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Photodynamic inactivation of foodborne and food spoilage bacteria by curcumin

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BIOGRAFIA

Camila Benedetti Penha nasceu no dia 14 de Outubro de 1987 na cidade de Jandaia do Sul, estado do Paraná. Graduou-se em Engenharia de Alimentos

pela Universidade Estadual de Maringá, PR, no ano de 2009. Tem especialização em Tecnologia e Qualidade de Alimentos pela Universidade Estadual de Maringá. Tem experiência nas áreas de Garantia e Controle de Qualidade, com ênfase em sistemas de gestão, atuando principalmente nos seguintes temas: Boas Práticas de Fabricação, APPCC, 5S e Auditoria Interna.

Dedico

Aos meus pais, Maria Luzia Benedetti e Dirceu Luiz Penha

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“Eu acredito em milagres... Eu acredito
em um mundo melhor para mim e para você.”

Ramones

APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um artigo científico.

Camila Benedetti Penha, Edineia Bonin, Alex Fiori da Silva, Noboru Hioka, Tania Ueda Nakamura, Benício Alves de Abreu Filho, Paula Aline Zanetti Campanerut-Sá, Jane Martha Graton Mikcha. Photodynamic inactivation of foodborne and food spoilage bacteria by curcumin.

GENERAL ABSTRACT

INTRODUCTION. Photodynamic inactivation (PDI) is a new and promising strategy to eradicate microorganisms such as Gram positive and Gram negative bacteria, yeasts, molds, viruses and parasites. This technique is based on the use of photosensitizer's (PSs) activated by an appropriate wavelength light. Among naturally occurring PSs, curcumin is a yellow pigment isolated from *Curcuma longa* and has been used as a spice since ancient times. Among its many biological activities are its antioxidant, antimicrobial, anti-HIV, anti-inflammatory and anticancer properties. The use of curcumin-mediated photosensitization has been reported against a range of bacteria and fungi.

AIMS. Evaluate antimicrobial photodynamic activity *in vitro* against pathogenic and spoilage bacteria using curcumin as a photosensitizer.

MATERIALS AND METHODS. Curcumin at 75 µM was used as a photosensitizer in the photodynamic inactivation experiments. The light source used was blue LED (λmax = 470 nm) and the light doses were calculated for the periods of 10 and 20 minutes of illumination. Standardized bacterial suspensions of Gram-positive bacterium *Staphylococcus aureus* ATCC 25923 and the Gram-negative bacteria *Aeromonas hydrophila* ATCC 7966; *Escherichia coli* ATCC 25922; *Salmonella enterica* serotype Typhimurium ATCC 14028 and *Pseudomonas aeruginosa* ATCC 27853 were made in 0.85% sterile saline using a McFarland Scale 0.5 and diluted to approximately 10^7 CFU/ml for use in the experiments. Aliquots of 50 µl of standardized bacterial suspension were incubated with 950 µl of curcumin solution at 75 µM in the dark for 10min. After incubation, 500 µl of the sample was illuminated with a blue LED. Two light exposure periods, 10 and 20 minutes, were evaluated, and the control was evaluated without light exposure. Afterwards serial dilutions of the treated and control samples were inoculated in trypticase soy agar (TSA; Difco) plates and incubated at 37°C/24h. The counting of colonies was carried out and the results of cell viability were expressed as log CFU/ml. The morphological changes of *S. aureus* and *A. hydrophila* induced by PDI with curcumin at 75 µM and irradiation by blue LED light for 10 minutes were examined by scanning electron microscopy. Cytotoxicity of PDI mediated-curcumin was evaluated using VERO cells, in similar conditions to bacterial photoinactivation.

RESULTS AND DISCUSSION. Light doses obtained were 139 J/cm² for 10 minutes of illumination and 278J/cm² for 20 minutes. Curcumin at 75 µM in the absence of light activation did not reduce bacterial counts and the exposure of the bacteria to blue led light had no effect on its viability. PDI mediated by curcumin at 75 µM in *S. aureus* revealed significant differences in cell viability compared with the control group ($p < 0.05$). Exposure to LED blue light for 10 minutes displayed a reduction of approximately 3.27 log CFU/ml while after 20 minutes a reduction of approximately 3.57 log CFU/ml was observed. Among evaluated Gram negative bacteria, *A. hydrophila* displayed more sensibility to the treatment. A significant decrease ($p < 0.05$) in counts of 3.33log CFU/ml was observed after 10 minutes (139 J/cm^2) of exposure. Additionally, complete

photoinactivation was obtained after 20 minutes (278 J/cm^2) of exposure to light. Reductions of 1.29 (p > 0.05) and 2.65 (p < 0.05) log CFU/ml were obtained for *E. coli* in light doses of 139 and 278 J/cm^2, respectively and for *S*. Typhimurium the reductions were approximately 1.26 and 1.81 log CFU/ml (p> 0.05), for 10 and 20 minutes of exposure to blue LED light, respectively. The treatment against *P. aeruginosa* exerted limited antimicrobial effects, resulting in only 0.24 and 0.3 log CFU/ml reductions with 10 and 20 minutes of exposure, respectively. Gram negative bacteria are significantly more resistant to PDI than Gram positive species, since present a complex outer membrane that work as a physical and functional barrier between the cells and the environment, while most Gram positive bacteria cells have a cell wall with a relatively high degree of porosity and permeability. The presence of S-layer in *A. hydrophila* could explain the sensibility and the best results to PDI by curcumin. The morphological changes of *S. aureus* induced by curcumin-mediated PDI showed a smooth cell surface when *S. aureus* was incubated with curcumin in the dark without LED exposure and with curcumin LED-irradiated for 10 minutes the cell membrane presented distortions and seemed shriveled and wrinkled. The morphological changes of the *A. hydrophila* induced by curcumin-mediated PDI showed the cell membrane with distortions and protrusion of small bubbles for the treatment at irradiation time of 10min while the control showed a smooth cell surface. The cytotoxicity of PDI mediated-curcumin in VERO cells showed a percentage of cell destruction of 13±0.05%, for both illumination times (10 and 20 minutes). Cytotoxicity assays are important for investigating the potential toxic effects of photodynamic therapy on the host cells and to avoid damage to basic cellular functions. An ideal PS should present no toxicity for the host cells and possess biological antimicrobial activity.

CONCLUSION. The PDI by curcumin was effective in reducing bacterial counts. *A. hydrophila* and *S. aureus* were the most susceptible and *P. aeruginosa* was the most resistant to PDI. Photodynamic inactivation could serve as a new and promising approach to controlling foodborne and food spoilage bacteria.

Keywords: photoinactivation, curcumin, LED, bacteria.

RESUMO GERAL

INTRODUÇÃO. Inativação fotodinâmica (PDI) é uma nova e promissora estratégia para erradicar microrganismos, tais como bactérias Gram positivas e Gram negativas, leveduras, bolores, vírus e parasitas. Esta técnica baseia-se na utilização de fotossensibilizadores (FS) ativados por uma luz de comprimento de onda apropriado. Entre os fotossensibilizadores naturais, a curcumina é um pigmento amarelo isolado a partir de *Curcuma longa* e tem sido utilizado como uma especiaria desde os tempos antigos. Entre as suas diversas atividades biológicas estão as suas propriedades antioxidante, antimicrobiana, anti-HIV, anti-inflamatória e anti-cancerígena. A curcumina absorve luz azul em uma faixa de espectro de absorção de 400-500 nm. Foi relatada a utilização de fotossensibilização mediada por curcumina contra uma gama de bactérias e fungos.

OBJETIVOS. Avaliar a atividade antimicrobiana *in vitro* da terapia fotodinâmica contra bactérias patogênicas e deteriorantes usando curcumina como um fotossensibilizador.

MATERIAL E MÉTODOS. Curcumina a 75 µM foi usada como fotossensibilizador nos experimentos de inativação fotodinâmica. A fonte de luz utilizada foi LED azul (λmáx = 470 nm) e as doses de luz foram calculadas para os períodos de 10 e 20 minutos de iluminação. A padronização das suspensões bacterianas da bactéria Gram-positiva *Staphylococcus aureus* ATCC 25923 e das bactérias Gram-negativas *Aeromonas hydrophila* ATCC 7966; *Escherichia coli* ATCC 25922; *Salmonella enterica* sorotipo Typhimurium ATCC 14028 e *Pseudomonas aeruginosa* ATCC 27853 foi realizada em solução salina estéril 0,85%, utilizando escala de McFarland 0,5 e estas foram diluídas a aproximadamente 10^7 UFC/ml para utilização nos experimentos. Alíquotas de 50 µl das suspensões bacterianas padronizadas foram incubadas com 950 µl de solução de curcumina a 75 µM no escuro durante 10 minutos. Após a incubação, 500 µl da amostra foram iluminados com um diodo emissor de luz azul. Dois períodos de exposição à luz, 10 e 20 minutos, foram avaliados, e o controle foi avaliado sem exposição à luz. Em seguida diluições em série das amostras tratadas e do controle foram semeadas em ágar tripticase de soja (TSA; Difco) e incubadas a 37°C/24h. A contagem das colônias foi realizada e os resultados de viabilidade celular foram expressos em log UFC/ml. As alterações morfológicas de *S. aureus* e *A. hydrophila* induzidas por PDI mediada por curcumina a 75 µM e irradiação por luz LED azul por 10 minutos foram examinadas por microscopia eletrônica de varredura. Citotoxicidade da PDI mediada por curcumina foi avaliada utilizando células VERO, em condições similares às da fotoinativação bacteriana.

RESULTADOS E DISCUSSÃO. As doses de luz obtidas foram 139 J/cm^2 durante 10 minutos e 278 J/cm^2 durante 20 minutos de iluminação. Curcumina a 75 µM na ausência de ativação de luz não reduziu as contagens bacterianas e a exposição da bactéria somente à luz LED azul não teve nenhum efeito sobre a sua viabilidade. PDI mediada por curcumina a 75 µM em *S. aureus* revelaram diferenças significantes na viabilidade celular em comparação com o grupo controle ($p < 0,05$). A exposição à luz LED azul

durante 10 minutos mostrou uma redução de aproximadamente 3,27 log UFC/ml enquanto que após 20 minutos, uma redução de aproximadamente 3,57 log UFC/ml foi observada. Entre as bactérias Gram negativas avaliadas, *A. hydrophila* mostrou maior sensibilidade ao tratamento. Uma redução significante ($p < 0,05$) de 3.33 log UFC/ml nas contagens foi observada depois de 10 minutos (139 J/cm^2) de exposição. Além disso, fotoinativação completa foi obtida depois de 20 minutos (278 J/cm^2) de exposição à luz. Reduções de 1,29 ($p > 0,05$) e 2,65 ($p < 0,05$) log UFC/ml foram obtidos para *E. coli* em doses de luz de 139 e 278 J/cm^2, respectivamente, e para *S.* Typhimurium as reduções foram de aproximadamente 1,26 e 1,81 log UFC/ml ($p > 0,05$), para 10 e 20 minutos de exposição à luz LED azul, respectivamente. O tratamento contra *P. aeruginosa* exerceu efeitos antimicrobianos limitados, resultando em apenas 0,24 e 0,3 log UFC/ml de redução com 10 e 20 minutos de exposição, respectivamente. Bactérias Gram-negativas são significativamente mais resistentes à PDI do que espécies Gram-positivas, uma vez que apresentam uma membrana externa complexa que funciona como uma barreira física e funcional entre as células e o meio ambiente, enquanto que a maioria das células de bactérias Gram-positivas tem uma parede celular com um grau relativamente elevado de porosidade e permeabilidade. A presença de uma camada S em *A. hydrophila* poderia explicar a sensibilidade e os melhores resultados para PDI por curcumina. As alterações morfológicas de *S. aureus* induzidas por PDI mediada por curcumina mostram uma superfície de células lisas quando *S. aureus* foi incubado com a curcumina no escuro, sem exposição ao LED, e com o tratamento com curcumina e irradiação por 10 minutos, a membrana celular apresentou distorções e enrugamento. As alterações morfológicas de *A. hydrophila* induzidas por PDI mediada por curcumina mostraram a membrana celular com distorções e saliência de pequenas bolhas para o tratamento no tempo de irradiação de 10 minutos, enquanto o controle mostrou uma superfície de células lisas. A citotoxicidade da PDI mediada por curcumina em células VERO mostrou uma porcentagem de destruição das células de 13 ± 0,05%, para ambos os tempos de iluminação (10 e 20 minutos). Os ensaios de citotoxicidade são importantes para investigar os potenciais efeitos tóxicos da terapia fotodinâmica no tratamento das células hospedeiras e para evitar danos para as funções celulares básicas. Um FS ideal deve apresentar nenhuma toxicidade para as células hospedeiras e possuir atividade antimicrobiana.

CONCLUSÕES. A inativação fotodinâmica mediada por curcumina foi eficaz na redução das contagens bacterianas. *A. hydrophila* e *S. aureus* foram mais susceptíveis e *P. aeruginosa* foi a bactéria mais resistente ao tratamento. Inativação fotodinâmica poderia servir como uma abordagem nova e promissora para controlar bactérias patogênicas de importância alimentar e deteriorantes de alimentos.

Palavras chaves: fotoinativação, curcumina, LED, bactéria.

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Photodynamic inactivation of foodborne and food spoilage bacteria by curcumin

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Abstract

The purpose of the present study was to evaluate the efficacy of photodynamic inactivation (PDI) of foodborne and food spoilage bacteria using curcumin and a blue light emitting diode (LED). Curcumin at 75 µM was used to photo-irradiate *Staphylococcus aureus* ATCC 25923, *Aeromonas hydrophila* ATCC 7966, *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 at light doses of 139 J/cm^2 and 278 J/cm^2. Curcumin-mediated PDI of *S. aureus* induced a significant reduction of approximately 3 log CFU/ml at both the light doses evaluated. Among Gram-negative bacteria, *P. aeruginosa* was the least susceptible to PDI, followed by *S*. Typhimurium, the counts of which were not significantly reduced. A significant reduction in *E. coli* counts was observed only at light dose of 278 J/cm^2. When photo-irradiated with curcumin at 278 J/cm^2, *A. hydrophila* was completely eradicated, while a significant decrease (3.33log CFU/ml) was observed in the bacterial counts at 139 J/cm^2. Scanning electron microscopy of *S. aureus* and *A. hydrophila* photo-irradiated showed morphological changes when compared to untreated samples. The cytotoxicity of curcumin LED-irradiated was evaluated in VERO cells and showed 13%±0.05 of cell destruction. Curcumin in combination with a blue LED light demonstrated be a potential candidate for PDI against foodborne and food spoilage bacteria.

Keywords: photoinactivation, curcumin, LED, bacteria.

1. Introduction

Microbial contamination of foods continues to be a major concern for public health, consumers, regulatory agencies and food industries throughout the world. Foodborne pathogens are responsible for numerous illnesses, which affect thousands of people, mostly children, pregnant women, babies, the elderly and people with vulnerable diseases, and can lead to death in many cases (WHO, 2015). The Center for Disease Control and Prevention (CDC) estimates that 48 million people become ill and 3.000 die due to foodborne diseases (CDC, 2015). Furthermore, spoilage bacteria cause food losses, with a significant economic, social and environmental impact (Lipinski et al., 2013).

Among the strategies used to ensure microbiological safety and food preservation are the use of chemical preservatives, thermal processes, such as pasteurization, other physical methods such as dehydration and irradiation, and new preservation treatments such as high hydrostatic pressure (Gonzalez & Barret, 2010). Photodynamic inactivation (PDI) is a new and promising strategy to eradicate microorganisms such as Gram positive and Gram negative bacteria, yeasts, molds, viruses and parasites (Alves et al., 2015). This technique is based on the use of photosensitizer's (PSs) activated by an appropriate wavelength light (Jiang et. al., 2014) that generates reactive oxygen species and leads to cell death (Alisson, Mota, Bagnato & Sibata, 2008). Several light sources such as lasers, LEDs and halogen lamps are currently used (Nagata et al., 2012). LEDs have been used as alternative light sources due to their low cost, wider emission bands, easy of use and greater flexibility in irradiation time (Costa et al., 2011).

The advantages of PDI are that no undesirable toxic chemicals are generated, the only energy required is the light source, there is a low probability of triggering the development of resistance in microorganisms and it can be potential applied in several areas: hospital, dental, industrial and environmental (Luksiene & Brovko, 2013; Alves et al., 2015).

Different PSs, including porphyrins, phthalocyanines, chlorophyllin and xanthene dyes have been tested against microorganisms. These are fundamentally defined as agents that produce singlet oxygen following light stimulation (Alison et al., 2008). Among naturally occurring PSs, curcumin is a yellow pigment isolated from *Curcuma longa*, and has been used as a spice since ancient times (Aggarwal et al, 2006). Among its many biological activities are its antioxidant, antimicrobial, anti-HIV, anti-inflammatory and anticancer properties (Arutselvi et

al., 2012; Singh et al., 2010; Aggarwal et al., 2006). Curcumin absorbs blue light in an absorption spectrum range of 400-500nm, and it can be used as a potential natural photosensitizer (Araújo et al., 2012).

The use of curcumin-mediated photosensitization has been reported against a range of bacteria and fungi, such as *Staphylococcus aureus* (Jiang et al., 2014), *Staphylococcus epidermidis* (Hegge, Bruzell, Kristensen, & Tønnesen, 2012) *Enterococcus faecalis* (Haukvik, Bruzell, Kristensen, & Tønnesen, 2009; Frota et al., 2015), *Streptococcus mutans* (Manoil et al., 2014; Paschoal et al., 2013, Soria-Lozano et al., 2015), *Streptococcus intermedius* (Haukvik et al., 2009), *Lactobacillus* spp. (Bulit et al., 2014), *Escherichia coli* (Haukvik et al., 2009), *Candida* spp. (Dovigo et al., 2011; Andrade et. al., 2013; Soria-Lozano et al., 2015) and *Aspergillus flavus* (Temba et al., 2016). Thus, the aim of this study was to evaluate antimicrobial photodynamic activity *in vitro* against foodborne and food spoilage bacteria using curcumin as a photosensitizer.

2. Materials and methods

2.1. Photosensitizer

The curcumin to be used as a photosensitizer was purchased commercially from Sigma-Aldrich, USA. A stock solution was prepared in dimethyl sulfoxide (DMSO; Merck, USA) at 1 mM and stored in the dark at -20°C. The working solution was diluted with phosphate buffered saline (PBS, pH 7.4) to obtain a concentration of 75 µM for use in photodynamic therapy experiments.

2.2. Light source

The light source used was blue LED (λmax = 470 nm, LED potency = 1.2 W), and the emission spectra were measured by a Varian-Cary Eclipse spectrofluorometer. The light doses were calculated as described by Yassunaka et al. (2015):

$$D_{Abs} = \frac{t}{} . \quad PAb \quad \lambda$$

Where A is the irradiated area of 1.77 cm^2, t is the exposure time of 10 and 20 minutes and P_{Abs} was calculated according to the following equation:

$P_{Abs} = X_{Abs} P_{LED\ Emitted}$, where X_{Abs} is the absorbed light fraction by the PS, P_{Abs} is the absorbed potency by the PS and $P_{LED\ Emitted}$ is the LED potency.

2.3. Bacterial strains and culture conditions

The Gram positive bacterium *Staphylococcus aureus* ATCC 25923 and the Gram negative bacteria *Aeromonas hydrophila* ATCC 7966; *Escherichia coli* ATCC 25922; *Salmonella enterica* serotype Typhimurium ATCC 14028 and *Pseudomonas aeruginosa* ATCC 27853 were used in the experiments.

The bacterial strains were stored at -20°C in brain heart infusion broth (BHI; Difco) containing 20% (v/v) glycerol, in the Laboratory of Food Microbiology of the State University of Maringá, UEM.

2.4. Photoinactivation assay

Bacterial strains were cultured in BHI at 37°C for 24h and plated on agar plates specific for each bacterium. Enteric Hektoen Agar (Difco, USA) was used for *S.* Typhimurium, *A. hydrophila* and *P. aeruginosa*; Eosin Methylene Blue Agar (Difco, USA) was used for *E. coli*, and Baird Parker Agar (Difco, USA) was used for *S. aureus*. After incubation at 37°C, the colonies were transferred to 5 ml of BHI and incubated at 37°C overnight. The cultures were harvested by centrifugation at 4500 g for 4 min, washed three times and resuspended in 1 ml of 0.85% sterile saline. Standardized bacterial suspensions were made in 0.85% sterile saline using a McFarland Scale 0.5 and diluted to produce approximately 10^7 CFU/ml for use in the experiments.

Aliquots of 50 µl of standardized bacterial suspension were incubated with 950 µl of curcumin solution at 75 µM in the dark for 10 minutes. After incubation, 500 µl of the sample was illuminated with a blue LED. Two light exposure periods, 10 and 20 minutes, were evaluated, and the control was evaluated without light exposure. The time of dark incubation and irradiation times were selected based on the results of preliminary studies (results not shown). Afterwards serial dilutions of the treated and control samples were inoculated in trypticase soy agar (TSA; Difco, USA) plates and incubated at 37°C/24h. The counting of

143 colonies was carried out and the results of cell viability were expressed as log CFU/ml. The
144 experiment was performed in triplicate.
145
146 *2.5. Scanning Electron Microscopy*
147 The morphological changes induced by PDI with curcumin were examined by scanning
148 electron microscopy in bacteria that present the best results in PDI. The control was performed
149 with *S. aureus* and *A. hydrophila* incubated with curcumin at 75 µM for 10 minutes in the dark,
150 and the treatments were performed with curcumin at 75 µM and irradiation by blue LED light for
151 10 minutes (139 J/cm^2). Briefly, control and treated bacterial strains were centrifuged and the
152 pellet was washed in sterile saline 0.85% and fixed with 2.5% glutaraldehyde (Sigma-
153 Aldrich,USA) in 0.1 M cacodylate buffer (SEM, USA) for 48h at 4°C. After further washing in
154 0.1M cacodylate buffer, 30 µl of the samples was smeared on glass coverslips and dehydrated
155 using a graded concentrations of ethanol (50, 70, 80, 90, 95 and 100%) for 15min. Finally, the
156 samples were critical-point dried in CO_2 and coated with gold for SEM examination with a
157 Shimadzu SS-550 (Tokyo, Japan) scanning electron microscope.

158 *2.6. Cytotoxicity assay*
159 The cytotoxicity of curcumin LED-irradiated was evaluated using *VERO* cells (ATCC
160 CCL – 81). The cells were cultured in plastic culture flasks (TPP) containing Dulbecco's
161 Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (SFB;
162 Gibco, USA) and 50 µg/ml gentamicin at 37°C in a humid atmosphere (Fischer Scientific model
163 Isotemp) containing 5% CO_2. VERO cells at a cellular density of 2.5x10^4 cells/100µl/well were
164 inoculated into 96 well tissue culture plates and incubated for 24 hours to obtain a confluent
165 monolayer. The cells were then washed with PBS, exposed to curcumin at 75 µM and incubated
166 in the dark for 10 minutes. Afterward, the cells were LED irradiated for 10 and 20 minutes.
167 Then, the cells were washed with PBS, fixed with 10% trichloroacetic acid for one hour and
168 stained with 0.4% sulforhodamine B for 30 minutes. The plates were washed with 1% acetic
169 acid to remove excess dye and color revelation occurred through the addition of 10mM Tris
170 Base. The absorbance of each well was read at 530 nm using a microplate enzyme-linked
171 immunosorbent assay reader. Control cells received only irradiation blue light. The absorbance

of the treated wells was compared with the control cells and the percentage of cell destruction was calculated. The experiment was performed in triplicate.

2.7. Statistical analysis

The results were expressed as mean ± SD and analyzed using analysis of variance (ANOVA) and the Tukey test with GraphPad Prism 6.0. The statistical analysis was performed with a 5% level of significance.

3. Results and discussion

3.1. Light doses

Light doses were calculated using equations that consider both the power of the light emitted (P_{em}) and the power absorbed (P_{Abs}) by curcumin at 75 µM (Figure 1). The values obtained were 139 J/cm² for 10 minutes of illumination and 278 J/cm² for 20 minutes.

As this equation considers P_{Abs} and the Pem to calculate the light dose, unlike other studies that used only the P_{LED} issued, differences in dose values have been found by different authors (Haukvik et al., 2009; Ribeiro et al., 2013; Jiang et al., 2014).

3.2. Photodynamic inactivation

PDI efficacy depends on the species of the bacteria, the PS and the light source used. The present study showed that curcumin in combination with a blue LED light had an effect on Gram positive and Gram negative bacteria *in vitro*. After illumination different levels of photodynamic inactivation were observed depending on the dose of light (139 J/cm^2 for 10 minutes and 278 J/cm^2 for 20 minutes) and bacteria evaluated. Furthermore, curcumin at 75 µM in the absence of light activation did not reduce bacterial counts and the exposure of the bacteria to blue led light had no effect on its viability (data not shown).

PDI mediated by curcumin at 75 µM in *S. aureus* revealed significant differences in cell viability compared with the control group ($p < 0.05$), as shown in Figure 2. Illumination for 10 minutes (139 J/cm^2) displayed a reduction of approximately 3.27 log CFU/ml while after 20 minutes (278 J/cm^2) a reduction of approximately 3.57 log CFU/ml was observed. The illumination times not differ significantly and 10 minutes was sufficient to obtain an efficient

reduction in *S. aureus* counts. The viability of *S. aureus* by PDI was also evaluated by Jiang et al. (2014) using different concentrations of curcumin (0, 0.5, 1, 1.5, 2 and 2.5 μM) and irradiation by blue LED with a light dose of 3 J/cm^2, reporting a maximum reduction of 2 log CFU/ml at the highest PS concentration utilized. Ribeiro et al. (2013) investigated the photodynamic effect of curcumin on methicillin-resistant (MRSA) compared to methicillin-susceptible *S. aureus* (MSSA). For MSSA, curcumin at 5, 10 and 20μM in combination with three light fluences (18.0, 25.5 and 37.5 J/cm^2) resulted in complete elimination of the bacteria. The association of lower curcumin concentrations (0.1 and 0.5 μM) irradiated with any of the light fluences showed reductions of approximately 2 log CFU/ml while for a curcumin concentration of 1 μM the reductions were approximately 5 log CFU/ml. For MRSA, the log reductions achieved were 6.7 and 8.3 for curcumin concentrations of 5 and 10 μM, respectively, in combination with a light dose of 37.5 J/cm^2. Curcumin at 20 μM combined with this same light dose caused total inactivation of bacterial suspensions. These authors showed more effective results using lower concentrations of curcumin and smaller light doses than those used in the present study, however, we do not know how they calculated their light dose and irradiation time, making comparison of the results difficult.

PDI mediated by curcumin was also evaluated in Gram negative bacteria. While several studies have evaluated photodynamic therapy using curcumin as a photosensitizer in microorganisms of dental and medical importance (Paschoal et al., 2013; Pileggi et al., 2013; Bulit et al., 2014; Manoil et al., 2014; Frota et al., 2015), little research is available regarding microorganisms with significance for the food industry. To our knowledge, no studies have reported the effects of PDI using curcumin for *A. hydrophila*, *S.* Typhimurium and *P. aeruginosa* although a study by Haukvik et al. (2009) described PDI mediated by curcumin in *E. coli*.

In the present study, *A. hydrophila* displayed more sensibility to the treatment. A significant decrease ($p < 0.05$) in counts of 3.33 log CFU/ml was observed after 10 minutes (139 J/cm^2) of exposure. Additionally, complete photoinactivation was obtained after 20 minutes (278 J/cm^2) of exposure to light (Figure 2). Similar results were reported by Yassunaka et al. (2015) who observed a reduction of 4.3log CFU/ml using erythrosine and irradiation by a green LED source after 10min of illumination and complete eradication after 20min of exposure. The results of Kussovski et al. (2009) also showed complete inactivation of a multidrug-resistant strain of *A.*

hydrophila by photodynamic therapy using a diode laser light source and modified cationic Zn(II)-phthalocyanine as PS. Pereira et al. (2014) using 10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py+-Me) as a PS and white light for irradiation achieved a maximum reduction of 5.3 log CFU/ml for *A. hydrophila* counts after 270 minutes of exposure to light, while in the present study 20 minutes was enough to eradication of this bacteria.

Reductions of 1.29 ($p > 0.05$) and 2.65 ($p < 0.05$) log CFU/ml were obtained in the present study when *E. coli* was treated with curcumin at 75 µM and LED irradiated in light doses of 139 and 278 J/cm^2, respectively (Figure 2). The phototoxic effects of curcumin at 2.5 and 25 µM in DMSO preparation against *E. coli* were evaluated by Haukvik et al. (2009) and resulted in a reduction of 3 log, at a light dose of 30 J/cm^2 with the highest concentration of PS utilized.

The combined treatment of curcumin and blue light irradiation (139 and 278 J/cm^2) was also examined in the present study for *S*. Typhimurium and caused a reduction of approximately 1.26 and 1.81 log CFU/ml ($p > 0.05$), respectively. Yassunaka et al. (2015) obtained similar results (approximately 1.5 log CFU/ml), when using photosensitization mediated by erythrosine and a green LED source. Brovko et al. (2014) exposed *S.* Typhimurium to fluorescent light for 30 minutes using phloxine B as a PS and the bacteria was unaffected by the treatment. The antibacterial effects of photosensitization on *S.* Typhimurium were also evaluated by Luksiene et al. (2013) showing reductions of 2.2 and 6.6 log CFU/ml, when treated with chlorophyllin (CHL) and 5-aminolevulinic acid hydrochloride (ALA), respectively, and irradiated for 40 minutes. When a combined treatment of CHL and ALA was applied, *S.* Typhimurium was inactivated to an undetectable level.

PDI mediated by curcumin against *P. aeruginosa* exerted limited antimicrobial effects, resulting in only 0.24 and 0.3 log CFU/ml reductions with 10 (139 J/cm^2) and 20 minutes (278 J/cm^2) of exposure, respectively. The resistance of *P. aeruginosa* to photodynamic inactivation has also been demonstrated by other authors who used other PSs and light sources. Yassunaka et al. (2015) and Ke et al. (2012) did not obtain an effective reduction using erythrosine as a PS and a green LED light source, and nor did Ke et al. (2014) and Vassena et al. (2014) using phthalocyanines as a PS and a halogen lamp and diode laser as light sources, respectively. Satisfactory results were reported by Hsieh et al. (2014) who observed complete photoinactivation of *P. aeruginosa* with a red LED light source (162 J/cm^2) for 90 minutes using

5-Aminolevulinic acid (ALA) at 10 mM as a PS. Treatment with 5 mM of ALA at the same light dose and irradiation time reduced the viable count by approximately 6.5 log. These authors also report that the antibacterial effect was reduced when they used lower concentrations of PS and short irradiation times. Brovko et al. (2014) also found a total inactivation of *P. aeruginosa* using Phloxine B activated by a white light during 30 minutes.

In general Gram negative bacteria are significantly resistant to PDI, while Gram positive species are more susceptible, since the main targets of PDI are the external bacterial structures. Gram negative bacteria present a complex outer membrane with two lipid bilayers that work as a physical and functional barrier between the cells and the environment, while most Gram positive bacteria have a cell wall constituted by peptidoglycan layers with a relatively high degree of porosity and permeability (Nagata et al., 2012; Pereira et al., 2014). Pereira et al. (2014) demonstrated that the composition and organization of bacterial external structures influenced the efficiency of PDI by a cationic porphyrin.In the present study, the best results were observed for *A. hydrophila*. The presence of S-layer in *A. hydrophila* (Pereira et al., 2014), could explain the sensibility of this bacteria to PDI by curcumin.

3.3. Scanning Electron Microscopy

The morphological changes of *S. aureus* induced by curcumin-mediated PDI were examined by scanning electron microscopy. Figure 3a show a smooth cell surface when *S. aureus* was incubated with curcumin in the dark without LED exposure. With curcumin treatment at irradiation times of 10 min (139 J/cm^2) the cell presented distortions and seemed shriveled and wrinkled (Figure 3b). Under the same conditions, the morphological changes of the *A. hydrophila* induced by curcumin-mediated PDI showed the cell membrane with distortions and protrusion of small bubbles for the treatment at irradiation time of 10min (139 J/cm^2), as exposed in figure 3d, while figure 3c shows the control with a smooth cell surface.

Yassunaka et al. (2015) found similar morphological changes in *S. aureus* induced by PDI using erythrosine as PS and illumination by green LED light. The morphology and surface characteristics of *Streptococcus mitis* treated withZnPPc4+phthalocyanine and UV–visible light source were analyzed by scanning electron microscopy by Spesia and Durantini (2013), and

showed the protrusion of small bubbles of various shapes, similar to the found in the present study, after incubation with 2 µM of the PSs and irradiation for 2h.

3.4. Cytotoxicity

The cytotoxicity of curcumin at 75 µM irradiated by blue LED light for 10 and 20 minutes was evaluated in VERO cells obtained 13%±0.05 of cell destruction, for both illumination times. In the same way, curcumin combined with blue LED resulted in photoinactivation of bacterial cells.

Bulit et al. (2014) exposed bacterial cells and human cell lines to the same conditions and showed that the concentration required to reduce mitochondrial activity by 50%, for murine odontoblast-like cells (MDPC), undifferentiated dental pulp cells (OD 21) and Human embryonic stem cells was 60 µmol/L, 30 µmol/L and 35 µmol/L of light-activated curcumin for 4 minutes, respectively. The phototoxicity for L929 fibroblasts was evaluated with curcumin and irradiation with a light fluence of 37.5 J/cm^2 by Ribeiro et al. (2013) and the metabolic activity of these cells decreased by 80% when exposed to 20 µM curcumin in combination with a blue LED light.

Cytotoxicity assays are important for investigating the potential toxic effects of photodynamic therapy on the host cells and to avoid damage to basic cellular functions. An ideal PS should present no toxicity for the host cells and possess biological antimicrobial activity (Paschoal et. al., 2013). It is important to note that the use of curcumin as a food additive is controlled by regulations such as European Parliament Directive 94/36/EC (EFSA, 2010) and is approved by the Brazilian Health Surveillance Agency, Ministry of Health, Brazil (ANVISA) for use in different foods (ANVISA, 2011).

4. Conclusion

The present investigation demonstrated that PDI by curcumin was effective in reducing bacterial counts. *A. hydrophila* and *S. aureus* were the most susceptible and *P. aeruginosa* was the most resistant to PDI. Photodynamic inactivation could serve as a new and promising approach to controlling foodborne and food spoilage bacteria.

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466 same conditions of photodynamic inactivation (blue LED light source and curcumin as PS).
467 *Ribeiro et al. (2013). This study evaluated photodynamic inactivation using similar conditions

468 to ours (blue LED light source and curcumin as PS).

469 *Yassunaka et al. (2015). It was the first study of our group that evaluated the antimicrobial

470 effect of photodynamic inactivation in bacteria of food importance. This study was used for light

471 dose calculations and standardization of the PDI methods in our laboratory.

Figures

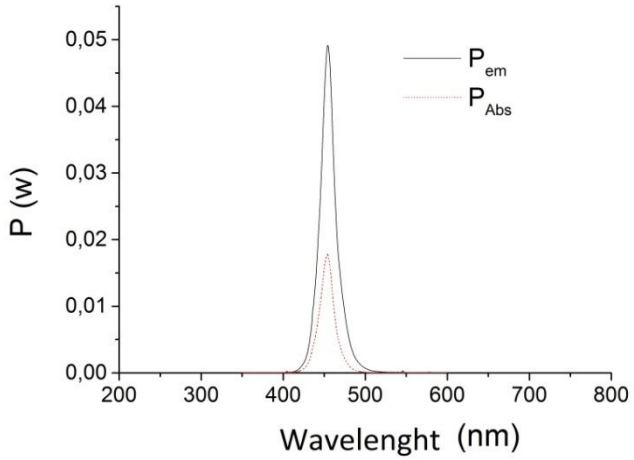

Figure 1. Light-emitting diode emitted potency (P_{LED} Emitted) and absorbed potency by curcumin (P_{Abs}) at 75 µM.

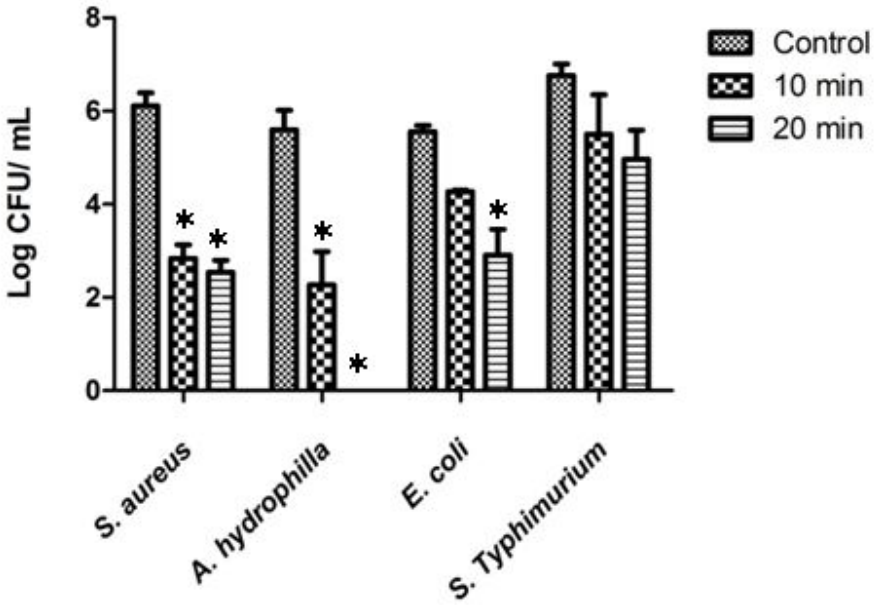

Figure 2. Photodynamic inactivation of Gram positive and Gram negative bacteria by curcumin at 75 µM and irradiation by blue LED light in times of 10 and 20 minutes. The controls were not exposure to the LED blue light. (*) Indicates significant difference between treatment and control sample.

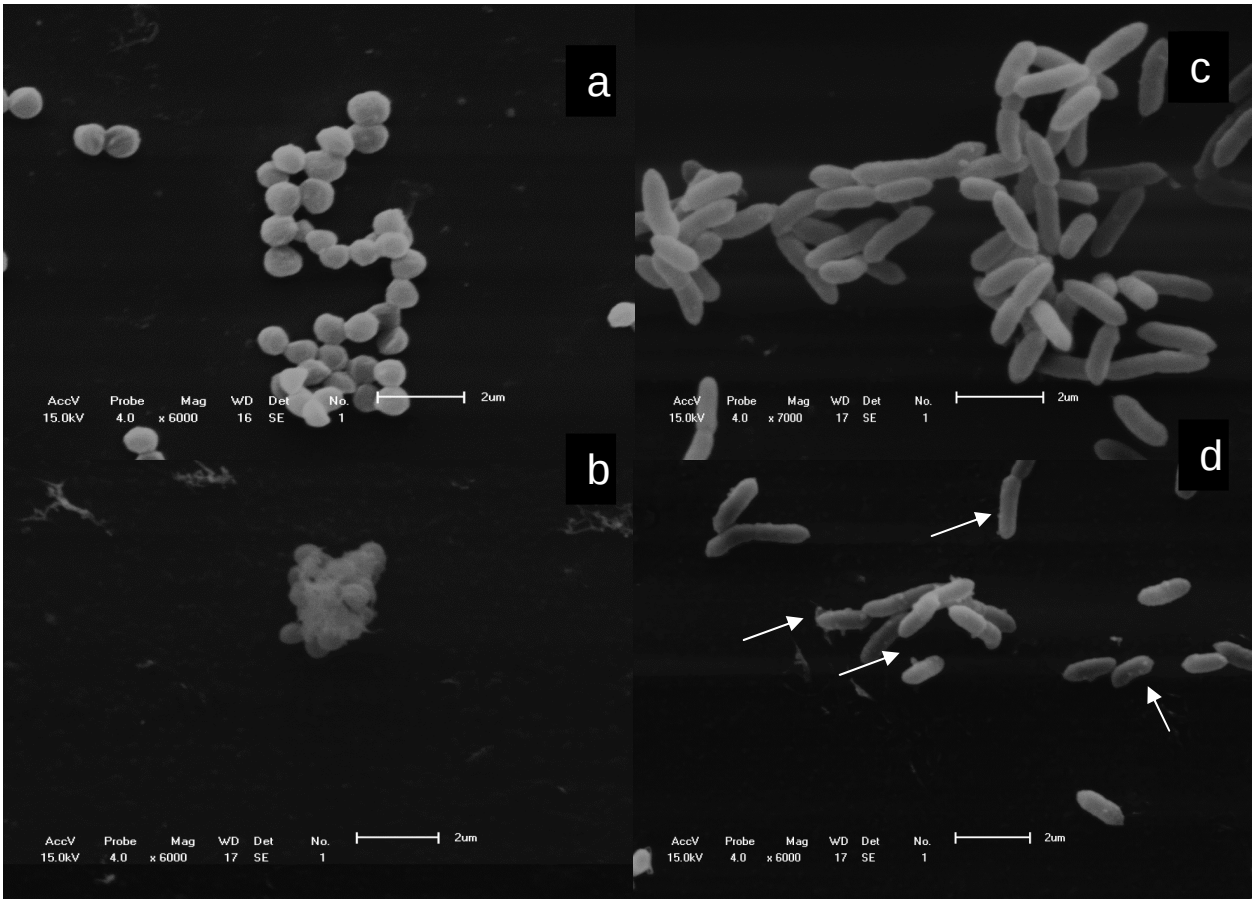

Figure 3. Scanning electron micrographs of cucurmin-mediated PDI of *S. aureus.* (a): *S. aureus* incubated with curcumin for 10min in the dark. (b): *S. aureus* treated by curcumin at 75 μM and irradiated with a light dose of 139 J/cm^2. (c): *A. hydrophila* incubated with curcumin for 10min in the dark. (d): *A. hydrophila* treated by curcumin at 75 μM and irradiated with a light dose of 139 J/cm^2. Arrows indicates the protrusion of small bubbles.