis of *S. aureus* (left side): negative control (bacteria with isopropyl alcohol) (A1), positive control (PS-L-) (A2), bacteria treated with 24.00 nM of RB and 15 min of illumination (A3); and of *S.* Typhimurium (right side): negative control (bacteria with isopropyl alcohol) (B1), positive control (PS-L-) (B2), bacteria treated with 38.00 µM of RB and 14.95 min of illumination, stained with SYTO 9 and PI. SYTO 9 fluorescence on the X-axis plotted against PI fluorescence on the Y-axis. Numbers in each gate represent the percentage of events.

When *S. aureus* was exposed to 24.00 nM of RB and 15 min of illumination, 92.3 % dead cells, 1.92 % injured cells, and 0.22 % live cells were obtained (Fig. 6 – B3). After exposure to 38.00 μM of RB and 14.95 min of illumination, *S.* Typhimurium showed 81.9 % dead cells and 15.87 % injured cells, as shown in Fig 6 – B3.

7. Discussion

aPDT is a novel, emerging technology that may be particularly useful in increasing microbial food control, with significant advantages over conventional methods (Luksiene & Brovko, 2013). Some authors showed good results in eliminate foodborne pathogens and food spoilage microorganisms in meat and seafood (Liu et al., 2016; López-Carballo Hernández-Muñoz, Gavara & Ocio, 2008; Wu et al., 2016) in extend the shelf-life and improve microbiological quality of fruits (Al-Asmari, Mereddy & Sultanbawa, 2018; Tao et al., 2019) and in decontaminate packing surfaces (Luksiene, Buchovec & Paskeviciute, 2009; Luksiene & Paskeviciute, 2011). However, it is necessary to say that the most researches obtained its results from experiments in a laboratory scale (Silva et al., 2018).

The present study demonstrates that RB in combination with green LEDs has a notable effect on *S. aureus* and *S.* Typhimurium cell viability. RB concentrations and illumination times used in the statistical experimental design for *S. aureus* and *S.* Typhimurium were generally low, proving the effectiveness of aPDT mediated by RB and green LED light against these microorganisms. This large photodynamic efficiency of RB was expected due to its high singlet oxygen quantum yields, with values around 0.79, which is relatively high among photosensitizers (Neckers & Valdes-Aguilera, 1993).

Some authors have studied the efficiency of aPDT with RB against several microorganisms (Cahan R, Schwartz R, Langzam Y & Nitzan Y., 2011; Decraene V, Pratten J & Wilson M. 2006; Freire et al., 2014; Gu et al., 2010; Vieira et al., 2018). However, such

studies did not involve green LED light, nor did they work with modified PS structures, and work with the RB encapsulated or added to other compounds.

Decraene V, Pratten J & Wilson M. (2006), working with a cellulose acetate-based coating incorporated with RB and toluidine blue, at 25 μM for each dye, and using a compact fluorescent light, achieved a reduction in *S. aureus* viability of 2.5 log CFU mL^{-1} and 6.3 log CFU mL^{-1} with lighting times of two and six hours, respectively. Cahan R, Schwartz R, Langzam Y & Nitzan Y. (2011), using RB immobilized on hydrophobic polymers and a white luminescent lamp as light source and illumination time of 24 hours, obtained reductions in *S. aureus* viability of approximately 5 log CFU mL^{-1}. Guo Y, Rogelj S & Zhang P. (2010) achieved a reduction in *S. aureus* counts of approximately 6 log CFU mL^{-1} using Rose Bengal-decorated silica nanoparticles at 3 μM and light with a 525 nm filter during 40 minutes (light dose of 33 J cm^{-2}).

There are a few studies using RB as PS against *Salmonella* spp. Dahl T, Midden WR & Neckers DC. (1988) obtained a reduction of approximately 90 % in the viability of *S.* Typhimurium using RB at 5 μM. However, they used a dark incubation time of 120 minutes and an illumination time of about 80 minutes, with a tungsten filament incandescent lamp. Brovko et al. (2009), using concentrations of RB of 50 and 500 μg mL^{-1}, illuminating for 30 minutes using a halogen lamp, obtained reductions > 6 Log CFU ml^{-1} in *S.* Typhimurium counts.

In our work, with the PS in its conventional form, we obtained better results, even with a lower RB concentration and illumination time than the mentioned studies. These results can be explained by the kind of light source used in our work. It is known that an ideal PS and light source for aPDT should have an appropriate combination of emission and absorption wavelengths (Fracalossi et al., 2016). The use of green LEDs provides a good overlap between the absorbance of the RB and the emission of LED light, which can be observed in our work,

where the hatched area in Fig. 7 shows the effectively overlapping between the spectral irradiance of the green light source and the RB spectral absorption.

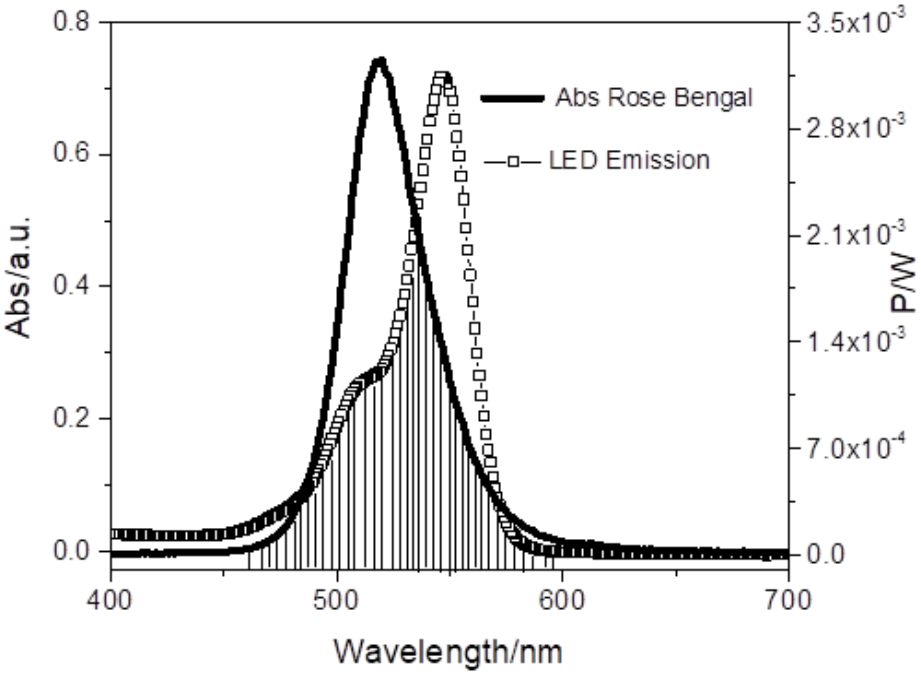

Fig. 7 – Light-emitting diode emitted potency (PLED Emitted) and absorbed potency by RB (PAbs). Absorbance (arbitrary unit); wavelength (nm); potency (Watt) (Abs Rose Bengal; LED emission).

Some studies have reported minimal effects of aPDT mediated by xanthene dyes on Gram-negative bacteria, compared with Gram-positive bacteria (Bonin et al., 2018; Cahan R, Schwartz R, Langzam Y & Nitzan Y., 2011; Decraene V, Pratten J & Wilson M. 2006; Yassunaka et al., 2015; Vieira et al., 2018). In our work, we reached total inhibition of *S.* Typhimurium; however, it required a concentration of RB approximately 1000 times greater than that used for *S. aureus* – this fact could be attributed to differences in the cell wall barrier properties of these bacteria.

The Gram-positive species, such as *S. aureus*, have a porous cell wall that allows penetration of most PS. Once the PS can penetrate the cell, it damages other targets such as proteins, enzymes, and DNA (Luksiene & Zukauskas, 2009; Waite & Yousef, 2009). In

contrast, Gram-negative bacteria such as *S.* Typhimurium have a cell wall that provides a physical and functional barrier to anionic compounds, such as RB, and hence the PS can be traversed and accumulated slowly (Dahl T, Midden WR & Neckers DC., 1988; Luksiene & Zukauskas, 2009).

In this work, RSM was applied to estimate the interaction effects of the PS concentration and illumination time on aPDT of *S. aureus* and *S.* Typhimurium. The application of the RSM showed that the concentration of PS and illumination time are important, but it is necessary to evaluate them separately to reflect their true influence on the response. In this sense, it was possible to observe that *S. aureus* was more susceptible to RB concentration and, that the photo inhibitory action increased in RB in a concentration-dependent manner. In agreement with this result, Manoil D, Filieri A, Schrenzel J & Bouillaguet S. (2016), evaluating *E. faecalis* photoinactivated by RB at 1 μM, 5 μM and 10 μM, and illuminating with a lamp emitting blue-light for 15, 60, and 240 seconds, also observed a greater influence of the RB concentration instead of illumination time.

S. Typhimurium was more susceptible to illumination time than PS concentration. This result could be related to *S.* Typhimurium cell walls providing a barrier to PS penetration, and, consequently, leading to difficulty in reaching intracellular targets. In this case, it would be necessary to provide more illumination time for the PS to generate singlet oxygen and cause cellular damage. According to Jemli M, Alouini Z, Sabbahi S & Gueddari M. (2002) to improve the efficacy of RB toward Gram-negative bacteria, it is necessary to increase the dye concentration and promote excessive light exposure.

Another important fact shown by RSM is that *S.* Typhimurium has its maximum inhibition with approximately 65 μM of RB, and in higher concentrations RB efficiency begins to decrease (Fig. 2B). This result may be related to the effect referred to as self-aggregation, which is commonly observed in xanthene dyes at high concentrations (Xu & Neckers, 1987).

It is known that the formation of aggregates affects the ability of a photosensitizer, because it modifies the photophysical properties of the dyes, the absorption spectrum and decreases the quantum yield of singlet oxygen ($\varphi_{\Delta 1}O_2$) (Valdes-Aguilera & Neckers, 1989). This result corroborates with the uptake of RB by *S.* Typhimurium which increased until RB at 38.00 μM, reduced with RB at 64.20 μM and stabilized, even when RB concentration increased to 75.05 μM.

Bacteria exposed to aPDT loses membrane integrity. This loss represents significant damage for cells, once multiple functions are linked to the plasma membrane (Joux & Lebaron, 2000; Jiang et al., 2013). With the flow cytometry technique is possible to detect different microbial subpopulations based on the integrity of the membrane, through the retention or exclusion of dyes (Manoil D, Filieri A, Schrenzel J & Bouillaguet S., 2016). Both *S. aureus* and *S.* Typhimurium treated with aPDT showed a high percentage of death or injured cells. The latter showed the simultaneous presence of SYTO-9 and PI in the cells promoted by a partial loss of membrane integrity.

The loss of membrane integrity of *S. aureus* and *S.* Typhimurium is consistent with reports from other studies with aPDT (Jiang et al., 2013; Deng et al., 2016 Manoil D, Filieri A, Schrenzel J & Bouillaguet S., 2016; Bonin et al., 2018), as well as with our findings by SEM and TEM. The treated groups showed morphological changes and internal cell structural alterations after photodynamic action. These findings demonstrate that membrane integrity damage may lead to cellular content leakage and can cause cell death.

The present study demonstrated that aPDT mediated by RB and green LED light was able to inactivate *S. aureus* and *S.* Typhimurium even at low light doses and PS concentrations. Increased permeability to propidium iodide, as well as morphological and internal cellular alterations after photodynamic action shown by *S. aureus* and *S.* Typhimurium indicate that cell membranes may be an important target of aPDT. The polynomial models developed could

provide accurate information on the combined influences of RB concentration and illumination time in aPDT treatment. The RSM showed that, for *S. aureus*, the RB concentration was the variable that most influenced the aPDT treatment, while illumination time had more influence on aPDT for *S.* Typhimurium. Hence, these findings provide important information to be considered in future research with respect to applying aPDT in the food industry.

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SUPPORTING INFORMATION

Supplementary material associated with this article can be found in the online version.

CONFLICT OF INTEREST

No conflict of interest declared.

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SUPPORTING INFORMATION

Response Surface Methodology can be used to predict photoinactivation of foodborne pathogens using Rose Bengal excited by 530 nm LED.

Adriele Rodrigues dos Santos*, Alex Fiori da Silva, Camila Fabiano de Freitas, Marcos Vieira da Silva, Evandro Bona, Celso Vataru Nakamura, Noboru Hioka, Jane Martha Graton Mikcha.

*Corresponding Author: Laboratório de microbiologia de alimentos, Departamento de análises clínicas e biomedicina, Universidade Estadual de Maringá, Avenida Colombo, 5790, zona 07, Maringá, 87020-900, PR, Brasil.

e-mail: adrielesantos@hotmail.com.

Table S1. Analysis of variance for the evaluation of the second-order polynomial model.

Source of variation	*Staphylococcus aureus*					*Salmonella* Typhimurium				
	Sum of squares	***df***	**Mean square**	***F*-ratio**	***p*-value**	**Sum of squares**	***df***	**Mean square**	***F*-ratio**	***p*-value**
Model	14.3425	3	4.7808	14.6167	0.0013	54.6354	3	18.2118	4.0662	0.0001
Residual	2.6166	8	0.3271			4.8110	8	0.6014		
Lack of fit	2.1833	5	0.4367	3.0232	0.1960	1.9717	5	0.3943	9.0134	0.8161
Pure error	0.4333	3	0.1444			2.8393	3	0.9464		
Total	16.9591	11				59.4465	11			

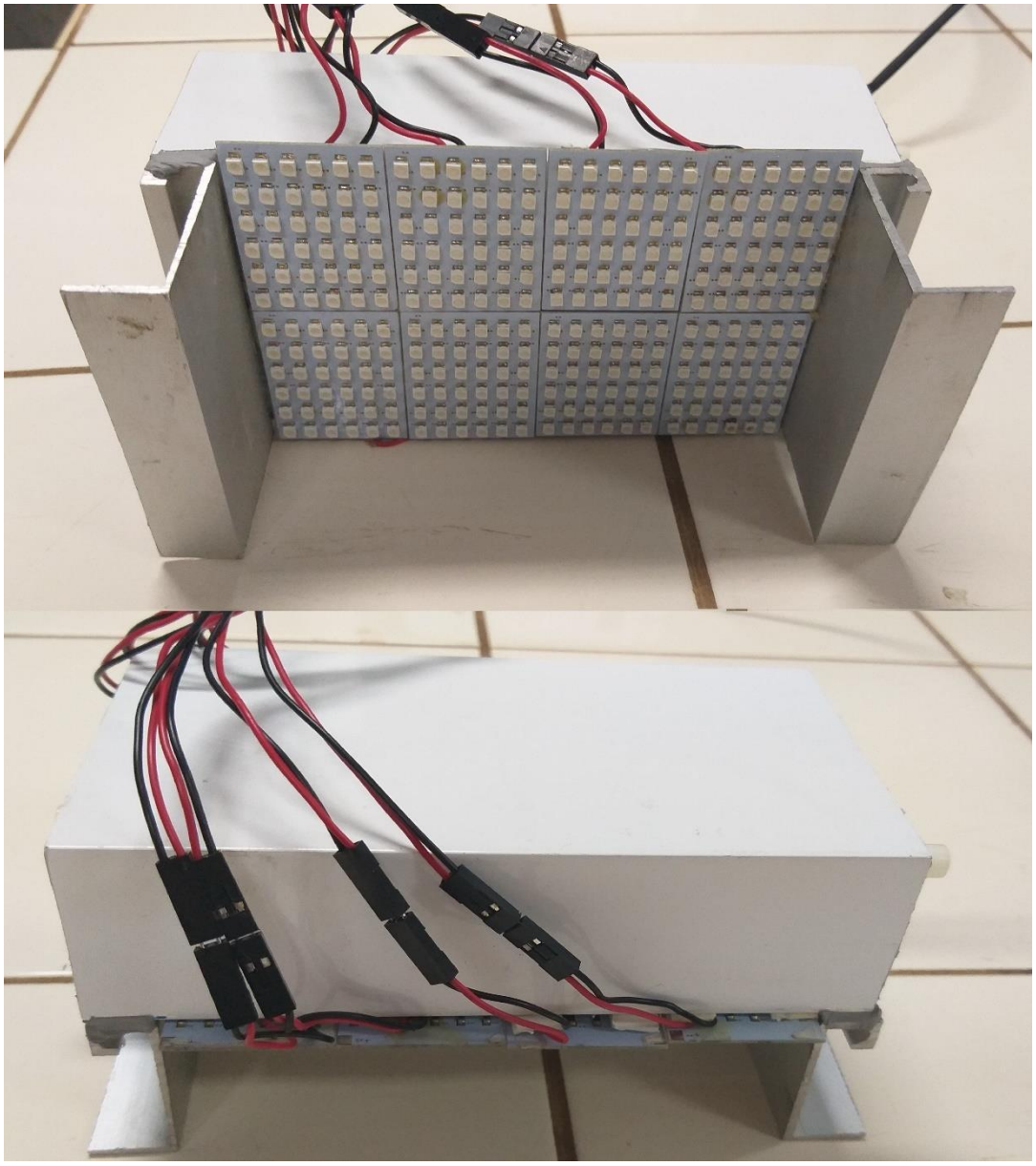
Coefficient of determination (R^2) – *S. aureus* = 0.8483 and *S.* Typhimurium = 0.9191;
Adjusted coefficient of determination (R^2_{adj}) – *S. aureus* = 0.7219 and *S.* Typhimurium = 0.8887

Table S2. Analysis of variance for the significant terms in the model

Independent variables*	*Staphylococcus aureus*					*Salmonella* Typhimurium				
	Sum of squares	***df***	**Mean square**	***F*-ratio**	***p*-value**	**Sum of squares**	***df***	**Mean square**	***F*-ratio**	***p*-value**
X_1	7.0768	1	7.0768	21.6366	0.0016	23.1046	1	23.1046	38.4189	0.0002
X_1^2	1.5461	1	1.5461	4.7270	0.0614	5.4992	1	5.4992	9.1442	0.0164
X_2	5.7194	1	5.7194	17.4864	0.0030	26.0316	1	26.0316	43.2861	0.0001

*X_1 – linear effect of PS concentration; X_2 – linear effect of illumination time; X_1^2 – quadratic effect of PS concentration; X_2^2.

Fig. S1. The homemade prototype of 530 ± 40 nm LED light system.



antibiotics

MDPI

Article

The Remarkable Effect of Potassium Iodide in Eosin and Rose Bengal Photodynamic Action against *Salmonella* Typhimurium and *Staphylococcus aureus*

Adriele R. Santos [1]*, Andréia F. P. Batista [1], Ana T. P. C. Gomes [2], Maria da Graça P. M. S. Neves [3], Maria Amparo F. Faustino [3], Adelaide Almeida [2,*], Noboru Hioka [4] and Jane M. G. Mikcha [5]*

[1] Postgraduate Program in Food Science, State University of Maringá, 87020-900 Maringá, Brazil; andreia.farias04@hotmail.com
[2] Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal; ana.peixoto@ua.pt
[3] QOPNA& LAQV-REQUIMTE and Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal; gneves@ua.pt (M.G.P.M.S.N.); faustino@ua.pt (M.A.F.F.)
[4] Department of Chemistry, State University of Maringá, 87020-900 Maringá, Brazil; nhioka@uem.br
[5] Department of Clinical Analysis and Biomedicine, State University of Maringá, 87020-900 Maringá, Brazil
* Correspondence: adrielesantos@hotmail.com (A.R.S.); aalmeida@ua.pt (A.A.); jmgmikcha@uem.br (J.M.G.M.)

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Abstract: Antimicrobial photodynamic therapy (aPDT) has been shown as a promising technique to inactivate foodborne bacteria, without inducing the development of bacterial resistance. Knowing that addition of inorganic salts, such as potassium iodide (KI), can modulate the photodynamic action of the photosensitizer (PS), we report in this study the antimicrobial effect of eosin (EOS) and rose bengal (RB) combined with KI against *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus.* Additionally, the possible development of bacterial resistance after this combined aPDT protocol was evaluated. The combination of EOS or RB, at all tested concentrations, with KI at 100 mM, was able to efficiently inactivate *S.* Typhimurium and *S. aureus.* This combined approach allows a reduction in the PS concentration up to 1000 times, even against one of the most common foodborne pathogenics, *S.* Typhimurium, a gram-negative bacterium which is not so prone to inactivation with xanthene dyes when used alone. The photoinactivation of *S.* Typhimurium and *S. aureus* by both xanthenes with KI did not induce the development of resistance. The low price of the xanthene dyes, the non-toxic nature of KI, and the possibility of reducing the PS concentration show that this technology has potential to be easily transposed to the food industry.

Keywords: xanthene derivatives; photodynamic inactivation; inorganic salt; antimicrobial resistance; *Salmonella*

1. Introduction

The access to safe food is considered as an important requirement to guarantee the quality of human life in modern society [1,2]. In fact, outbreaks of foodborne diseases are one of the main causes of morbidity and mortality being considered as an international public health problem [3], causing significant social and economic impacts [4]. According to the World Health Organization (WHO), it is estimated that more than 600 million people get sick as the result of unsafe food consumption [5,6]. One of the emerging problems related with foodborne bacteria is the increase of antibiotic resistance. It is known that changes in the patterns of food consumption (the preference for fresh and minimally processed foods), alterations in the globalization of the food market, and the emergence of multidrug resistant (MDR) bacteria have turned the control of foodborne diseases into a challenge [7,8]. According to the Centre for Disease Control and Prevention [9], about 400,000 people per year are affected by foodborne infections caused by MDR bacteria in the United States. Multidrug resistant *Salmonella* spp. and *Staphylococcus aureus* are a cause of concern since they have been isolated from meat, poultry, and dairy [10-14].

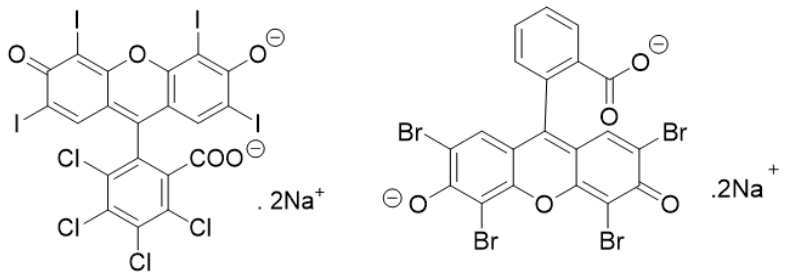
Nowadays, it is assumed that the development of novel antibiotics will not solve the MDR bacteria problem, since microorganisms may find new pathways of resistance to these new molecules. Therefore, efforts should be made towards the development of more efficient, non-toxic, and noninvasive antimicrobial methods to apply to the hosts. Importantly, these new methods should not induce the development of antimicrobial resistance [15-17]. Toward this end, antimicrobial photodynamic therapy (aPDT) has been considered as a promising non-antibiotic approach to inactivate foodborne bacteria [18-23].

aPDT involves the use of a photosensitizer (PS) that when excited by light reacts with molecular oxygen producing reactive oxygen species (ROS) such as singlet oxygen and/or hydroxyl radicals, superoxide, and hydrogen peroxide [15,23]. These ROS can react with biological molecules (e.g., proteins, lipids, and nucleic acids) causing microbial death [16,24,25]. This technique presents several advantages when compared with the use of traditional antimicrobials, showing to be efficient independently of the antimicrobial resistance profile and to prevent further development of resistance even after several cycles of treatment [15-17,26]. This approach has been efficient to inactivate several microorganisms, such as gram-negative and gram-positive bacteria [18,19,21], fungi [15,27-29], and viruses [15,30], and to degrade the matrix of microbial biofilms and kill the resident bacteria [16,31,32].

An ideal PS is a molecule that is present, in general, with a high quantum yield of singlet oxygen (Φ_Δ), low photo-bleaching yield, high affinity for the targeted site, and high stability [33,34]. Xanthene dyes have been considered good PSs to induce bacterial photoinactivation due to their low price, high molar absorptivity, and high singlet oxygen quantum yield (Φ_Δ) [18,33,35]. The xanthene dyes, rose bengal (RB) and eosin Y (EOS) (Figure 1), have already proven to be effective against gram-positive and gram-negative bacteria [19,20,31,36], however, these dyes showed to be more effective against gram-positive bacteria. This limitation can be overcome by the use of different organic salts such as sodium bromide, sodium azide, sodium thiocyanate, and potassium iodide (KI) [36-38]. Recently, some studies have demonstrated that the combinations of PSs and the inorganic salt KI improve the efficiency of aPDT [15,36,39-41]. Some xanthene dyes are approved for use in drug, cosmetic, and medical applications, and as food additives [19,31], while the safety of KI has been reported by a Food and Drug Administration (FDA) document [42].

aPDT certainly is a promising tool to inactivate food and food surfaces. However, to adopt and implement photoinactivation in the food industry, a variety of factors, both those related to aPDT and those related to the food matrix, need to be evaluated [3,43]. Most of the studies with aPDT in food matrices or food-related contamination have been done at laboratory scale, and have focused on fruits, vegetables, and poultry, or food contact surfaces [3,43]. Tao et al. [22] applied different concentrations of curcumin in fresh-cut Fuji apple inoculated with *Escherichia coli*. The fruits were illuminated with a 420 nm LEDs on both sides, at a 4 cm distance from the LED. The authors observed a reduction in *E. coli* population, as well in the activity of the enzymes

933 polyphenol oxidase and peroxidase. Aurum and Nguyen [44] achieved a 2 log inactivation of *E.*
934 *coli* on grapes treated with curcumin at 1.6 μM. The grapes were immersed in curcumin solutions
935 containing the inoculum for 60 min and the samples were afterwards irradiated with a blue LED
936 light (465–470 nm). Luksiene and Paskeviciute [45], using Na-Chl at 0.75 μM and a 405 nm LED,
937 tested the efficacy of PDI against *Listeria monocytogenes* Ly 56 cells attached on polyolefine. They
938 observed that the aPDI were able to eliminate a 4 log CFU/cm^2 of bacterial population. The results
939 of these studies showed that no negative effects were observed in the food matrices [22,44].
940 Therefore, the aim of this work was to investigate the antimicrobial photodynamic effect of
941 the xanthene dyes RB and EOS combined with the inorganic salt KI against *Salmonella*
942 Typhimurium and *Staphylococcus aureus.* Additionally, the aim was to evaluate the bacterial
943 resistance induced by the combination of RB/EOS, KI, and light irradiation.
944



Rose Bengal (RB) **Eosin Y (EOS)**

945

946 **Figure 1.** Chemical structures of rose bengal (RB) and eosin Y (EOS).

947

948 **2. Results and Discussion**

949 *2.1. Photostability Assay*

950 The absorption spectra of EOS (Figure 2A) and RB (Figure 2B) before and after being
951 irradiated for 15 min, under the conditions used in the photodynamic assays (vide infra) show a
952 slight decrease in the maximum absorption intensity (ca 6% and 2%). The decrease is dependent
953 on the irradiation time and these results are in agreement with the work of Rabello et al. [46],
954 where it was reported that EOS has a higher tendency to suffer photobleaching than RB. In future
955 research the absorption spectra of the combined use of xanthenos dyes with KI may be conducted
956 to better ascertain the use of these PSs in association with green LED light to control bacterial
957 contamination.

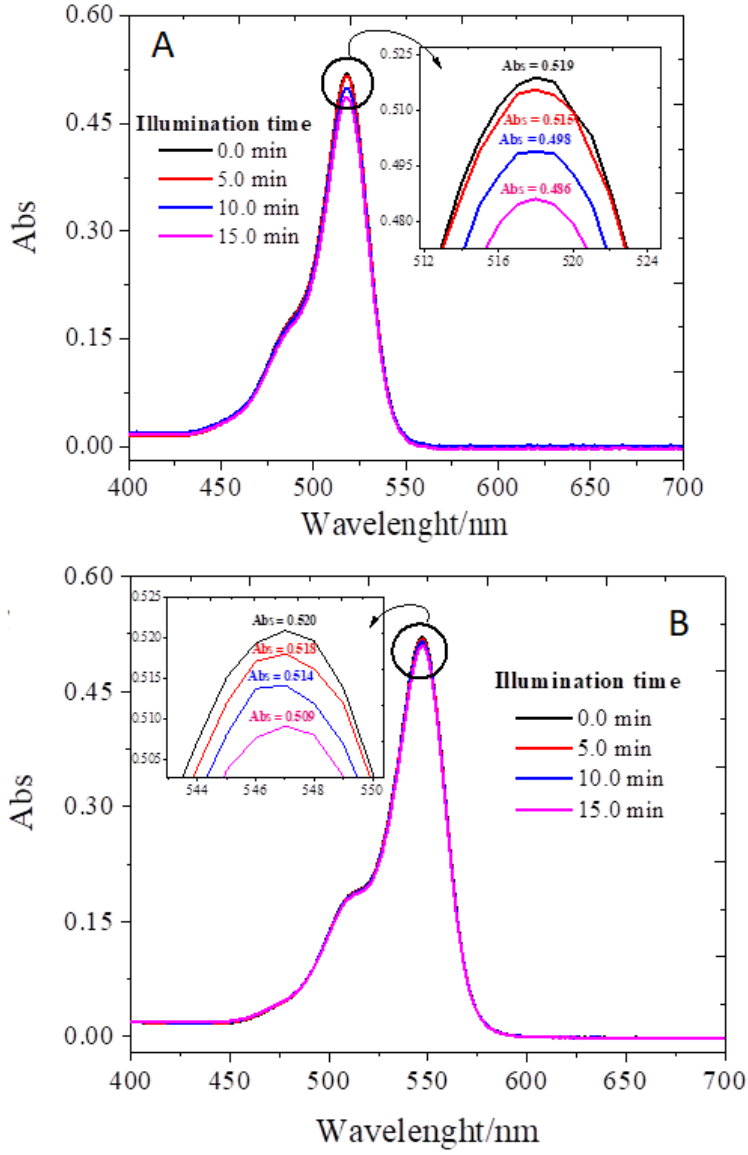


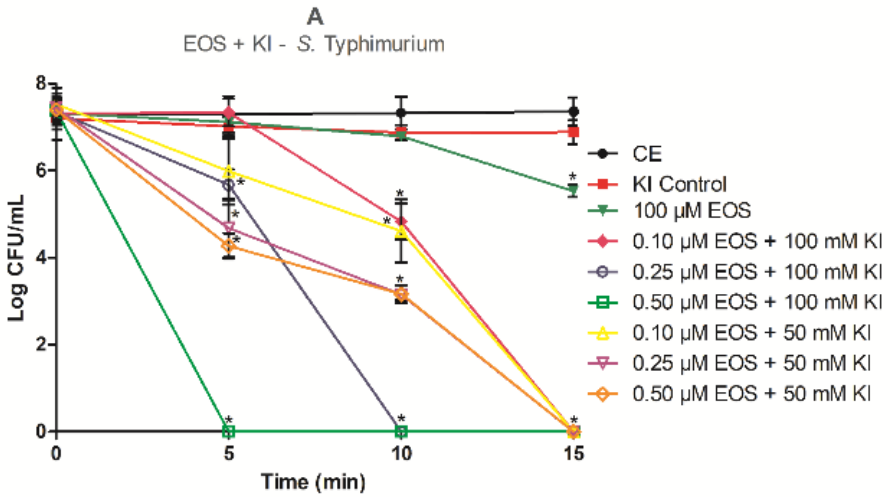
Figure 2. Photobleaching of EOS (**A**) and RB (**B**) without KI in PBS illuminated by a set of LEDs (10 mW/cm^2 and a wavelength of 530 ± 40 nm) for a period of 0, 5, 10, and 15 min.

2.2. Photodynamic Inactivation Assays

Although the xanthene derivatives dyes RB and EOS, in aqueous buffer solution, show high singlet oxygen quantum yield (Φ_Δ = 0.75 for RB, and Φ_Δ = 0.57 for EOS), which is enough to inactivate gram-positive bacteria, at neutral pH, they are dianionic protolytic molecules [33]. This is a limitation for the photoinactivation of gram-negative bacteria, once they are mostly impermeable to anionic or neutral charged dyes [41]. However, recently Hamblin described an efficient photoinactivation of *E. coli* in the presence of an anionic porphyrin combined with KI [37]. Having this mind, we have decided to study if the photodynamic effect of EOS and RB towards *S. aureus* and *S.* Typhimurium is potentiated by KI. The concentration of RB, EOS, and KI, as well as the irradiation times were chosen based on previous studies of our research group [15,19,31]. So, RB was tested at 10.0 nM alone and, at 5.0 nM, 7.5 nM, and 10 nM combined with KI, for *S. aureus* (a gram-positive bacterium) and at 50 μM alone and, 0.10 μM, 0.25 μM, and 0.50 μM with KI, for *S.* Typhimurium (a gram-negative bacterium). The concentrations of EOS for the assays in the absence of KI were 1.0 μM for *S. aureus* and 100 μM for *S.* Typhimurium. In the presence of KI, the EOS concentrations tested were 0.10 μM, 0.25 μM, and 0.50 μM for both *S.*

aureus and *S.* Typhimurium. In these assays the KI concentrations used were of 50 mM and 100 mM for both bacteria. The results obtained are presented in Figure 3. The dark control samples (PSs + KI in the dark (DC)) and the light control (bacteria strains only irradiated with LED (LC)) (data not shown) had no reductions on bacterial population compared with bacterial control group (bacterium strain only in PBS). The KI control also did not show differences in the *S. aureus* and *S.* Typhimurium cells reduction when compared with the control group ($p < 0.05$), as shown in Figure 3A–D.

The results obtained for the inactivation of *S.* Typhimurium mediated by EOS and RB alone show that these PSs have a limited efficacy in the photoinactivation of this gram-negative bacterium (Figure 3A, B). When EOS was used alone (Figure 3A), even at 100 μM and after an irradiation period of 15 min (light dose of 9.0 J/cm^2), the reduction in the survival of *S.* Typhimurium cells was only about 2 log ($p < 0.05$). Bonin et al. [19] has shown that EOS irradiated for 15 min with green light (530 ± 40 nm) promoted a slight reduction of about 1 log in *S.* Typhimurium survival using the EOS at 10 μM. These results show that increasing the concentration of EOS led to different photoinactivation profiles of *S.* Typhimurium cells. When RB alone was used (Figure 3B) it was possible to observe the total inactivation of *S.* Typhimurium cells with a concentration of 50 μM and 15 min of irradiation (9.0 J/cm^2). Silva et al. [31] also achieved the complete inactivation of *S.* Typhimurium cells with a small irradiation time (5 min.), but they used RB at 75 μM. So, even reaching the inactivation of *S.* Typhimurium cells until the detection limit of the method with RB, it was necessary for a high concentration of the PS, and this could be a barrier to its application in the food industry.



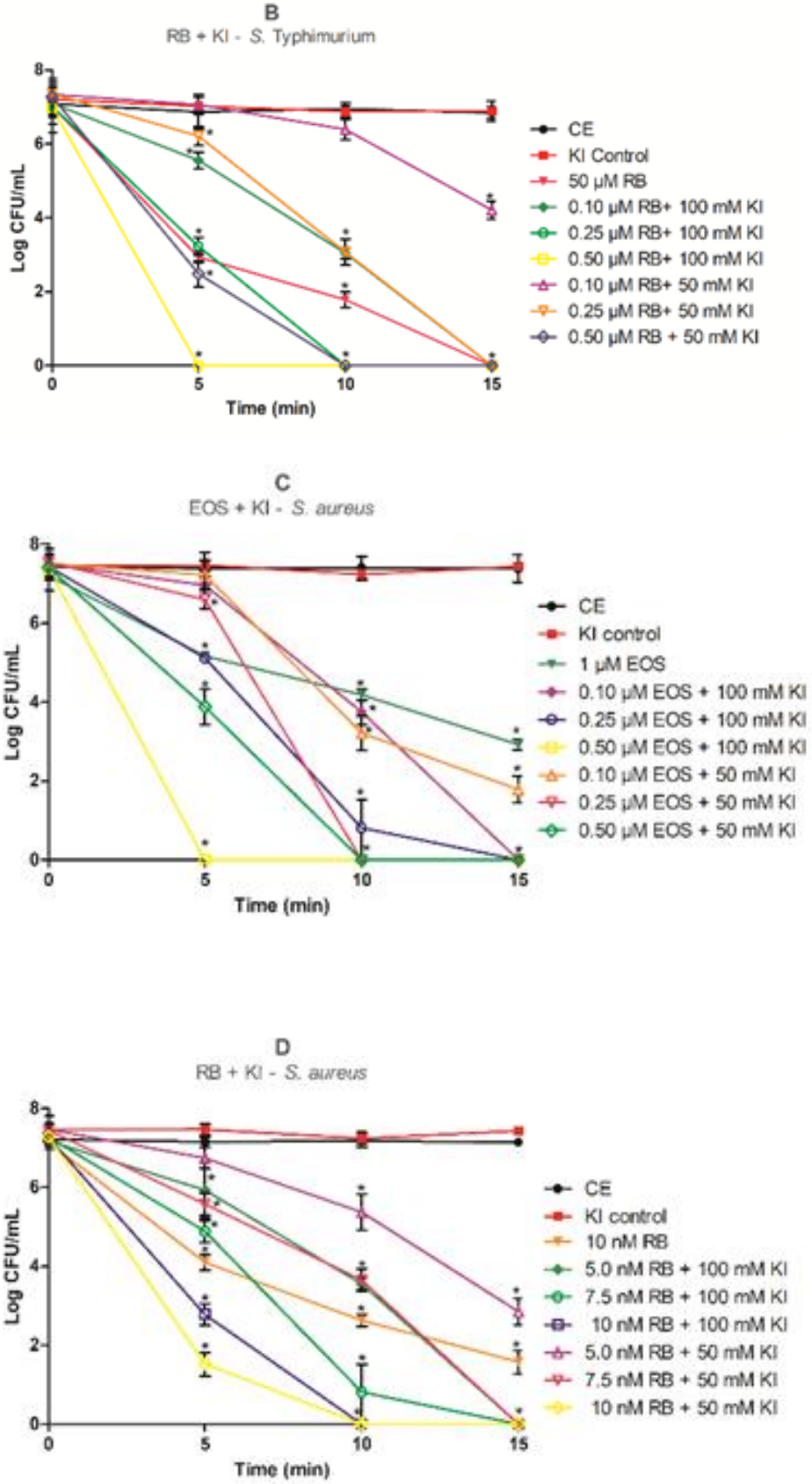


Figure 3. Effect of different times of irradiation and concentrations of EOS and RB combined with KI in the inactivation of *Salmonella* Typhimurium (**A**, **B**) and *Staphylococcus aureus* (**C**, **D**) cells. Samples were incubated in the dark for 10 min and then subjected to 5, 10, or 15 min of green (530 ± 40 nm) LED light exposure. The control group represents the cells in phosphate-buffered saline

(PBS). Data are presented as mean values and the error bars indicate the standard deviation. * $p < 0.05$. Lines just combine the experimental points.

The combined effect of EOS and RB with KI against *S.* Typhimurium (Figure 3A, B) show that this combination is effective in the photoinactivation of this gram-negative bacterium. For the combination 0.10 μM EOS with 100 mM KI total inactivation was observed after 15 min of irradiation (light dose of 9.0 J/cm^2), while for the combinations 0.25 μM EOS + 100 mM KI and 0.50 μM EOS + 100 mM KI the limit detection of the methodology was achieved after 10 min (6.0 J/cm^2) and 5 min (3.0 J/cm^2) of irradiation, respectively ($p < 0.05$). With the combination EOS + KI it was possible to completely inactivate the *S.* Typhimurium cells using a PS concentration 1000 times smaller. These data show that KI effectively potentiates EOS in aPDT against this gram-negative bacterium. For RB, our results also show an aPDT effect surprisingly high, promoting a reduction in the RB concentration up to 200 times against *S.* Typhimurium (Figure 3B). In this case it was possible to inactivate *S.* Typhimurium until the detection limit of the method for all combinations of RB with 100 mM KI as shown in Figure 3B ($p < 0.05$). With the concentration of RB 0.50 μM with 100 mM KI no culturable cells were recovered even after 5 min of light exposure (3.0 J/cm^2). In agreement with previous studies [38,39], it was also possible to observe that the photoinactivation rate increases with the increase of the PS or KI concentration or with the time of irradiation ($p < 0.05$; Figure 3). Our results are in accordance with Wen et al. [41] and Vieira et al. [36] that showed a great improvement in the action of the xanthene dye RB against gram-negative bacteria with the addiction of KI.

The use of EOS and RB alone in aPDT treatment against *S. aureus* cells proves to be more effective than for *S.* Typhimurium (Figure 3C, D). Nevertheless, it was not possible to achieve complete inactivation of the bacterium cells, even with the longest irradiation time (15 min; 9.0 J/cm^2). On the other hand, Bonin et al. [19] used 5.0 μM of EOS alone to achieve the total inhibition of the bacterium at 5 min of light exposure. While Silva et al. [20] reported that it was necessary to use 25 nM RB alone to achieve total inhibition of *S. aureus* cells with the same time of light exposure (5 min).

Additionally, the combination of EOS and RB with KI also achieved a good improvement in the photoinactivation action against *S. aureus* (Figure 3C, D). When experiments were performed with EOS at 0.10 μM and 0.25 μM with 100 mM KI a total inactivation was observed after 15 min (9.0 J/cm^2) of irradiation. When the concentration of EOS was increased for 0.50 μM no cultivable cells were recovered after 5 min (3.0 J/cm^2) of irradiation ($p < 0.05$; Figure 3C). Instead, for EOS alone at 1 μM and 5 min (3.0 J/cm^2) of irradiation, a reduction of about only 2 logs was achieved. In the photoinactivation mediated by RB with 100 mM KI, the total inactivation of *S. aureus* was observed for all PS studied concentrations ($p < 0.05$; Figure 3D). When it was used RB at 10 nM with 100 mM KI the total inactivation of the bacterium cells was achieved with 10 min (6.0 J/cm^2) of irradiation ($p < 0.05$). While when the RB was used alone, in the same concentration and time of irradiation, it was observed that it achieved a reduction of about 3 logs in the *S. aureus cells* ($p < 0.05$).

In our aPDT studies, namely when KI was used, the necessary PS concentration to inactivate the bacteria was very low, which probably would not affect the food. However, in a near future, further experiments, using food matrices, are needed in order to evaluate the potential of this combined aPDT approach in food industry.

It was possible to observe that the effect of KI was more pronounced when combined with EOS rather than RB, as expected. According to Huang et al. [47] when a PS already has a pronounced activity, such as RB, further improvements are more difficult to be achieved. But as EOS has a lower activity on its own, KI easily improved its photodynamic effect.

Some research groups that studied the use of PSs with KI in aPDT stated that, when a PS is used in combination with KI, it is necessary to have lower PS concentration or lower light exposure than when used in non-combined strategies [15,36,38-41,47-49]. The potentiated effect of RB by KI was studied in the photoinactivation of *E. coli* and *S. aureus* [36,41]. *E. coli* cells exposed

to RB with KI and a light dose of 10 J/cm² (540 ± 15 nm) were reduced in more than 6 logs, while when KI was omitted, less than 1 log of killing was found [41]. Vieira et al. [36] also demonstrated that RB alone showed no photoinactivation effect in *E. coli* cells, but that the addition of KI provided the reduction of the cells until the detection limit of the method was reached. Methicillin-resistant *Staphylococcus aureus* (MRSA) was reduced about 2 logs with 100 nM of RB alone plus light (20 J/cm²), but when KI (100 mM) was added eradication of cells at 20 J/cm² was observed [41]. Importantly, to our knowledge, there are no reports of the combined use of EOS with KI against *S. aureus* and *S.* Typhimurium.

Some studies have shown that KI also enhances the effect of other PS classes [36,38-40,47-49]. The potentiated effect of KI was observed for porphyrin-based PSs, Photofrin [40] and, for a formulation constituted by five cationic porphyrin derivatives [15], in the photoinactivation of gram-negative and gram-positive bacteria. The combined effect of MB and KI for the photoinactivation of *E. coli* and *S. aureus* was also shown [36,39]. These authors observed that the addition of KI increased the bacterial killing in 4 logs for *S. aureus* and 2 logs for *E. coli [39]*, as well as reduced the time of light exposure of 150 min to 30 min for *E. coli* inactivation [36]. So, when comparing our results with these results, we can say that our findings are in line with them.

All the aforementioned studies helped to elucidate how KI acts in the potentiation of aPDT. Huang et al. [40] proposed that, for porphyrins, the reaction mechanism occurred via singlet oxygen ($^1O_2$), once they observed an increase of the oxygen consumption when Photofrin was irradiated in the presence of KI, as well the generation of hydrogen peroxide. Rose bengal and EOS show high singlet oxygen quantum yield and they operate predominantly via the type II photochemical pathway, as well as porphyrins. So, for these dyes this extra killing effect of KI is caused by several parallel reactions that initiates with the reaction of $^1O_2$ with KI [15,36-38,40,41,49] (Figure 4). These reactions could produce free iodine (I_2/I_3^-) and hydrogen peroxide (H_2O_2), that are stable species, as well as the short-lived reactive iodine radicals ($I_2^{\bullet-}$). The stable species (I_2/I_3^- and H_2O_2) are mostly involved in the photokilling of gram-negative bacteria [37]. This could be explained because the thin cell wall of gram-negative bacteria allows iodine species to penetrate and kill them easier, in comparison with other microbial cells with thicker cell walls [41], while the short-lived radicals ($I_2^{\bullet-}$) were most involved in the photokilling of gram-positive bacteria [37].

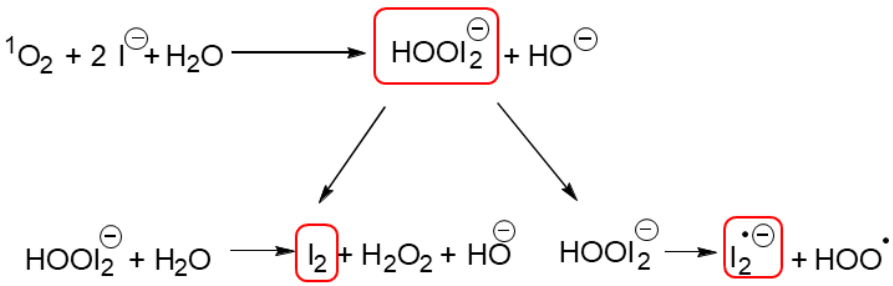


Figure 4. Schematic representation of the decomposition of peroxyiodide into free iodine (I_2/I_3^-) and hydrogen peroxide (H_2O_2) or iodine radicals ($I_2^{\bullet-}$) (elaborated according with the literature [24,37–40,43–45]).

Some authors suggest that free iodine must reach a threshold concentration to be microbicidal and that this amount of free iodine produced is directly related to the amount of singlet oxygen produced, as well as the concentration of iodide anion present in the solution [36,37]. It is believed that due to the very short lifetime of singlet oxygen, the probability of being quenched by iodide is higher when the iodide concentration is high, thus the iodide concentration is important in aPDT [40].

When we used KI at 50 mM, a half of the usual KI concentration, in combination with EOS or RB, a strong potentiate effect in the photoinactivation of *S.* Typhimurium and *S. aureus* is still observed, compared to the PSs alone [19,20] (Figure 3; $p < 0.05$). However, it was possible to observe that when EOS at 0.50 μM with KI at 50 mM was used, an additional time of light exposure of 10 min (9.0 J/cm^2) are needed, in comparison with the same EOS concentration with KI at 100 mM, to totally photoinactivate *S.* Typhimurium (Figure 3A). When the EOS concentration was reduced to 0.10 μM with KI at 50 mM it was not necessary an addition of light exposure to reach the total photoinactivation of *S.* Typhimurium cells, compared with KI at 100 mM. For the combination of RB at 0.25 μM and 0.50 μM with KI at 50 mM, it was observed that was necessary to use higher irradiation times to reach the total photokilling of *S.* Typhimurium cells (>7 log of reduction), compared with the KI at 100 mM (Figure 3B). The assays performed with the lowest RB concentration (0.10 μM) in the presence of KI at 50 mM showed a reduction of approximately 3 logs even after an irradiation time of 15 min (total light dose 9.0 J/cm^2). Wen et al. [41] eradicated *E. coli* cells using KI at 25 mM and a light dose of 10 J/cm^2, however, a RB concentration 100 times higher was used. These results suggest that if the concentration of KI is reduced, it is necessary to increase the PSs concentration or the light exposure time to achieve the same photoinactivation profile.

When EOS at 0.50 μM combined with KI at 50 mM was used against *S. aureus* it was possible to observe a similar profile, to photoinactivate *S.* Typhimurium; it was necessary to increase the time of light exposure to achieve total inhibition of the bacterium (Figure 3C). A reduction of approximately 6 log was achieved when the KI concentration was halved and it was used at the lowest EOS concentration (0.10 μM) with a light exposure of 15 min (9.0 J/cm^2). When we tested the highest RB concentration (10 nM) in combination with KI at 50 mM, total inactivation of *S. aureus* cells was observed after 10 min of light exposure (Figure 3D). This result was the same observed for RB at 10 nM but with KI at 100 mM. It is known that the xanthene derivatives weakly binds to most microorganisms. In this case, some authors suggest that concentrations up to 100 mM of KI are necessary to have an improvement in the PS action [38,40,41]. In our study we really achieved a great improvement in the PSs' action when KI was used at 100 mM, but for lower KI concentrations, high inactivation rates was also achieved.

2.3. aPDT Resistance Assays

Actually, multidrug resistant bacteria are one of the most serious health problems in world. It is known that some multidrug resistant foodborne pathogens have been found in food for human consumption [7,10-14]. In this sense, aPDT can be a promising alternative once it affects a high number of microbial targets simultaneously, thus preventing the development of bacterial resistance [16,36]. In order to evaluate the potential development of bacteria resistance to aPDT treatment mediated by RB or EOS with KI, ten cycles of photoinactivation under similar conditions to the ones applied for the photoinactivation profile determination were performed. Thus, concentration of PS + KI and the irradiation time used were chosen based on the reduction of ca. 50% in the CFU levels. After each cycle of aPDT, the *S.* Typhimurium or *S. aureus* colonies, that survived to the performed photoinactivation cycle, were aseptically removed from the TSA plates and re-suspended in PBS, and then submitted to the same photoinactivation protocol. The results obtained are presented in Figure 5.

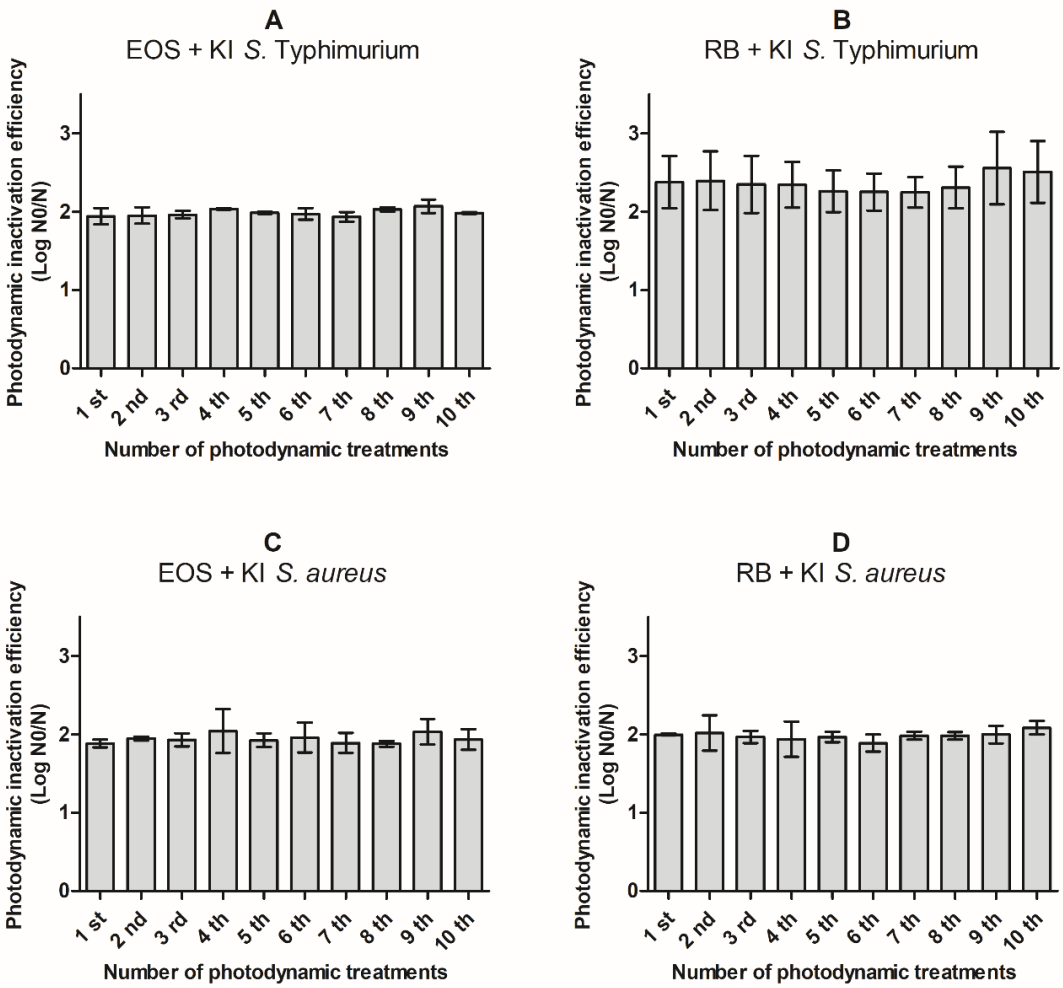


Figure 5. Photodynamic inactivation efficiency of ten consecutive cycles of *S.* Typhimurium (up), and *S. aureus* (down) by 0.10 μM of eosin (EOS) with 100 mM of KI (**A**, **C**), 0.10 μM of rose bengal (RB) with 100 mM of KI (**B**), and 5.0 nM of rose bengal (RB) with 100 mM of KI (**D**) after 10 min of irradiation with green LED light (530 ± 40 nm). N0 represents the plaque counts of bacterial cells before the irradiation; N represents the plaque counts after the cycle treatment; error bars indicate the standard deviation.

The results showed that there was no significant increase ($p < 0.05$) in resistance of *S.* Typhimurium to photosensitization after 10 consecutive sessions of 10 min with EOS or RB at 0.10 μM and KI at 100 mM (Figure 5 A, B). Similar behavior was observed for *S. aureus* cells, where no significant increase ($p < 0.05$) was observed in the resistance to photodynamic action after 10 aPDT cycles (10 min) with EOS at 0.10 μM or RB at 5 nM and KI at 100 mM (Figure 5 C, D). Lauro et al. [50] stated that the development of bacterial resistance could be detected by important reductions on the bacterial photoinactivation efficiency among experiments. These results clearly show the aPDT protocol with both EOS and RB with KI against *S.* Typhimurium and *S. aureus* does not induce development of resistance.

Some studies were conducted to determine if bacterial resistance occurs after several consecutive aPDT treatments [16,51]. Tavares et al. [51] also did not observe development of *E. coli* resistance by 10 cycles of 25 min of irradiation (white light 4.0 mW/cm^2) with 5.0 μM of Tri-Py$^+$-Me-PF. The same conclusions were reported by Bartolomeu et al. [16] working with three strains of *S. aureus* treated with Tetra-Py$^+$-Me at 5.0 μM and illuminated by white light (4.0 mW/cm^2) by 10 consecutive cycles of aPDT. However, to our knowledge there are no published

results that determine if bacterial resistance occurs when the PS is used in combination with KI after several consecutive aPDT treatments.

3. Materials and Methods

3.1. Bacterial Strains and Culture Conditions

Salmonella enterica serotype Typhimurium (ATCC 14028) and *Staphylococcus aureus* (ATCC 25923) stored at −20 °C in Brain and Heart Infusion Broth (BHI, Difco, Becton Dickinson, Sparks, MD, USA) with 20% glycerol, was used in this study. The bacteria were sub cultured in Hektoen Enteric Agar (Difco, Becton Dickinson, Sparks, MD, USA) for *S.* Typhimurium and Baird Parker Agar (Difco) for *S. aureus*, and prior to experiments, they were grown overnight at 37 °C in BHI (Difco, Becton Dickinson, Sparks, MD, USA). Then, the microorganisms were harvested by centrifugation (5000× *g* for 5 min) and washed three times with 0.85% saline solution. The inoculums were adjusted to approximately 1×10^7 colony-forming units (CFU) per mL and used in the experiments [20].

3.2. Photosensitizers and LED Light Source

A stock solution of RB and EOS (Sigma Aldrich, Darmstadt, Germany) at 1.0 mM was prepared in PBS pH 7.2, filter sterilized, standardized in a spectrophotometer (UV-Vis Beckman Coulter DU *800) and kept in the dark under refrigeration until use [19].

The green LED homemade device prototype has 252 LEDs appropriately arranged on a plate of 13 cm length × 8 cm width, with a distance from the microplate surface of 3.5 cm. The prototype has an irradiance of 10 mW/cm^2 and a wavelength of 530 ± 40 nm. The spectral emission of the LEDs system was obtained using a spectrofluorimeter (Varian Cary Eclipse, San Diego, CA, USA). The absolute irradiance of the LEDs was evaluated with a Spectroradiometer USB2000+RAD (Ocean Optics, Winter Park, FL, USA).

3.3. Photostability Assay

The photostability of EOS and RB was evaluated in PBS. The samples were continuously illuminated by a set of LEDs (10 mW/cm^2 and a wavelength of 530 ± 40 nm) for a period of 0, 5, 10 and 15 min and the LED system were adapted to the Varian Cary-60 spectrophotometer. This spectrophotometer works with phase-modulated radiation, allowing the experiment to be conducted without interference from external radiation. So, 2.0 mL of the aqueous solution containing the dyes were added in a quartz cuvette (1.0 cm optical pathway). The LED system was positioned at the top of the cuvette and the spectral reading was initiated using the kinetic method of the equipment. Finally, the spectral variations were properly evaluated [46].

3.4. Photodynamic Inactivation Assays

The photoinactivation assays were performed according to Silva et al. [20]. In a 24-well plate 500 μL of bacterial suspension with different concentrations of RB or EOS with KI were kept in the dark for 10 min to promote the PS + KI binding to bacterial cells before irradiation. Simultaneously, four control groups were also evaluated: positive control (C), containing only the bacterial inoculum in PBS without illumination; light control (LC), containing only the bacterial inoculum in PBS exposed to the same light conditions as the samples; KI control (KIC), containing the bacterial inoculum in PBS + KI exposed to the same light protocols and; dark control (DC) containing the inoculum and PS + KI without illumination. After incubation, the samples, LC and KIC were exposed to the green LED light for 5, 10 and 15 min.

Finally, samples from each well were serially diluted in 0.85% saline solution and plated in duplicate onto Tryptic Soy Agar (TSA, Difco, Becton Dickinson, Sparks, MD, USA). The plates

were incubated at 37 °C for 24 h and the CFU/mL was counted. Experiments were carried out in duplicate and repeated three times in independent experiments.

3.5. aPDT Resistance Assays

In order to verify the development of resistance to aPDT treatment with RB with KI and EOS with KI, ten cycles of photoinactivation under similar conditions were performed. The concentration of PS + KI and the irradiation time used were chosen based on the reduction of ca. ~50% in the CFU levels. After each cycle of aPDT, the *S.* Typhimurium or *S. aureus* colonies, that survived to the previous cycle of photoinactivation, were aseptically removed from the TSA plates and re-suspended in PBS, and then underwent the same photoinactivation protocol. The optical density of both bacteria suspension, before each assay, was measured to prevent differences in the aPDT efficiency. The aPDT efficiency was expressed as log N0/N, where N0 and N represent the colony counts before and after the irradiation, respectively. Three independent assays in duplicate were performed [16].

3.6. Statistical Analysis

Statistical analysis was performed by using one-way ANOVA and the Tukey multiple comparison test (GraphPad Prism 7.0). The level of statistical significance was set at $p < 0.05$. All experiments were carried out in duplicate and repeated at least three times in independent experiments.

4. Conclusions

The present study demonstrated that addition of KI at both concentrations tested (50 mM and 100 mM) can strongly potentiate the aPDT mediated by the xanthene derivatives EOS and RB. The use of KI allowed a drastic reduction of the PSs concentration (at least 500 times) and promoted the inactivation even of the gram-negative bacterium *S.* Typhimurium, a bacterium which is not so prone to inactivation with xanthene dyes when used alone. It was also confirmed that *S.* Typhimurium and *S. aureus* did not develop resistance mechanisms when submitted to consecutive cycles of aPDT protocol in the presence of EOS and RB with KI. Therefore, the effective inactivation of both bacteria without development of resistance, the low price of the xanthene dyes, the nontoxic nature of KI and the possibility of greatly reducing the EOS and RB concentrations allow the development of a very promising alternative to control foodborne pathogens, forecasting its ease of potential transposition to the food industry.

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