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Photodynamic Inactivation of Food Pathogen *Listeria monocytogenes*

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Summary

The aim of this study is to examine the possibility to inactivate food pathogen *Listeria monocytogenes* by nonthermal antimicrobial treatment – photosensitization. *L. monocytogenes* was incubated with 5-aminolevulinic acid (ALA) (7.5 mM) for 0–2 h to produce endogenous photosensitizers and then illuminated with visible light. The LED-based light source used for the illumination of *L. monocytogenes* emitted light at λ=400 nm with energy density of 20 mW/cm^2. The illumination time varied from 0 to 20 min, and a total energy dose reached 0–24 J/cm^2. The obtained results reveal that *L. monocytogenes* can effectively produce endogenous porphyrins after incubation with 7.5 mM ALA. Subsequent illumination of cells with visible light significantly decreased their viability *in vitro* (4 log). After adhesion of *Listeria* to the surface of packaging material and following photosensitization, the surface-attached bacterial population was inactivated by 3.7 log. In addition, most resistant *Listeria* biofilms are susceptible to this treatment. Their inactivation reached 3.1 log under certain experimental conditions. The cells and biofilms of Gram-positive bacteria *L. monocytogenes* ATCL3C 7644 could be effectively inactivated by ALA-based photosensitization in the solution as well as adhered onto the surface of packaging material in a nonthermal way.

Key words: photosensitization, nonthermal inactivation of *L. monocytogenes*, biofilms

Introduction

Most people are routinely exposed to *Listeria* with no health consequences, although *L. monocytogenes* and *L. ivanovii* are highly pathogenic (*1*). The foodborne illness caused by these bacteria is known as listeriosis. *L. monocytogenes* is a primary cause of food-related mortality and morbidity (*2*). It primarily affects pregnant women, newborn, and elderly people with weakened immune system (*3*). The Center for Disease Control and Prevention estimates that 2500 cases of listeriosis account for about $200 million in monetary loss per year in the USA (*4*). *L. monocytogenes* is responsible for 3.8 % of foodborne illness-related hospitalizations and 27.6 % of foodborne disease-related deaths (*5*).

One of the specific and striking features of *L. monocytogenes* is its adaptation to stress (extreme environmental conditions), such as high salt mass fraction (10 % NaCl) or broad pH range (pH=4.5–9). The bacterium is capable of growing even at 1.7 °C (*6*). Another threat caused by these bacteria is their extremely strong adherence to different surfaces in food processing industry (stainless steel, polypropylene, aluminium, glass). As biofilms are more resistant to antibacterial treatment than planktonic cells, they make a lot of trouble in food industry (*7*). For instance, *Listeria* has been isolated from such surfaces as conveyor belts, floor drains, condensate, storage tanks, hand trucks, and packaging equipment (*8*).

Most of the conventional food safety technologies provoke thermal or chemical effects, which usually induce undesirable physical and chemical changes in the food and reduce its quality. Thus, the development of modern, nonthermal, ecologically friendly and cost-effective anti-

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microbial technology is necessary. In our opinion, one of them could be photosensitization (*9*).

Photosensitization, as one of the modern biophotonic technologies, is based on the interaction of three nontoxic agents: photoactive compound (photosensitizer), light and oxygen (*10*). Numerous investigators have confirmed that different microorganisms including bacteria, viruses and microfungi are destroyed after their treatment with an appropriate photosensitizer and light (*11–15*). The killing capacity of this treatment depends on many factors, including physiological state of bacteria, cellular structure and organization, physicochemical properties of photosensitizers and their capacity to accumulate inside the cell. For instance, due to the complicated cell wall structure, Gram-negative bacteria accumulate less photosensitizers and subsequently their susceptibility to photosensitization is lower (*13,16*).

Few results are published on photodynamic inactivation of food pathogen *Listeria monocytogenes* (*17*). Three photosensitizers are used in this study: tetra(*N*-methyl-4-pyridyl)porphine tetratosylate salt (TMPyP), toluidine blue O (TBO), and methylene blue trihydrate (MB). The efficiency of all the tested photosensitizers was the following: TBO>MB>TMPyP, but none of them was able to inactivate *Listeria* totally. Moreover, none of the evaluated photosensitizers had a significant role in dealing with food safety problems.

It is well established that most bacteria use the heme biosynthetic pathway to produce porphyrins from precursor 5-aminolevulinic acid (ALA), but with different intensity. These endogenous porphyrins represent a mixture of coproporphyrin, uroporphyrin and protoporphyrin, and after excitation with λ=400 nm light, they can produce photocitotoxic effects in bacterial cells (*9,13*).

Meanwhile, no data exist on the possibility to inactivate the troublesome food pathogen *L. monocytogenes* by exploitation of intrinsic bacterial metabolism to produce endogenous photosensitizers (porphyrins) from those exogenously applied (ALA).

The aim of this study is to evaluate the efficiency of ALA-based photosensitization for inactivation of different physiological forms of food pathogen *L. monocytogenes* both *in vitro* and when adhering to the surface of packaging materials.

Material and Methods

Chemicals

Stock solution of 5-aminolevulinic acid hydrochloride (ALA) (Fluka, Israel) was prepared by dissolving ALA in 0.1 M phosphate buffered saline (PBS), pH=7.2, to which NaOH was added up to the concentration of 0.2 M to adjust the pH of the solution to 7.2. ALA stock solution was made instantly before use and sterilized by filtration through 0.20-μm filter (Roth, Karlsruhe, Germany) (*18*).

Bacterial growth

Listeria monocytogenes ATCL3C 7644 was kindly provided by the National Veterinary Laboratory (Vilnius, Lithuania). The bacterial culture was grown at 37 °C and maintained on the tryptone soy agar supplemented with 0.6 % yeast extract (TSYEA; Liofilchem, Italy).

The bacterial culture was grown overnight (approx. 14 h) at 37 °C in 20 mL of tryptone soy medium supplemented with 0.6 % yeast extract (TSYE; Liofilchem, Italy), with agitation at 120 rpm (Environmental Shaker-Incubator ES–20; Biosan, Latvia). After that, this culture was diluted 20 times with fresh medium (A=0.164) and grown at 37 °C to approx. $1.16 \cdot 10^9$ CFU/mL, A=0.9, in a shaker (at 120 rpm). Bacterial absorbance was determined in a 10.01-mm glass cuvette at λ=540 nm (Helios Gamma & Delta spectrophotometers; ThermoSpectronic, Cambridge, UK). Afterwards, the bacteria were harvested by centrifugation (20 min, 5000×g) and resuspended to approx. $5.8 \cdot 10^9$ CFU/mL of the final concentration in 0.1 M PBS (pH=7.2). This stock suspension was diluted to approx. 10^7 CFU/mL and used immediately for the photosensitization experiments.

Photosensitization

Aliquots of 10 mL of bacterial suspension (approx. 10^7 CFU/mL in 0.1 M PBS buffer) were incubated in a 50-mL plastic bottle for cell culture cultivation in the dark at 37 °C, in the shaker (at 120 rpm) with 7.5 mM ALA for different periods (0–2 h) (*19*). After incubation, 150-μL aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and illuminated (0–20 min). LED-based light source, constructed by optoelectronics group in our institute, emitted light with light power density of P=20 mW/cm^2 and wavelength λ=400 nm (peak half-width 10 nm).

Fluorescence measurements

In order to evaluate the ability of *Listeria* to produce endogenous photosensitizers from ALA, fluorescence spectra of endogenously produced porphyrins were studied. The cell suspensions for measurements were prepared as follows: cells (10^7 CFU/mL in 0.1 M PBS, pH=7.2) were incubated in the dark at 37 °C with 7.5 mM ALA for 0–120 min. Then, 2-mL aliquots of bacterial suspensions were withdrawn by centrifugation (10 min, 5000×g) and after that resuspended in the same amount of cold PBS in order to stop the release of photosensitizer from the cells. These resuspended cells were used for cell-bounded porphyrin fluorescence measurements (*19*). PerkinElmer LS 55 fluorescence spectrophotometer (Beaconsfield, UK) was used for the detection of fluorescence. Scan range parameters were as follows: excitation wavelength 390 nm, emission 590–750 nm, excitation slit 2.5 nm, emission slit 15 nm, and scan speed 200 nm/min.

Photoinactivation of bacteria adhering to the surface of packaging material

Packing yellow trays (polyolefine, a mixture of polyethylene and polypropylene) were provided by LINPAC (West Yorkshire, UK). In order to simplify the experiments and minimize illumination square, the packaging coupons for photosensitization experiments were cut into 2.5×4 cm pieces. To adhere the bacteria to the surface, each packaging sample was soaked in 25 mL of *L. mono-*

cytogenes suspension (approx. 10^7 CFU/mL). The soaked samples were kept in a sterile place for 30 min for better attachment of the bacterial cells. Then, appropriate packing coupons with the adhered bacteria were incubated in the dark with 7.5 and 10 mM ALA for 15 min. The control samples were incubated with PBS (pH=7.2) buffer. After incubation with ALA, all packaging samples were dried in a laminar flow hood at room temperature for 20 min. The dried samples were placed in the treatment chamber and exposed to light for 15 min. The control samples were not illuminated.

Photoinactivation of bacterial biofilms on the surface of packaging material

L. monocytogenes biofilms were prepared according to the method of Pan *et al*. (*20*). The packaging samples (2.5×4 cm) were placed in sterile plastic tubes to keep them separated from each other. *L. monocytogenes* cell suspension (approx. 10^7 CFU/mL) was added until all samples were completely submerged for 3 h at 37 °C. Afterwards, the cell suspension was removed by aspiration and the samples were washed separately three times with sterile PBS to exclude weakly adhered cells. The plastic coupons were then transferred into plastic tubes containing 25 mL of TSYE medium, with each tube containing one sample. These tubes were incubated at 22.5 °C for 48 h for biofilm development. Then the samples were washed three times with sterile PBS positioned in plastic tubes containing 25 mL of 7.5 and 10 mM ALA solutions, and incubated in the dark for 30 min. The control coupons were incubated with sterile PBS. After incubation with ALA, all packaging samples were dried in a sterile place at room temperature for 20 min. The dried samples were placed in the treatment chamber and exposed to light for 15 min up to the dose of 18 J/cm^2. The control samples were not illuminated.

Bacterial cell survival assay

In order to detach bacteria from the surface, all packaging samples were placed in a sterile 100 BagPage with 30 mL of 0.1 M sterile PBS buffer and washed for 1 min with a BagMixer (model MiniMix 100 VP, Interscience, France). The antibacterial effect of photosensitization on *L. monocytogenes* was evaluated by the spread plate method. Thus, 100 μL of appropriate dilutions of bacterial test culture after photosensitization, using the spread plate method, were surface inoculated on the separate TSYEA plates. Afterwards, the bacteria were in the incubator for 24 h at 37 °C. 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 one. Planctonic cells and biofilms adhered to plastic coupons were expressed in CFU/cm^2.

Temperature measurements

Precision Celsius temperature sensors (Delta Ohm, Padova, Italy) were used for temperature measurements as they have an advantage over linear temperature sensors calibrated in Kelvin; the user is not required to subtract a large constant voltage from its output to obtain convenient temperature scaling, and the sensor does not require any external calibration or trimming to provide typical accuracies of ±0.25 °C at room temperature.

Statistical analysis

Bacterial populations were transformed from CFU/mL and CFU/cm^2 into log/mL and log/cm^2, respectively. Each experiment was carried out in triplicate. Standard deviation was estimated for every experimental point and shown as error bars. Sometimes the bars were too small to be visible (S.E.=0–0.32). The fluorescence data were analysed with Origin v. 7.5 software (OriginLab Corporation, Northampton, MA, USA).

Results

Detection of endogenous porphyrins synthesized from ALA in L. monocytogenes

The production of endogenous porphyrins from ALA by other pathogens was investigated in our previous studies (*21,22*). Using the same methodology, we tried to find whether Gram-positive bacterium *L. monocytogenes* produces endogenous porphyrins from extrinsically applied ALA as well. For this purpose, the cells were incubated with 7.5 mM ALA in the dark for 0–2 h. Afterwards, in order to detect the production of endogenous porphyrins, the fluorescence emission spectra in the region of 590–680 nm were analysed (this spectral region is attributed to the presence of endogenously synthesized porphyrins (*22*).

The data presented in Fig. 1 indicate that relative fluorescence intensity of endogenous porphyrins increases with the increase of incubation time with ALA. For instance, fluorescence intensity is very low after 2 min of incubation with ALA, but after 30 min of incubation, it becomes more significant. Following the increase of incubation time to 2 h, fluorescence intensity increased more than 10 orders of magnitude. As our task was to

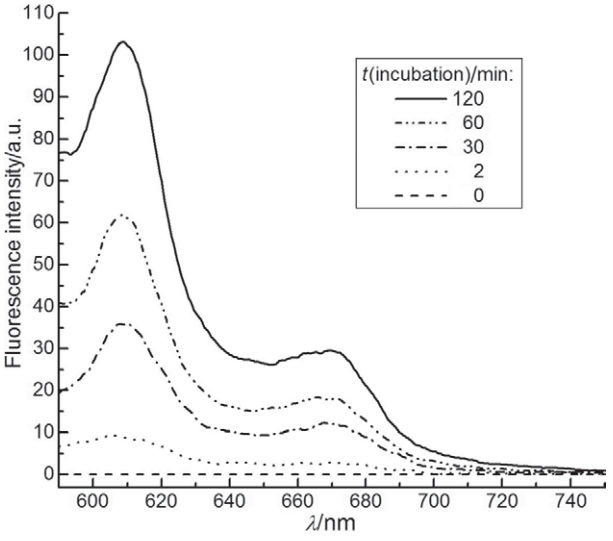

Fig. 1. Fluorescence spectra of endogenous porphyrins produced by *Listeria monocytogenes* ATCL3C 7644 after incubation with 7.5 mM ALA for different time intervals

investigate if *Listeria* can produce endogenous porphyrins in general, we did not go deeper into spectral analysis of what type of endogenous porphyrins this bacterium was producing.

Inactivation of L. monocytogenes by ALA-based photosensitization in vitro

Experimental data reveal that traditionally neither the incubation of cells with ALA (dark toxicity of ALA) nor light alone change the viability of *L. monocytogenes* (data not shown). Even incubation with ALA and subsequent illumination decrease the survival fraction fairly sharply, especially when longer incubation times are used. Clear dependence of inactivation efficiency on the illumination time (light dose) as well as time of incubation with ALA (or concentration of produced endogenous porphyrins) was observed. The number of killed *L. monocytogenes* reached even 4 orders of magnitude, when 20 min of illumination time (24 J/cm^2 of light dose) and 2 h of incubation time were used. It is evident that at a given illumination dose the inactivation effect can be modified by ALA incubation time (Fig. 2).

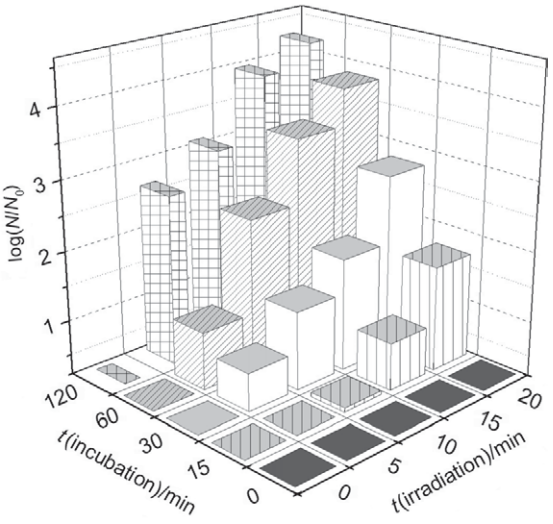

Fig. 2. Inactivation of *Listeria monocytogenes* ATCL3C 7644 by 7.5 mM ALA-based photosensitization when different illumination time and time of incubation with ALA were used

It is important to note that according to our mathematical modelling in previous paper (*19*), no resistant cells were left after photosensitization treatment.

Comparative susceptibility of L. monocytogenes cells and biofilms to ALA-based photosensitization

In the next stage, *Listeria* cells were adhered to the surface of packaging material. The amount of *L. monocytogenes* cells adhered onto one coupon of packaging material reached over 4.6 log. After that coupons of packaging material with the adhered bacteria were soaked in 7.5 and 10 mM ALA solution and illuminated with light (λ=400 nm). Illumination time reached 15 min and a total light dose did not exceed 18 J/cm^2. The data shown in Fig. 3a clearly indicate that the inactivation efficiency of photosensitization treatment depends on the ALA concentration and varied between 2.6–3.7 log under certain experimental conditions.

Afterwards, bacterial biofilms were adhered to the surface of packaging material. Approximately 5.9 log CFU/cm^2 of biofilm-associated cells were adhered onto one plastic coupon. The treatment of biofilm-associated cells with 7.5 and 10 mM ALA in the dark did not exhibit any cytotoxic effect against bacterial cells. Light alone had no toxic effect either. Incubation with ALA alone and subsequent illumination of these bacteria with light (λ=400 nm) reduced significantly the formation of biofilms. Depending on the used ALA concentration (7.5 or 10 mM), inactivation of biofilm-associated cells increased from 1.7 to 3 log, respectively (Fig. 3b).

Since our aim was to investigate the susceptibility of cells and biofilms to ALA-based photosensitization at minimal working prodrug concentration, we did not try to evaluate the effect of higher ALA concentrations.

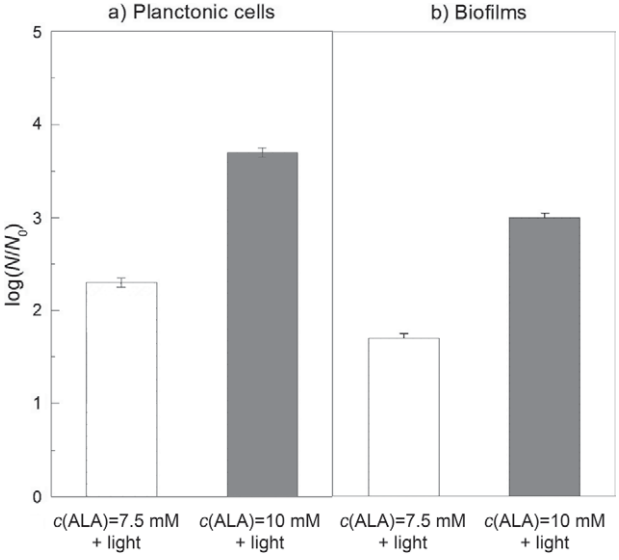

Fig. 3. Susceptibility of *Listeria monocytogenes* ATCL3C 7644 to ALA-based photosensitization: (a) cells and (b) biofilms adhering to the surface of packaging material. Illumination time 15 min, total light dose 18 J/cm^2. Control, untreated sample (log (N/N_0)=0)

Measurements of temperature during photosensitization-based inactivation of Listeria

One of the tasks in this study was to find experimental algorithm for non-thermal inactivation of *Listeria* by photosensitization. In order to check whether inactivation conditions are non-thermal, dynamics of temperature inside the chamber was measured with precise thermometer every 2 min. Data presented in Fig. 4 clearly indicate that temperature in the chamber of LED-based light source increased very slowly up to 24 °C. Temperature was practically unchanged from 14 min of the treatment up to 20 min. Even after 20 min of illumination, temperature in the chamber did not exceed 25 °C.

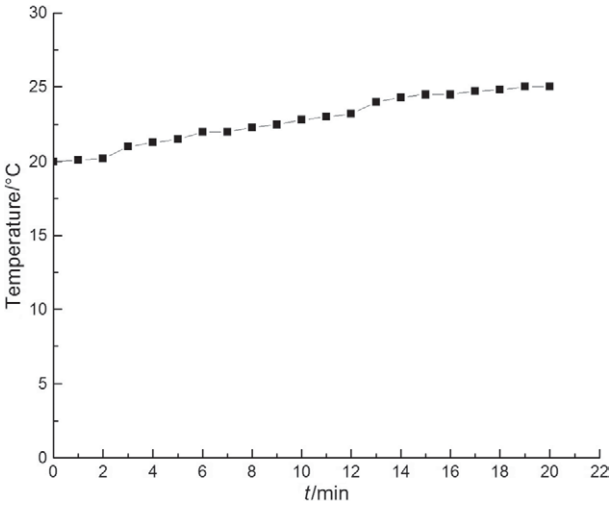

Fig. 4. The increase of temperature in the chamber of LED-based light source during 20 min of illumination

Discussion

Food pathogen *Listeria monocytogenes* was found in raw milk, cheese, ice cream, raw vegetables, fermented raw meat sausages, raw and cooked poultry, raw meat (all types), raw and smoked fish (*6*,*20*,*23*). The risk is the highest in ready-to-eat food, as bacteria may contaminate the cooked products before packaging, during transportation, or during post-cooking handling (*24*).

Many attempts have been made to inactivate food pathogen *L. monocytogenes* using emerging food safety technologies. Ultrahigh hydrostatic pressure inactivated *Listeria* by 2.76 log in the liquid (*24*). Pulsed UV light technology can inactivate *Listeria* by 6 log *in vitro*, but some thermal effects take place (*25*,*26*) where pulsed electric field treatment is ineffective (*27*). High dose of ionizing radiation (0.380 to 0.682 kGy) reduced the amount of *L. monocytogenes* cells by 6.4–8.6 log CFU/mL, but the irradiation values were somewhat higher than usable in food industry (*28*).

The main antibacterial agents in food industry used to avoid the formation of biofilms are chemical sanitizers like hypochlorite, iodine, ozone and chloramines (*29*). According to Jeyasekaran *et al*. (*30*) the efficiency of sodium hypochlorite on the inactivation of *L. monocytogenes* biofilm adhered on plastic material is not sufficient: 100 and 200 ppm hypochlorite gave 2.0 and 3.0 log reduction of bacterial viability, respectively. Antibacterial efficiency of ozone exhibited the same killing efficiency as hypochlorite (*31*). Another chemical sanitizer peroxide reduced the biofilm formation on the packaging surface by 2.0 log.

The first results obtained in this study on the inactivation of *Listeria* cells by ALA-based photosensitization *in vitro* look promising. Due to suitable production of endogenous porphyrins from exogenously applied ALA (Fig. 1) and following illumination with light, *L. monocytogenes* can be inactivated by 4 log (Fig. 2). The efficiency of inactivation strongly depends on the used ALA concentration, time of incubation with ALA and light dose delivered to the bacteria. Hence, there are enough ways to increase the inactivation efficiency of ALA-based photosensitization, if necessary.

In order to compare susceptibility of cells and biofilms to ALA-based photosensitization, *Listeria* cells were adhered to the surface of packaging material. The data presented in Fig. 3a reveal that inactivation of cells after photosensitization can reach 2.3–3.7 log. No measurable changes were detected in the cell inactivation after their incubation with 7.5 and 10 mM ALA solution or illumination alone. The biofilms of *Listeria* under the same experimental conditions exhibit lower susceptibility to ALA-based photosensitization (Fig. 3b). Inactivation of biofilms by 1.7–3.1 log indicates that this treatment has a potential to combat biofilms, usually more resistant to environmental effects than their planktonic counterparts (*32*).

According to the obtained results, ALA-based photosensitization can inactivate *Listeria* biofilms to a lesser extent than the cells adhered to the same surface of packaging material. This effect can be explained by polysaccharide matrix acting as a diffusion barrier for photosensitizer and reducing its accumulation inside bacteria. Moreover, a high concentration of extracellular polysaccharides in biofilms may reduce the quantity of light reaching the bacteria, thereby decreasing the effectiveness of the photosensitizing process (*33*,*34*).

The application of photosensitization is considered for decontamination of food. Not every photosensitizer that can be used against cancer or infections is possible to use for food decontamination. First of all every photosensitizer interacts with food matrix. ALA solution itself is colourless and odourless, thus its spraying on food matrix (for instance fruits and vegetables) will not change the organoleptic properties. Besides, our previous experiments on decontamination of wheat sprouts by ALA indicated that ALA could stimulate the growth of wheat seedlings and roots without impairing the vigour of germination and the viability of seeds (*35*). Moreover, 5-ALA increased the rate of photosynthesis (chlorophyll content) and the activities of antioxidant enzymes, which could be associated with enhanced cellular capacity to detoxify reactive oxygen species (*35*). In addition, ALA is an essential precursor of such tetrapyrrole compounds as vitamin B12 and hemes, which serve as prosthetic groups of respiratory enzymes and chlorophyll in plants (*36*). Suitable ALA concentrations have promotive effects on the growth rates and photosynthesis. For instance, crop yields were enhanced by the application of ALA at the leaf stage for rice, barley, potato and garlic (*37*). In addition, foliar application of ALA (100 mg/mL) on date palm has promoting effects on the fruit mass, volume and sugar content (*38*).

Conclusions

The data presented in this study clearly indicate for the first time that food pathogen *L. monocytogenes*, which is most resistant to chemical and physical antibacterial treatments, can be inactivated by ALA-based photosensitization due to high production of endogenous photosensitizers in the presence of ALA. Moreover, *Listeria* vegetative cells and biofilms adhered to the surface of packaging material after photosensitization were in-

activated by 3.7 and 3.1 log, respectively. In this context, photosensitization may serve as an effective tool to combat both antimicrobial and biofilm-related resistance. Results presented in this study support the data of our previous papers and indicate that the main food pathogens, microfungi and yeasts, which can be found in form of spores and biofilms, are susceptible to photosensitization and can be inactivated *in vitro* as well as when attached to the different surfaces.

This phenomenon could serve as a background for further development of a novel nonthermal or hurdle technology for decontamination of foods (for instance ready-to-eat fruits and vegetables) or food-related surfaces. Undoubtedly, photosensitization will not be as universal as ionizing radiation, but in some special cases, for some special food matrices, it has a good potential.

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