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Novel Approach to Effective and Uniform Inactivation of Gram-Positive *Listeria monocytogenes* and Gram-Negative *Salmonella enterica* by Photosensitization

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Summary

The aim of this work is to compare the antimicrobial efficiency of aminolevulinic acid-based photosensitization (ALA-photo) and chlorophyllin-based photosensitization (CHL-photo) against Gram-positive *Listeria monocytogenes* and Gram-negative *Salmonella enterica*, to assess some quantitative inactivation parameters of their survival curves and to propose a strategy for their effective inactivation. Cells were incubated with CHL ($7.5 \cdot 10^{-5}$–$7.5 \cdot 10^{-8}$ M) or ALA (7.5 mM) and then illuminated with visible light (λ=400 nm). The analysis of bacterial survival curves indicates that *Salmonella* is more resistant to CHL-photo than to ALA-photo. The shoulder of the inactivation curve was longer and the population reduction suddenness was smaller than that after ALA-photo. *Listeria* was more sensitive to CHL-photo than to ALA-photo. No shoulder of the survival curves of *Listeria* was detected after CHL-photo. Examination of bacterial survival curves enabled the determination of the optimal experimental set-up for combined ALA- and CHL-photo treatments. The obtained results indicate that both *Salmonella* and *Listeria* were inactivated to an undetectable level when combined treatment was applied. Thus, combined ALA- and CHL-photo treatment may be an effective tool to increase effective inactivation of both Gram(+) and Gram(–) food pathogens. Experimental results and the analysis of survival curves suggest innovative approach to combat Gram(+) and Gram(–) bacteria by photosensitization in effective and uniform way.

Key words: photosensitization, chlorophyllin, aminolevulinic acid, *Salmonella enterica*, *Listeria monocytogenes*

Introduction

Recent report from the World Health Organization has concluded that the incidence of foodborne diseases is a growing public health problem in both developed and developing countries (*1*). Contaminated food consumed in the United States causes an estimated 48 million illnesses, 128 000 hospitalizations, and 3000 deaths annually (*2*).

Human listeriosis infections are mostly (98 %) foodborne and account for about 2500 cases of illness and approx. $200 million in monetary loss in the US annually (*3*). *Salmonella* infection is one of the most common foodborne infections, resulting in an estimated $365 million in direct medical costs annually (*2*). In 2001, 1.4 million cases of salmonellosis in the USA (*4*) and 157 822 cases of this disease in the EU were estimated (*5*). One of the reasons of such statistics can be the increasing number of multidrug-resistant *S. enterica* isolates on a

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global scale: some strains are usually resistant to at least five antimicrobial agents including ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (*6*).

A new approach to inactivate pathogenic and harmful microorganisms in a cost-effective, non-thermal and environmentally friendly way is highly needed. From this point of view, photosensitization might serve as a promising tool to decontaminate some foods and food-related surfaces.

Photosensitization is a treatment involving the administration of a photoactive compound that selectively accumulates in the target cells which are then illuminated. The interaction of two non-toxic elements, photoactive compound (endogenous porphyrins produced in the cell from ALA or exogenous photosensitizers, for instance chlorophyllin) and visible light, in the presence of oxygen results in a plethora of cytotoxic reactions and consequently induces selective destruction of target microorganism (*7*,*8*). Some work has been done on the photoinactivation of food pathogens using chlorophyllin (*9–12*) and the precursor of endogenous photosensitizers, ALA (*13–16*). Data indicate that chlorophyllin as negatively charged photosensitizer is effective against Gram(+) bacteria spores and biofilms, but Gram(–) bacteria are less susceptible to this treatment. Resistance of these bacteria to photosensitization-based inactivation by negatively charged or neutral photosensitizers has challenged the search for other approaches in order to overcome this problem. On the contrary, Gram(–) bacteria are enough susceptible to ALA-based photosensitization (*13–16*).

The aim of this work is to compare the antimicrobial efficiency of ALA-based photosensitization and CHL-based photosensitization against Gram(+) and Gram(–) bacteria, to assess some quantitative inactivation parameters of their survival curves and propose an experimental set-up for the combination of these two treatments.

Materials and Methods

Chemicals

Stock solution of chlorophyllin (Roth, Karlsruhe, Germany) was prepared by dissolving in 0.9 % NaCl up to the concentration of $7.5{\cdot}10^{-5}$–$7.5{\cdot}10^{-8}$ M. Stock solution of 5-aminolevulinic acid hydrochloride (Fluka, Sigma-Aldrich, Rehovot, Israel) was prepared by dissolving ALA in 0.1 M phosphate-buffered saline (PBS, pH=7.2) up to the concentration of 0.2 M, and NaOH was used to adjust the pH of the solution to 7.2. ALA stock solutions were made instantly before use and sterilized by filtration through 0.20-μm filter (Roth). All used CHL and ALA concentrations were based on previous works (*9–16*).

Bacterial strains and culture conditions

Listeria monocytogenes $ATC_{L3}C$ 7644 was kindly provided by the National Veterinary Laboratory (Vilnius, Lithuania). *Salmonella enterica* serovar Typhimurium strain DS88 [SL5676 SmR (pLM32)], resistant to tetracycline, was kindly provided by Prof. D.H. Bamford (University of Helsinki, Helsinki, Finland).

The *Listeria* cultures were grown overnight (approx. 14 h) at 37 °C in 20 mL of tryptone soya medium supplemented with 0.6 % yeast extract (TSYE; Liofilchem s.r.l., Roseto degli Abruzzi, Italy), with agitation at 120 rpm (Environmental Shaker-Incubator ES-20, Biosan, Riga, Latvia). This culture was then diluted 20 times with the fresh medium ($A_{540\ nm}$=0.164) and grown at 37 °C to approx. $1.16{\cdot}10^9$ colony forming units (CFU) per mL, $A_{540\ nm}$= 0.9 in a shaker (120 rpm; Biosan). Bacterial absorbance was determined in a 1-cm glass cuvette at λ=540 nm (Heλios Gamma & Delta spectrophotometers, ThermoSpectronic, Cambridge, UK). Afterwards, the bacteria were harvested by centrifugation (20 min, 5000×*g*) and resuspended to the final concentration of approx. $5.8{\cdot}10^9$ CFU/mL in 0.1 M PBS (pH=7.2). This stock suspension was diluted with PBS to approx. 10^7 CFU/mL and immediately used for the photosensitization experiments.

The *Salmonella* culture was grown overnight (approx. 14 h) at 37 °C in 20 mL of Luria-Bertani medium (LB; Liofilchem), with aeration at 120 rpm (Environmental Shaker-Incubator ES-20, Biosan). The overnight bacterial culture grown in LB medium was diluted 20 times with the fresh LB medium ($A_{540\ nm}$=0.164) and grown at 37 °C to the mid-log phase (approx. $5{\cdot}10^8$ CFU/mL, $A_{540\ nm}$=1.3) in a shaker (120 rpm). Bacterial absorbance was determined in a 1-cm glass cuvette at λ=540 nm (Heλios Gamma & Delta spectrophotometer, ThermoSpectronic). Cells were then harvested by centrifugation (10 min, 5000×*g*) and resuspended in 1 mL of 0.1 M PBS (pH=7.2) to give approx. $2.5{\cdot}10^9$ CFU/mL. This stock suspension was diluted to approx. 10^7 CFU/mL and immediately used for the photosensitization experiments.

CHL-based photosensitization in vitro

A volume of 10 mL of the solution of bacterial cell suspension with appropriate concentrations of CHL ($7.5{\cdot}10^{-5}$–$7.5{\cdot}10^{-8}$ M) was incubated in the dark in 50-mL plastic tubes at 37 °C for 2 min. After incubation, 150-μL aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light for different periods (0–40 min). A light-emitting diode (LED)-based light source (constructed at the Institute of Applied Sciences of Vilnius University, Vilnius, Lithuania) emitted light of λ=400 nm with intensity of 20 mW/cm^2 on the surface of samples (distance 10 cm). Light dose was calculated as light intensity multiplied by time. Light power density measurements were performed with a light energy measured by 3sigma meter (Coherent, Santa Clara, CA, USA) equipped with a pyroelectric detector. No thermal effects were detected under these experimental conditions.

ALA-based photosensitization in vitro

Aliquots (10 mL) of bacterial suspension (approx. 10^7 CFU/mL in 0.1 M PBS buffer, pH=7.2) with 7.5 mM ALA were incubated in a 50-mL plastic bottle for cell culture cultivation in the dark at 37 °C. For the following experiments, the cells were incubated in the shaker (120 rpm; Environmental Shaker-Incubator ES-20, Biosan) for different periods of time. After incubation, 150-μL

aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light.

Bacterial cell survival assay

The antibacterial effects of photosensitization on *S. enterica* and *L. monocytogenes* were evaluated by the spread plate method. Thus, 100 μL of bacterial test culture after photosensitization were surface inoculated on the separate Luria-Bertani agar (LBA) plate. Afterwards, the bacteria were kept in the thermostat at 37 °C for 24 h. The surviving cell populations were enumerated and expressed as N/N_0, where N_0 is the number of CFU/mL in the untreated culture and N is the number of CFU/mL in the treated culture.

Statistical analysis

Complete experimental design was repeated 3–5 times for different batches. A standard error was estimated for every experimental point and marked in the figures as an error bar. Sometimes the bars were too small to be visible. The data were analyzed with Origin v. 8 software (OriginLab Corporation, Northampton, MA, USA) and the approximation parameters R^2_{adj} (adjusted coefficient of determination) and RMSE (root mean square error) were used.

Results

Inactivation of Salmonella by CHL-based and ALA-based photosensitization in vitro

The obtained results indicate that incubation of *Salmonella* with CHL ($7.5 \cdot 10^{-7}$ M) for 2 min had negligible effect on their viability. The illumination of bacteria with visible light (405 nm) due to photosensitization reduced their population. It was mentioned in our previous study that the variation of incubation time with CHL from 2 to 60 min had no impact on photoinactivation level for *Salmonella* (*11*). The reduction of the number of surviving cells after 10 min of treatment (light dose 7.2 J/cm^2) was negligible, 20 min of illumination time (14.4 J/cm^2) reduced the surviving bacterial fraction by approx. one log, 30 min (21.6 J/cm^2) by approx. 2.1 log, and 40 min (28.8 J/cm^2) by approx. 2.2 log. Very weak dependence of bacterial viability on illumination time longer than 30 min (21.6 J/cm^2) was observed. This means that *Salmonella* incubated with CHL and then illuminated with light is rather resistant to photosensitization. A nonlinear sigmoidal decrease of surviving *Salmonella* population was found.

ALA-photo-based inactivation of *Salmonella* depends strongly on the incubation and illumination time: 2 min of incubation and 10 min of illumination (7.2 J/cm^2) decreased the viability of bacteria by approx. 1.7 log, 2 min of incubation and 30 min of illumination (21.6 J/cm^2) by approx. 4.5 log, and 2 min of incubation and 40 min of illumination (28.8 J/cm^2) by approx. 6.6 log. It must be mentioned that the incubation time of bacteria with ALA included the illumination time. When cells were incubated with ALA for 60 min and then illuminated as described above, the surviving bacteria reached 3.3, 6.1 and 6.8 log.

Inactivation of Listeria by photosensitization in vitro

Incubation of *Listeria* with CHL ($7.5 \cdot 10^{-7}$ M) for 2 min had negligible effect on the survival. Bacterial population was drastically reduced after illumination with visible light (405 nm). The data indicate that CHL-photo-based inactivation curve of *Listeria* did not possess any shoulder and strongly decreased as the illumination time increased. After 1 min of illumination (0.7 J/cm^2), the number of surviving *Listeria* cell count decreased by 3.5 orders, after 2 min of illumination (1.4 J/cm^2) the cell count decreased by 4.7 orders of magnitude, and 5 min illumination (3.6 J/cm^2) killed pathogens by 7 log. This means that Gram(+) food pathogen *Listeria* is more susceptible to CHL-based photosensitization than Gram(–) *Salmonella*.

It is clear that the fraction of surviving bacteria decreased by approx. 0.5 log after 10 min of illumination and by approx. 1.7 log after 20 min of treatment with ALA-photo when 2-minute incubation time was used. More effective inactivation (approx. 3.8 log) of *Listeria* was obtained when 60-minute incubation time was used. Thus, it is obvious that *Listeria* is less susceptible to ALA-based photosensitization than *Salmonella*.

Mathematical analysis of bacterial survival curves after CHL-photo and ALA-photo treatment

Mathematical analysis of bacterial survival curves after photosensitization treatment was done using the following formula:

$$\log\frac{N(t)}{N_0} = N_r\left(1 - \frac{1}{1+(t/\tau)^p}\right) \qquad /1/$$

where N_0 and N are an initial number of cells and a number of cells after treatment, respectively, N_r is the number of resistant cells, t is irradiation time, parameters p and τ describe bacterial reparation activity at the beginning of illumination (shoulder on the curve) and reduction suddenness of the bacterial population, respectively. The relative number of resistant cells of both pathogens, N_{res}, was calculated as follows: $\log(N_{res}/N_0)$. This model was used for description of microbial inactivation by ALA-based photosensitization (*13*).

Eq. 1 describes a survival curve concave for $t>0$ if $p\leq 1$ and a sigmoidal function if $p>1$. In the second case the time coordinate of the inflexion point is:

$$t_{inf} = \tau\left(\frac{p-1}{p+1}\right)^{1/p} \qquad /2/$$

The maximal rate of bacterial population decrease is at the inflexion and equals:

$$r_m = -\frac{N_r}{4p\cdot\tau}(p-1)^{1-1/p}(p+1)^{1+1/p} \qquad /3/$$

The obtained value is called reduction suddenness. The shoulder of the inactivation curve is defined as a time point where the tangent to sigmoidal curve at the inflexion intersects with the upper asymptote and can be found as follows:

$$t_s = \tau\left(\frac{p-1}{p+1}\right)^{1+1/p} \qquad /4/$$

It follows from Eqs. 2 and 4 that:

$$t_s = \frac{p-1}{p+1}t_{inf} \qquad /5/$$

The shoulder is increasing function of τ and $p>1$.

For approximation of experimental data the Nonlinear Curve Fitter in Origin v. 8 was used. To evaluate the goodness of approximation, the adjusted coefficient of determination (R^2_{adj}) and root mean square error (RMSE) between the experimental data and those predicted by the mathematical model were applied. Inactivation of both bacteria can be adequately described by Eq. 1 with good characteristics of the approximation: $R^2_{adj}>0.98$ and RMSE<0.83, excluding the case of ALA-photo treatment of *Salmonella* after 60 min of incubation when RMSE=2.39. It is obvious that Eq. 1 successfully describes the different shapes of the observed survival curves (Figs. 1 and 2).

Thus, in order to describe the susceptibility of *Salmonella* to ALA-photo, the following values of key inactivation parameters were calculated: the number of resistant cells N_r=-7, the shoulder parameter of the inactivation curve t_s=5.59 (2 min of incubation) and 2.57 (60 min of incubation), parameter of the reduction suddenness of the bacterial population r_m=0.35 (2 min of incubation) and 0.51 (60 min of incubation). The results indicate that the inactivation of *Salmonella* by CHL-photo can be described by following parameters N_r=-2.2, t_s=14.42, r_m=0.19. This means that *Salmonella* after CHL-photo has about 3.2 times more resistant cells in comparison with ALA-photo. In addition, the shoulder parameters of the inactivation curve were about 2.6 and 5.6 times larger than those for *Salmonella* inactivated by ALA-photo. Moreover, the reduction suddenness of the bacterial population was about 1.8 and 2.7 times lower than that of *Salmonella* incubated for 2 and 60 min with ALA, respectively, and then illuminated. Prolongation of the incubation time with ALA from 2 to 60 min reduced the shoulder about 2.2 times but increased the reduction suddenness about 1.5 times. In particular, Fig. 1 shows that the shoulder of inactivation curve of *Salmonella* after CHL-photo is longer than that of ALA-photo.

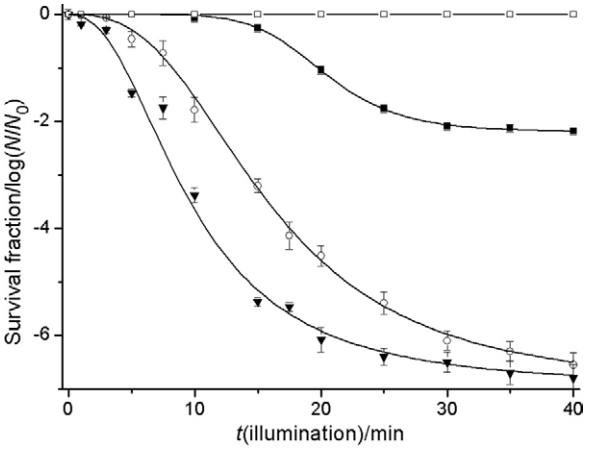

Fig. 1. Inactivation of *Salmonella enterica* with $7.5\cdot10^{-5}$ M CHL-based photosensitization (■) and with $7.5\cdot10^{-3}$ M ALA-based photosensitization: □ – control, ○ – 2 min of incubation with ALA, ▼ – 60 min of incubation with ALA. Solid line – fit of the model in Eq. 1

Comparative analysis of inactivation of *Listeria* after CHL-photo and ALA-photo indicates that this pathogen is more susceptible to the first treatment (Fig. 2). The fraction of surviving bacteria decreased by approx. 0.5 log after 10 min of illumination and by approx. 1.7 log after 20 min of treatment with ALA-photo (2 min of incubation time), whereas the survival of *Listeria* treated with CHL-photo decreased very sharply: 1, 2 and 4 min of illumination reduced the viability of bacteria by approx. 3.5, 4.7 and 6.5 log, respectively. The inactivation of *Listeria* with ALA-photo can be described using the following parameters: N_r=-7, t_s=6.22 and r_m=0.13 (2 min of incubation) and t_s=0.12 and r_m=0.31 (60 min of incubation). Reduction suddenness grew about 2.3 times when the incubation with ALA was prolonged from 2 to 60 min. For the inactivation of *Listeria* after CHL-photo the approximation procedure gave the following parameter values of Eq. 1: N_r=-11.62, p=0.86 and τ=3. Value $p>1$ indicates that the inactivation curve from Eq. 1 is concave for $t>0$ and does not possess inflexion point. Value N_r=-11.62 denotes a very high reduction rate of *Listeria* after CHL-photo.

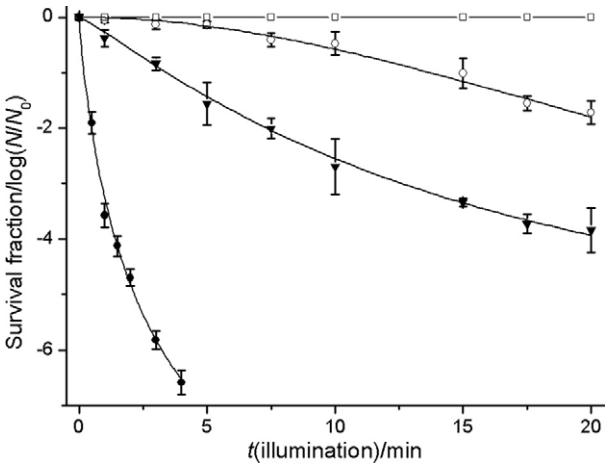

Fig. 2. Inactivation of *Listeria* with $7.5\cdot10^{-7}$ M CHL-based photosensitization (●) and with $7.5\cdot10^{-3}$ M ALA-based photosensitization: □ – control, ○ – 2 min of incubation with ALA, ▼ – 60 min of incubation with ALA. Solid line – fit of the model in Eq. 1

Inactivation of Salmonella and Listeria by combined CHL-photo and ALA-photo treatment

Data presented in Fig. 3 reveal that *Salmonella* population incubated for 2 min in $7.5\cdot10^{-3}$ M ALA and $7.5\cdot10^{-5}$ M CHL and then illuminated can be reduced from 7.4 log in the control to 0.7 log in the treated sample. Further prolongation of incubation time to 60 min reduced *Salmonella* counts to an undetectable level. *Listeria* incubated for 2 min with a cocktail of $7.5\cdot10^{-3}$ M ALA and $1.5\cdot10^{-7}$ M CHL and then illuminated was reduced to an undetectable level as well, without any need to prolong the incubation time to 60 min. Data presented in Fig. 3

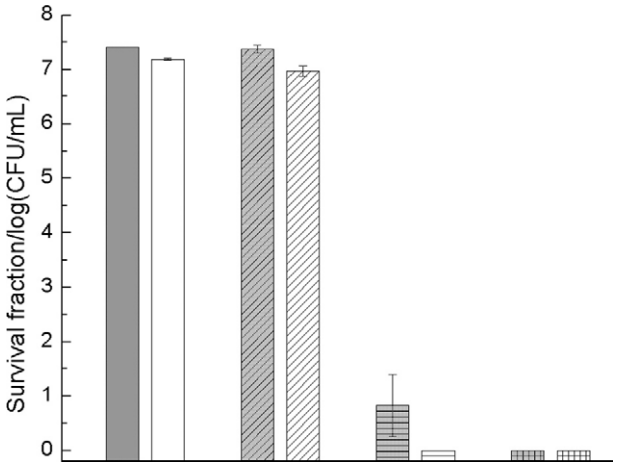

Fig. 3. Inactivation of *Salmonella enterica* and *Listeria monocytogenes* with CHL-based and ALA-based photosensitization. *S. enterica*: control; c(ALA)=7.5·10^{-3} M, c(CHL)=7.5·10^{-5} M: t(incubation)=2 min, t(incubation)=2 min+light, t(incubation)=60 min+light; *L. monocytogenes*: control; c(ALA)=7.5·10^{-3} M, c(CHL)=1.5·10^{-7} M: t(incubation)=2 min, t(incubation)=2 min+light, t(incubation)=60 min+light

reveal that combining CHL- and ALA-based photosensitization using optimized experimental set-up enabled to kill effectively and uniformly both Gram(+) and Gram(–) bacteria.

Discussion

We compared the antimicrobial efficiency of aminolevulinic acid-based photosensitization (ALA-photo) and chlorophyllin-based photosensitization (CHL-photo) against *L. monocytogenes* and *S. enterica* and assessed some quantitative inactivation parameters of their survival curves. Bacterial survival curves indicate that *Salmonella* is more resistant to CHL-photo than to ALA-photo, while *Listeria* was more sensitive to CHL-photo than to ALA-photo. Based on the experimental results and quantitative analysis of survival curves, we suggested an innovative approach to combat *L. monocytogenes* and *S. enterica* by photosensitization in an effective and uniform way.

Photosensitization-based inactivation of pathogenic and harmful microorganisms exhibits unique properties, since it is efficient, environmentally friendly and cost-effective. In our opinion, the main disadvantage of this treatment is high resistance of Gram(–) bacteria to photosensitization. One of the ways to increase the sensitivity of Gram(–) bacteria to photosensitization is the use of positively charged photosensitizers. Other authors used membrane-disintegrating agent polymyxin B nanopeptide in order to increase the penetration of negatively charged photosensitizer (*7*). Last but not least way to overcome this problem is the usage of endogenous photosensitizers produced in bacteria from exogenously applied 5-aminolevulinic acid (ALA) (*17*). *Listeria monocytogenes*, *Bacillus cereus* and *Salmonella enterica* spores and biofilms produce endogenous porphyrins in sufficient amounts and can be destructed by ALA-based photosensitization: *Bacillus* and *Salmonella* to an undetectable level, whereas *Listeria* just 4 orders of magnitude (*13–16*). Lower susceptibility of *Listeria* to ALA-photo can be explained by special resistance and recovery mechanism of these bacteria. These data are in line with the results described by Fotinos *et al*. (*17*), who found that both Gram(+) (*S. aureus*) and Gram(–) bacteria (*E. coli*, *P. aeruginosa*) are able to produce endogenous porphyrins, and that inactivation efficiency mostly depends on the concentration of produced endogenous porphyrins.

A lot of work has been done on bacterial inactivation of CHL-based photosensitization. Chlorophyllin is a water–soluble food additive (E140) known for its antimutagenic and anticarcinogenic properties (*18*), exhibiting high antioxidant capacity (*19*). Results obtained in the previous work of Luksiene and Paskeviciute (*10,11*) indicate that inactivation of Gram(+) *Listeria* or *Bacillus* on the surface of packaging material by CHL-based photosensitization is fairly effective and can significantly clean the surface from the attached pathogens, spores and biofilms. Preliminary data indicate that this treatment is less effective against Gram(–) bacteria.

Data obtained in the present study allow us to compare the susceptibility of Gram(+) and Gram(–) food pathogens to CHL- and ALA-photo. For instance, inactivation of Gram(–) food pathogen *Salmonella* by 2 log required 2 orders higher CHL concentration (7.5·10^{-5} M) and 10 times longer illumination time (Fig. 1) than inactivation of Gram(+) pathogen *Listeria*, which was diminished after photosensitization by 7 log *in vitro* at very low photosensitizer concentration (7.5·10^{-7} M) and short illumination time (4 min) (Fig. 2).

The question arises whether it is possible to find a common mathematical formula which after determination of its parameters could help to evaluate the susceptibility of Gram-positive and Gram-negative bacteria to both ALA- and CHL-based photosensitization and to use that formula for optimal combination of both treatments. However, the first models of microbial inactivation were developed on the assumption that bacterial inactivation follows a first order kinetics. Deviations from log-linear curves have been observed by numerous authors (*20–24*). Three kinds of deviation have been observed: sigmoid, concave and convex shapes. Since the shapes of sigmoid survival curves are similar to those of growth curves, some growth models such as the logistic, the Gompertz or the Baranyi were adapted to model bacterial inactivation (*23,25–27*). To model non-sigmoid survival curves, the Weibull model is often used, which can describe concave, convex and linear shapes. This model has been used in a number of studies to describe thermal (*28–30*) or nonthermal bacterial inactivation (*26,27,31*). In our previous work (*14*), Weibull model was used to describe ALA-based inactivation of Gram(+) bacterium *Bacillus cereus*:

$$\log\frac{N(t)}{N_0} = k \cdot t^q \qquad /6/$$

where t is illumination time, $N(t)$ is bacterial number at time t, N_0 is initial number of bacteria, and k and q are the scale and shape parameters, respectively.

Inactivation of *Listeria* for t<20 can be successfully described by the Weibull model. The difference between

survival curves of both models (Eqs. 1 and 6) is negligible. Similar are the approximation parameters: $R^2_{adj}>0.986$ and RMSE<0.83 for the model described by Eq. 1 and $R^2_{adj}>0.978$ and RMSE<0.79 for Weibull model. Time coordinate of the inflexion points calculated for fitted curves determined by Eqs. 1 and 2 is 19.27 (2 min of incubation time) and 1.66 (60 min of incubation). Inactivation curve of *Listeria* after ALA-photo did not possess inflexion point with positive time coordinate. It can be assumed (with small error) that the curves are convex or concave in the whole investigated time interval. Therefore, the Weibull model gives a good approximation of experimental data: convex curve with q=1.65 (ALA-photo, 2 min of incubation time), concave curves with q=0.72 (ALA-photo, 60 min of incubation time) and q=0.52 (CHL-photo).

Because of a small number of parameters, Weibull function in Eq. 6 cannot describe sigmoidal inactivation curves of *Salmonella*. Thus, in order to describe the inactivation of Gram(+) and Gram(–) bacteria by ALA- and CHL-photo, Eq. 1 was used. The parameters calculated from this formula allowed us to find experimental conditions at which effective (7 log) and uniform inactivation of both Gram(+) and Gram(–) bacteria can be achieved.

Thus, taking into account the above-mentioned quantitative evaluations of bacterial inactivation, we found specific experimental conditions and optimized inactivation of both *Salmonella* and *Listeria* by combined CHL- and ALA-photo treatment. Data presented in Fig. 3 reveal that combining chlorophyllin- and aminolevulinic acid-based photosensitization using optimized experimental set-up enabled to kill effectively and uniformly both Gram(+) and Gram(–) bacteria. For instance, inactivation of *S. enterica* after incubation with $7.5\cdot10^{-3}$ M ALA and $7.5\cdot10^{-5}$ M CHL for 2 min had no impact on the bacterial survival, but 2 min of incubation with $7.5\cdot10^{-3}$ M ALA and $7.5\cdot10^{-5}$ M CHL followed by illumination reduced the surviving population to less than one log. Prolongation of incubation time to 60 min diminished the survival of the population to an undetectable level. Inactivation of *L. monocytogenes* after incubation with $7.5\cdot10^{-3}$ M ALA and $1.5\cdot10^{-7}$ M CHL for 2 min as well as for 60 min followed by illumination reduced the survival of the population to an undetectable level as well. Of course, there is no advantage of the use of ALA-photo against *Listeria* and of CHL-photo against *Salmonella* separately.

Data obtained in this study indicate that *Salmonella* and *Listeria* naturally distributed on different surfaces can be eliminated by this combined treatment. Meanwhile, to answer the question whether all Gram(–) and Gram(+) bacteria can be destroyed by combining ALA-photo with CHL-photo additional studies must be done in the future.

Conclusions

The susceptibility of the investigated Gram(+) *Listeria monocytogenes* and Gram(–) *Salmonella enterica* food pathogens to ALA-based and CHL-based photosensitization is specific. *Salmonella enterica* was more resistant to CHL-based photosensitization than to ALA-based photosensitization, while *Listeria monocytogenes* was more susceptible to CHL-based photosensitization than to ALA-based treatment. Quantitative evaluation of bacterial inactivation curves allowed us to make hypothesis that cocktails of the two compounds (CHL and ALA) would be an effective tool to enhance photosensitization efficiency against naturally distributed Gram(+) and Gram(–) food pathogens and to inactivate them in a more uniform way. Experimental data confirmed that combining CHL- and ALA-based photosensitization enables the inactivation of the investigated Gram(+) *L. monocytogenes* and Gram(–) *S. enterica* bacteria by 7 log.

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