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ORIGINAL ARTICLE

Novel approach to control *Salmonella enterica* by modern biophotonic technology: photosensitization

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5-*aminolevulinic acid,* decontamination technology, pathogen control, photosensitization, *Salmonella*.

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Abstract

Aims: Salmonellosis is one of the most common foodborne diseases in the world. The aim of this study was to evaluate the antibacterial efficiency of 5-aminolevulinic acid (ALA) based photosensitization against one of food pathogens *Salmonella enterica*.
Methods and Results: *Salmonella enterica* was incubated with ALA (7·5 mmol l^{-1}) for 1–4 h and afterwards illuminated with visible light. The light source used for illumination of *S. enterica* emitted light λ = 400 nm with energy density 20 mW cm^{-2}. The illumination time varied from 0 to 20 min and subsequently a total energy dose reached 0–24 J cm^{-2}. The data obtained indicate that *S. enterica* is able to produce endogenous photosensitizer PpIX when incubated with ALA. Remarkable inactivation of micro-organisms can be achieved (6 log) after photosensitization. It is obvious that photosensitization-based inactivation of *S. enterica* depends on illumination as well as incubation with ALA time.
Conclusion: ALA-based photosensitization can be an effective tool against multi-drug resistant Gram-negative bacteria *S. enterica* serovar Typhimurium.
Significance and Impact of the Study: Experimental data and mathematical evaluations support the idea that ALA-based photosensitization can be a useful tool for the development of nonthermal food preservation technology in future.

Introduction

Recently, there has been an increased concern about the microbial safety of the world food. The contamination of food can occur anywhere along the way 'from farm to fork'. *Salmonella enterica* is one of the most important foodborne pathogens in many countries (Dooley and Roberts 2000). The Center for Disease Control and Prevention (CDC) estimates that 1·4 million cases of *salmonellosis occur annually in the USA* (Center for Disease Control and Prevention 2004). The European Union reported 157 822 cases of human salmonellosis in the year of 2001 (Mbata 2005). One of the reasons of such statistics can be the increasing number of multidrug resistant *S. enterica* isolates on a global scale: some strains are usually resistant to at least five antimicrobial agents ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (An expert report 2006).

The major existing technologies for food preservation have various shortages, for example, thermal effects, which usually induce different physical and chemical changes in the food. Many of potential food safety technologies are still at a research stage. Not one of them can provide 100% assurance of food safety. Thus, reduction of micro-organism survival by 1–2 log provides appropriate improvements in the microbiological control of the product. In this context, modern biophotonic technology based on photosensitization (photodynamic therapy in treatment of infectious diseases) might serve as a promising food decontamination tool (Luksiene 2005; Luksiene *et al.* 2005).

Photosensitization is the result of a combined effect of three nontoxic agents: photosensitizer, light and oxygen. Different micro-organisms die after their treatment with appropriate photosensitizer and visible light (Hamblin and Hasan 2004). Various studies demonstrated that

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Gram-negative bacteria were significantly more resistant to photosensitization than Gram-positive bacteria (Kennedy and Pottier 1992; Nitzan *et al.* 2004). The point is that due to the more complicated cell wall structure, most photosensitizers, being anionic aromatic molecules, cannot penetrate inside these micro-organisms. Thus, it seems reasonable to exploit cell metabolism for the production of endogenous photosensitizers using well-known precursor 5-aminolevulinic acid (ALA). ALA is a naturally occurring metabolite during haeme synthesis in eukariotic as well as prokaryotic cells, which induces the production of endogenous photosensitizer protoporphyrin IX (PpIX), uroporphyrin and coproporphyrin (Fig. 1) (Malik *et al.* 1992; Grönlund-Pakkanen *et al.* 1997). It is important to note that there is no experimental evidence on the methabolic pathway for the production of endogenous porphyrins in food pathogen *S. enterica* and no data about the possibility to inactivate this Gram-negative bacterium by ALA-based photosensitization.

The present work focuses on the evaluation of a new possibility to inactivate *S. enterica* by ALA-based photosensitization. For this purpose, production of endogenous porphyrins from exogenously applied ALA and inactivation of *S. enterica* by following illumination must be evaluated.

Materials and methods

Chemicals

Stock solution of ALA (Fluka, Jerusalem, Israel) was prepared by dissolving ALA in 0·1 mol l^{-1} PBS (pH 7·2) up to the concentration 0·2 mol l^{-1} and NaOH was used to adjust 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, Karlsruhe, Germany) (Luksiene *et al.* 1999).

Bacterial growth

The target bacteria, *Salmonella enterica* serovar Typhimurium strain DS88 [SL5676 SmR (pLM32)] resistant to tetracycline, were kindly provided by Prof. D. H. Bamford (University of Helsinki, Finland).

The bacterial culture was grown overnight (~14 h) at 37°C in 20 ml of Luria-Bertani medium (LB) (Liofilchem, Roseto degli Abruzzi, Italy), with aeration of 120 rev min^{-1} (Environmental Shaker–Incubator ES-20; Biosan, Riga, Latvia). The overnight bacterial culture grown in LB medium was 20 times diluted by the fresh LB medium (OD_{540} = 0·164) and grown at 37°C to the mid-log phase [5 × 10^8 colony forming units (CFU) ml^{-1},

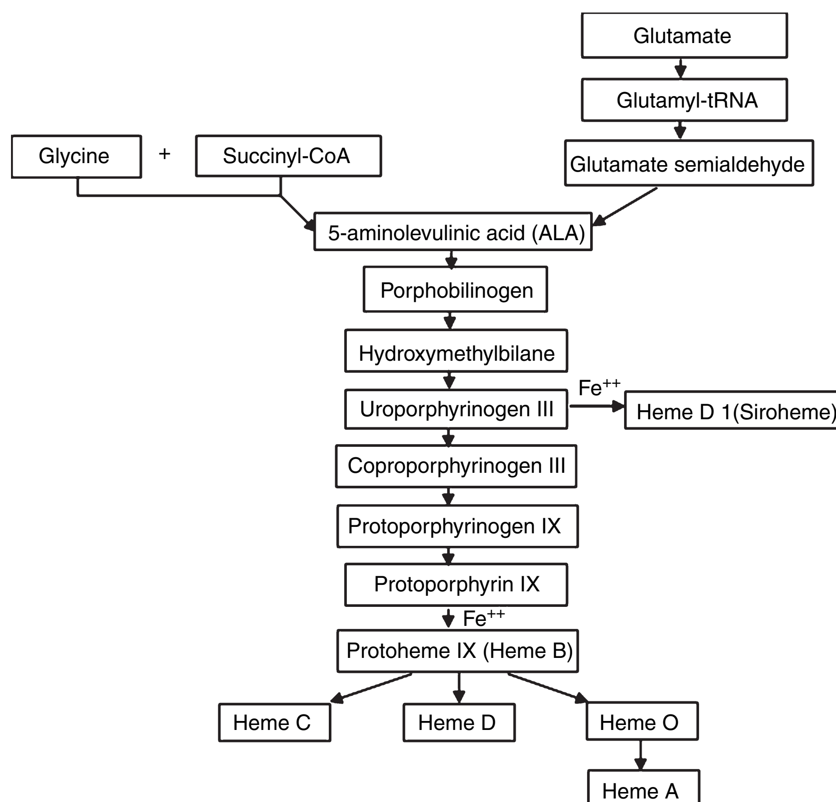

Figure 1 Bacterial biosynthetic pathway of haeme (adapted from Hamblin and Hasan 2004).

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OD_{540} = 1·3] in a shaker (120 rev min^{-1}). Bacterial optical density was determined in a 10·01 glass cuvette at $\lambda = 540$ nm (Heλios Gamma & Delta spectrophotometers; ThermoSpectronic, Waltham, MA, UK). Cells were then harvested by centrifugation (10 min, 5000 ***g***) and resuspended in a 1 ml of 0·1 mol l^{-1} PBS (pH 7·2), to give ~2·5 × 10^9 CFU ml^{-1}. This stock suspension was diluted accordingly to ~1 × 10^7 CFU ml^{-1} and immediately used for the photosensitization experiments.

Photosensitization

Aliquots (10 ml) of bacterial suspension [~1 × 10^7 CFU ml^{-1} in 0·1 mol l^{-1} PBS (pH 7·2)] with 7·5 mmol l^{-1} concentration of ALA were incubated in a plastic 50 ml bottle for cell culture cultivation in the dark at 37°C. For the following experiments, the cells were incubated in the shaker (120 rev min^{-1}) (Environmental Shaker–Incubator ES-20) for different periods (0–60 min). After corresponding incubation time, 150 μl aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light for different time (0–20 min) (Nitzan *et al.* 2004). LED based light source for illumination was constructed in the Institute of Applied Sciences of Vilnius university and emitted light $\lambda = 400$ nm with intensity 20 mW cm^{-2} at the surface of samples. Light dose was calculated as light intensity multiplied on irradiation time. Light power density measurements were performed with a light energy measure by 3 Sigma meter ('Coherent') equipped with piro-electrical detector J25LP04. No thermal effects were detected at the exploited experimental conditions.

Fluorescence measurements

The cell suspensions for measurements of endogenous porphyrins from ALA were prepared as follows. Cells [~1 × 10^7 CFU ml^{-1} in 0·1 mol l^{-1} PBS (pH 7·2)] were incubated in the dark at 37°C and 7·5 mmol l^{-1} ALA concentration for the indicated time. Then 2 ml aliquots of bacterial suspension were withdrawn and afterwards used for fluorescence measurements. Perkin-Elmer model LS-55 fluorescence spectrophotometer was used for the fluorescence detection. Scan range parameters are as follows:

1. Excitation wavelength – 390 nm
2. Emission – 590–750 nm
3. Excitation slit – 2·5 nm
4. Emission slit – 15 nm
5. Scan speed (nm min^{-1}) – 200.

Evaluation of endogenous porphyrins, produced by cells was performed according methodology described in our previous work (Luksiene *et al.* 2001). The fluorescence data were analysed with Origin 7·5 software (OriginLab Corporation, Northhampton, MA, USA).

Bacterial cell survival assay

The antibacterial effect of photosensitization on *S. enterica* was evaluated by the spread plate method. Thus, 100 μl of appropriate dilutions of bacterial test culture after photosensitization, exploiting the spread plate method, was surface inoculated on the separate Luria-Bertani Agar (LBA) plate. Afterwards the bacteria were kept in the thermostat for 24 h at 37°C. The surviving cell populations were enumerated and expressed as $N\ N_0^{-1}$ where N_0 is the number of CFU ml^{-1} in the untreated culture and N is the number of CFU ml^{-1} in the treated culture. Bacterial populations were transformed from CFU ml^{-1} into $\log_{10}$ ml^{-1}. The experiments were carried out in triplicate for each set of exposure. A standard error was estimated for every experimental point and marked in a figure as an error bar. Sometimes the bars were too small to be visible.

Results

Firstly, we tried to find out if *S. enterica* is able to produce endogenous porphyrins from ALA. To achieve it, ALA-induced metabolic pathway must take place in bacterial cells. Fluorescence spectroscopy of endogenous porphyrins in the cells usually is used to detect this process (Luksiene *et al.* 2001). For this purpose, the cells were incubated in the dark with ALA (7·5 mmol l^{-1}) at 37°C in 0·1 mol l^{-1} PBS at pH 7·2 for 0–240 min. The production of endogenous porphyrins was demonstrated by the fluorescence emission peaks in the region 580–700 nm (excitation, $\lambda = 390$ nm) (Fig. 2). It is necessary to note that the time of incubation of bacteria with ALA is an important factor for the production of endogenous porphyrins. A short incubation time with ALA (2 min) enables bacteria to just start porphyrins synthesis, usually estimated by peak at 610–630 nm, whereas 2 h incubation time can significantly increase relative production of endogenous porphyrins.

The photosensitization of *S. enterica* was performed as follows. Bacteria at an exponential growth phase were incubated with 7·5 mmol l^{-1} ALA for different times (0–60 min) in the dark. In the next step, bacteria were irradiated by light ($\lambda = 400$ nm, energy density 20 mW cm^{-2}). The inactivation efficiency was evaluated by the viability test, counting colony forming units following 24 h after treatment. No significant viability decrease was observed after the incubation of bacteria in the dark (data not shown). Just following illumination of cells with increasing light doses (0–24 J cm^{-2}) diminishes

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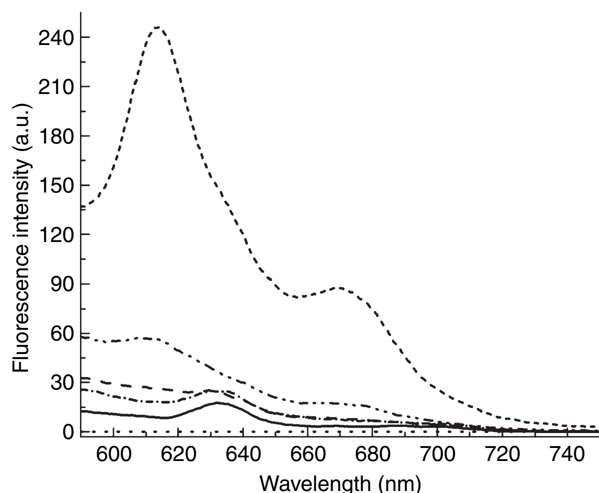

Figure 2 Fluorescence spectra of endogenous porphyrins produced by *Salmonella enterica* Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2) after incubation with 7·5 mmol l^{-1} ALA for different time interval: (····) control; (——) –2 min; (-·-·-) –15 min; (– – ·) –30 min; (–···–) –60 min; (- - - - -) –120 min.

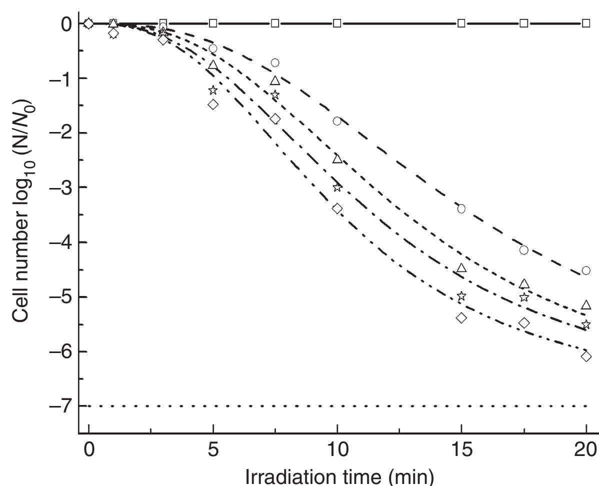

Figure 3 Inactivation of *Salmonella enterica* Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2) by 7·5 mmol l^{-1} ALA - based photosensitization as function of irradiation time, when different incubation with ALA time was used: (–□–) control; (- ○ -) –2 min; (· ·△· ·) –15 min; (-☆·) –30 min; (-◇·) –60 min. A - log value of relative cell number, when all bacteria are killed or resistance bacteria are survived.

bacterial viability in dose-dependent manner (Fig. 3). The obtained data clearly indicate, that inactivation of Salmonella depends strongly on incubation with ALA time. Another important factor in the determining inactivation of bacteria is irradiation time: 5 min irradiation decreases viability of cells by 0·5–1·5 log, whereas longer irradiation time (20 min) by 4·5–6 log. The increase of incubation time up to 1 h and the following irradiation up to 20 min diminishes the viability of *S. enterica* up to six orders of magnitude.

From data presented in Fig. 3, it is obvious that dependency of surviving bacteria on irradiation time (energy dose) exhibits nonlinear sigmoidal behaviour. We propose a mathematical equation describing microbial inactivation after ALA-based photosensitization. According to this model, the reduction of a relative number of the bacterial population N can be expressed by this equation:

$$\log \frac{N}{N_0} = A \cdot \left[1 - \frac{1}{1 + (t_R/\tau_R)^p} \right] \quad (1)$$

where N_0 and N are correspondingly an initial number of cells and a number of cells after treatment; A = parameter which describes the number of resistant cells; t_R = irradiation time; p = parameter which reflects the shoulder on the inactivation curve and describes bacterial reparation activity at the beginning of irradiation (shoulder on the curve, Fig. 3); τ_R = the time parameter which is specific to bacterial strain and describes reduction suddenness of the bacterial population which started to decrease with an increasing irradiation time (after shoulder in the Fig. 3).

The fitting of experimental data points by eqn (1) was depicted in Fig. 3 (curves).

Experimental data fit to the equation and confirmed that 40 min irradiation time is effective enough to destruct nearly all micro-organisms, which can form colonies (data not shown). After mentioned illumination time, the relative number of surviving cells can be expressed by the equation:

$$\log \frac{N}{N_0} = A; \quad (2)$$

and does not depend on the irradiation time any more. This extrapolation result is in line with experimentally obtained one and is of great importance in developing a novel antibacterial technology.

As a rule, the initial population of bacteria consists of the cells resistant to the treatment ($N_0 \cdot 10^A$), the cells which after the incubation time and following irradiation can repair damage and survive (N_{repair}), and the cells which are very sensitive to the treatment due to the absence/loss of repair systems (N_{sensit}). From the technological point of view the most important is the possibility to reduce the number of ($N_0 \cdot 10^A$) and N_{repair} bacteria. Equation (1) proposes us parameter τ_R which can be specific for every bacterial strain and describes the reduction of population ($N_{repair} + N_{sensit}$). The parameter τ_R depends on bacteria incubation time t_i in ALA. This dependence is presented in Fig. 4 and can be approximated by exponential decay.

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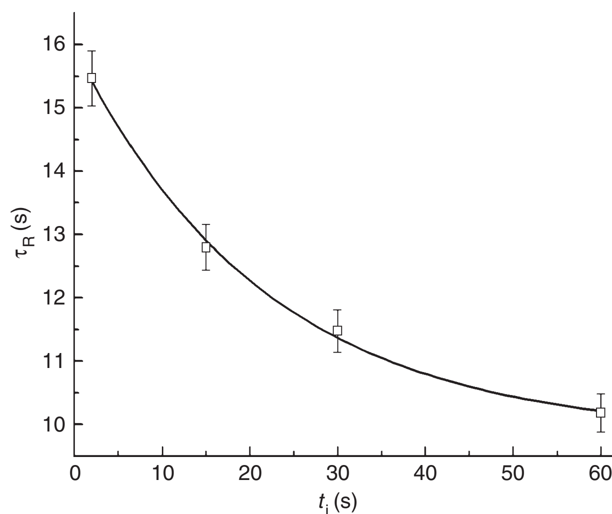

Figure 4 Time parameter τ_R dependency on incubation time approximates by first order exponential decay.

τ_R dependency on t_i allows us to evaluate the optimal incubation time for the best inactivation of (N_{repair} + N_{sensit}) the bacterial population. It is evident that there is no point to increase the incubation time more than $t_i \approx 80$ min because it will not change enough the specific bacterial inactivation parameter τ_R any more and subsequently will not change the efficiency of treatment.

Discussion

Despite the tremendous progress in microbiology, food-borne diseases continue to be one of important problems in the world. The development of modern, ecologically friendly and cost-effective antimicrobial technologies, which are integral to food production and manufacturing, is obvious. Benefits from these technologies are numerous, ranging from providing high quality and good physical condition crops to safe food products (An expert report 2006). In this context, modern light technologies, including high power pulsed light or photosensitization might serve as promising antibacterial tools (Gomez-Lopez *et al.* 2007; Luksiene *et al.* 2007).

Since the beginning of the 20th century it is well known that certain micro-organisms can be killed by the combination of dye and appropriate light (Luksiene 2005). It was found that a fundamental difference exists in the susceptibility to photosensitization between Gram-positive and Gram-negative bacteria. There is a good deal of evidence that the main targets which photosensitization can damage in the cell are DNA and the cytoplasmic membrane.

There are myriads of Gram-positive bacteria (Nitzan *et al.* 2004) and few microfungi (Luksiene *et al.* 2005) which might be destructed by the photosensitization-based technology. It is well known that neutral or anionic photosensitizers can efficiently accumulate in the cell and after irradiation inactivate Gram-positive bacteria. High susceptibility of these bacteria is based on the structure of their cytoplasmic membrane surrounded by a porous layer of peptidoglycan and lipoteichoic acid, allowing photosensitizer to cross (Nitzan *et al.* 1992). The envelope of Gram-negative bacteria consists of an inner cytoplasmic and outer membranes which are separated by the peptidoglycan-containing periplasm. The outer membrane forms a physical and functional barrier for different compounds. To overcome this problem, Nitzan *et al.* used polycationic peptide polymixin B nanopeptide (PMBN) to increase the permeability of the outer membrane of Gram-negative bacteria (Wilson 1993).

A second approach for inactivation of Gram-negative bacteria can be the usage of positively charged photosensitizers (Dahl *et al.* 1988). According to the literature, inactivation of *S. enterica* was performed using several photosensitizers (Table 1). Ozkanca *et al.* (2002) demonstrated photoinctivation of *S. typhimurium* LT2 by toluidine blue (TB) and light. A 1·5 log reduction in *Salmonella* cell viable count was observed after the 8 h treatment.

Peckaityte *et al.* (2005) used meso-tetra (3-hydroxyphenyl) chlorine (mTHPC), cationic tetrakis (*N*-ethylpyridinium-4-yl) porphyrin tetratosylate (TN-Et-PyP), zinc

Table 1 *Salmonella strains sensitive to photosensitization*

Salmonella strain	Photosensitizer	Author
S. Typhimurium	Rose Bengal	Dahl *et al.* (1988)
S. Typhimurium LT2	Toluidine blue	Ozkanca *et al.* (2002)
S. enterica serovar *Typhimurium* strains DS88 (SL5676 Smr pLM2); *S. enterica* serovar *Typhimurium* strains SL1102 (rfaEb)	Meso-tetra(3-hydroxyphenyl)chlorine; Cationic tetrakis (*N*-ethylpyridinium-4-yl) porphyrin tetratosylate; Zinc phthalocyanine tetrasulfonate; Aluminium phthalocyanine tetrasulfonate	Peckaityte *et al.* (2005)

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phthalocyanine tetrasulfonate (ZnPcS4) and aluminium phthalocyanine tetrasulfonate (AlPcS4) for inactivation of several Gram-negative bacteria. In this case, *S. enterica* was most sensitive to TN-Et-PyP: 95% of cells were killed and just 5% remained alive after 30 min irradiation.

By no means, the main premise trying to find a way for inactivation of Gram-negative food pathogens is photosensitizer. It must be pure compound with stable shelf-life, water soluble and easy to synthesize. Moreover, photosensitizer must be included in the list of food additives or represent food constituent. Taking this into consideration, the exploitation of bacterial metabolism to produce endogenous photosensitizer seems promising. It is well established that most bacteria use the haeme biosynthetic pathway to produce porphyrins from precursor 5-aminolevulinic acid. These porphyrins represent mixture of coproporphyrin, uroporphyrin and protoporphyrin (Hamblin and Hasan 2004) and after excitation with $\lambda = 400$ nm light can produce photocitotoxic effects in bacterial cells.

The data obtained in this study clearly indicate for the first time, that multi-drug resistant food pathogen *S. enterica* can be inactivated by nonchemical, environmentally friendly and effective technology: ALA-based photosensitization. It becomes obvious that *S. enterica* can produce endogenous porphyrins from ALA and the following irradiation inactivates this pathogen by six orders of magnitude.

Intrinsically, our previous data (Luksiene *et al.* 2005, 2006) as well as the ones obtained in this study, support the idea that important food pathogens localized on the surface of different food matrix or food processing equipment might be eliminated in the future by the photosensitization-based technology which pretends to be completely safe, reproducible, nonmutagenic, cost-effective and environmentally inert.

Conclusions

The present data clearly indicate for the first time that food pathogen *S. enterica*, resistant to many antimicrobials, in the presence of ALA can produce endogenous porphyrins which after irradiation with light can induce lethal effects in bacteria and decrease their viability by six orders of magnitude. Moreover, the experimental data, as well as mathematical evaluations clearly indicate that there is a new opportunity to inactivate *S. enterica* in a very efficient way leaving insignificant amount of cells.

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