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INACTIVATION OF FOOD PATHOGENS BY PHOTOACTIVATED CHLOROPHYLLIN: MECHANISM OF ACTION, OPTIMIZATION AND POSSIBLE APPLICATIONS

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VILNIAUS UNIVERSITETAS

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MAISTO PATOGENŲ INAKTYVACIJA FOTOAKTYVUOTU CHLOROFILINU: POVEIKIO MECHANIZMAS, OPTIMIZAVIMAS IR PRITAIKYMO GALIMYBĖS

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ABBREVATION LIST

$^{\bullet}OH$ – hydroxyl radicals

$^{1}O_2$ – singlet oxygen

ALA – 5-aminolevulinic acid hydrochloride

APPT – atmospheric pressure plasma treatment

CDC – Center of Disease Control and Prevention

cfu – colony forming units

Chl–CHS – chlorophyllin chitosan conjugate

Chl – sodium chlorophyllin (not copperized chlorophyll sodium salt)

CHS – chitosan

$CO_3^{\bullet-}$ – carbonate radical anions

DG18 – dichloran glycerol medium

ECDC – European Centre for Disease Prevention and Control

EDTA – ethylene diamine tetraacetic acid

EFSA – European Food Safety Authority

EPR – electron spin resonance

ERS – Economic Research Service

ESRGR – European strawberry and raspberry genetic resources

EU – European Union

FV – fruits and vegetables

HCl – hydrochloric acid

HHP – high hydrostatic pressure

HIFU – high intensity focused ultrasound

Hip – hypericin

HPPL – high power pulsed light

IR – ionizing radiation

LB – Luria-Bertani medium

LBA – Luria-Bertani Agar

MB – methylene blue

NaN_3 – sodium azide

NaOCl – sodium hypochlorite

ND – not detectable limit

$O_2^{\bullet-}$ – superoxide anion

$O_2^{\bullet-}$ – superoxide anion

OD – optical density

PBS – phosphate-buffered saline

PDA – potato dextrose agar medium

PDT – photodynamic therapy

PEF – pulsed electric field

PS – photosensitizer

RB – Rose Bengal

Rev/min – revolution per minute

ROS – reactive oxygen species

SEM – scanning electron microscopy

TBO –toluidine blue O

TSYE – Tryptone soya medium supplemented with 0.6 % yeast extract

TSYEA – Tryptone soya agar supplemented with 0.6 % yeast extract

US – United States

UV – ultraviolet light

WHO – World Health Organization

WMO – World Meteorological Organization

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INTRODUCTION

Recent report from the World Health Organization (WHO) has concluded that the incidence of foodborne diseases is a growing public health problem in both developed and developing countries (1). The center of Disease Control and Prevention (CDC) reported 48 million illnesses, 128000 hospitalizations, and 3000 deaths every year due to foodborne illness caused by pathogenic microorganisms (2). Fresh produce has been increasingly implicated as the vehicle of pathogen transmission and became the second leading cause of foodborne illnesses, which poses a $77.7 billion economic burden in the US annually (3).

Gram-negative *Salmonella enterica* is one of the most important foodborne pathogens (4). Actually, about 1 million people were infected with non-typhoidal *Salmonella*, resulting in 19336 hospitalizations and 378 deaths in the US yearly (5), whereas 96883 cases of human salmonellosis in 2011 were reported in the EU. The highest confirmed case rates were reported in the Czech Republic (80.69 cases per 100000 population), Slovakia (71.70) and Lithuania (70.70) (6). Gram-positive *Listeria monocytogenes* is a primary cause of food-related mortality and morbidity (mortality rates as high as 30%) (7). According to CDC approximately 1600 illnesses and 260 deaths due to listeriosis occur annually in the US (6), whereas 1524 cases of listeriosis in 2011 were reported in the EU (5). *Bacillus cereus* have been identified as the cause of 2050 cases of food-borne illnesses in the US (8).

Strawberry (*Fragaria* × *ananassa* Duch.) is a major crop with 4–5 millions in tons of production worldwide, whereas European production of strawberries reaches 1.1 Mt annually (9). Gray mold (*Botrytis cinerea*) is one of the most economically important postharvest pathogens that causes postharvest loses of strawberries. This phytopathogenic mold may cause 30–40% losses of the harvest if no chemical control is applied (10). Meanwhile, 63% of strawberries contain multiple pesticide residues (11).

In this context, modern non-thermal and nonchemical technology based on photosensitization (photodynamic therapy in treatment of infectious diseases) might serve as a promising fruit decontamination tool (12,13).

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 and visible light, in the presence of oxygen results in a plethora of cytotoxic reactions and consequently induces selective destruction of target microorganism (14,15). However, ALA is a rather expensive compound and it requires a longer time to produce endogenous porphyrins thus raising the cost of every ALA-based decontamination technology (14).

Our data indicate that chlorophyllin (Chl) as negatively charged photosensitizer (PS) is effective against Gram-positive bacteria, but Gram-negative bacteria are less susceptible to this treatment. This disadvantage of Chl-based photosensitization limits its more wide application.

In order to increase susceptibility of Gram-negative food pathogens to Chl-based photosensitization Chl–Chitosan (CHS) conjugate were performed.

The aim of the dissertation work was to increase susceptibility of Gram-negative food pathogen *Salmonella enterica*, Gram-positive *Listeria monocytogenes, Bacillus cereus* and gray mold *Botrytis cinerea* to photosensitization treatment and to investigate the mode of action of Chl-based photosensitization and photoactivated Chl–CHS conjugate. Moreover, it was important to apply this antimicrobial treatment for decontamination of food-related surfaces and some fruits.

The following **tasks** have been formulated:

1. Inactivation of *L. monocytogenes*, *B. cereus*, *S. enterica* by ALA-based photosensitization *in vitro* and on the surface of packaging material.

2. Evaluation of the antimicrobial effect of Chl-based photosensitization against Gram-negative (*S. enterica*), Gram-positive (*L. monocytogenes, B. cereus*) bacteria and gray mold (*B. cinerea*) *in vitro* and on the surface of packaging material.
3. Estimation of the antimicrobial efficiency of negatively-charged Chl-based photosensitization combining it with antimicrobial properties of positively-charged CHS by immobilization of Chl into CHS polymer.
4. Investigation of the mode of action of Chl-based photosensitization and photoactivated Chl–CHS conjugate.
5. Application of this conjugate for microbial control of strawberries and investigation of different quality attributes of treated berries.

Novelty and importance of this work

1. For the first time food pathogens were inactivated by photosensitization using never tested before Chl which was well known as food additive (E140 ii), but never as photosensitizer.
2. For the first time decontamination of food packaging (polyolefine) with attached food pathogens was sterilized (3.7–4 log inactivation) using ALA-based photosensitization and Chl-based photosensitization.
3. For the first time antimicrobial properties of Chl-based photosensitization were combined with that of CHS to combat Gram-negative bacteria and moulds.
4. For the first time Chl–CHS coating and visible light were applied for decontamination of strawberries. Results indicate that shelf-life of strawberries prolonged 40% without any negative effects on strawberry nutritional value or organoleptic properties.

Statements presented for defence

1. Food pathogens can be effectively inactivated by ALA-based photosensitization *in vitro* and on the surface of packaging material.
2. Gram-negative bacteria and harmful molds are more resistant to Chl-based photosensitization than Gram-positive bacteria.
3. The susceptibility of the Gram-negative food pathogens to Chl-based photosensitization can be increased combining it with antimicrobial properties of positively-charged CHS, immobilizing Chl into CHS polymer.
4. Photoactivated Chl–CHS can be a useful tool for the future development of edible photoacive antimicrobial coatings which can preserve strawberries and prolong their shelf-life without any negative impact on total antioxidant activity, visual appearance, color, weight and radical-based fundamental changes.

Contribution of the author

The author performed majority of experiments presented in the dissertation and analyzed the obtained results. The conclusions of the dissertation were achieved by the author's discussion with the supervisor of the doctoral studies prof. dr. habl. Ž. Lukšienė. The author was very active in the interpretation of results and prepared several drafts of publications (2, 4, 8, 9, 10, 11, 12, 13), also contributed to preparation process of others drafts. The author has also contributed to the preparation of a number of scientific posters presented at the national and international scientific conferences, some of which she presented by herself (9, 12, 14).

The author acknowledges that this dissertation would have never been finished without contribution of his colleagues, listed as co-authors in the publications related to the dissertation.

1. STATE – OF – THE – ART

1.1. Food safety problems

Microbiological food safety is an increasing worldwide problem. More than 250 different foodborne diseases have been described. Most of these diseases are infections, caused by a variety of bacteria, viruses, and parasites that can be foodborne (2). Despite tremendous progress in technology and biomedical science the number of reported food-borne infections continues to rise. Health experts estimate that food-borne illnesses in the US cost 77,7 billion US dollars in direct medical expenses and lost productivity (3). CDC estimates that each year 31 major pathogens acquired in the US caused 9.4 million episodes of foodborne illness, 55961 hospitalizations, and 1351 deaths. Most (58%) illnesses were caused by norovirus, followed by nontyphoidal *Salmonella* spp. (11%), *Clostridium perfringens* (10 %), and *Campylobacter* spp. (9%). Leading causes of hospitalization were nontyphoidal *Salmonella* spp. (35%), norovirus (26%), *Campylobacter* spp. (15%), and *Toxoplasma gondii* (8 %). Leading causes of death were nontyphoidal *Salmonella* spp. (28%), *T. gondii* (24%), *Listeria monocytogenes* (19%), and norovirus (11%) (2). In the year 2013 the European Food Safety Authority (EFSA) reported 5196 food-borne outbreaks, including water-borne outbreaks.

Salmonella enterica is one of the most important foodborne pathogens in many countries (4). Actually, about 1 million people are infected with non-typhoidal *Salmonella*, resulting in 19336 hospitalizations and 378 deaths in the US yearly (5), whereas 138469 cases of human salmonellosis in 2008 were reported in the EU (16). During the six-year period from 2008 to 2013 within the EU, the annual total number of *Salmonella* outbreaks has decreased markedly by 38.1% (17). 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 (18).

Clostridium botulinum, Bacillus cereus and *L. monocytogenes* are the main Gram-positive pathogens causing food-borne diseases (19). *L. monocytogenes* is highly pathogenic bacteria (20). The food-borne illness caused by these bacteria is known as listeriosis. *L. monocytogenes* is a primary cause of food- related mortality and morbidity (mortality rates as high as 30%) (7). It primarily affects pregnant women, newborns, persons of advanced age and people with weakened immune systems (21). According to CDC approximately 1600 illnesses and 260 deaths due to listeriosis occur annually in the US (22). In 2013, 27 EU member states reported 1763 confirmed human cases of listeriosis (17). As a natural biofilm community is functioning through collective behavior and coordinated activity, which assists survival of individual cells in stressful conditions, biofilms are more than 100 times resistant to antibacterial treatment than planktonic cells and make a lot of trouble in food industry (23). For instance, *Listeria* biofilms have been isolated from such surfaces as conveyor belts, floor drains, condensate, storage tanks, hand trucks, and packaging equipment (24).

B. cereus is spore-forming bacterium that is widely distributed in the environment, mainly in soil, from which it is easily spread to many types of foods, especially those of vegetable origin, as well as meat, eggs, milk, and dairy products (25). Heat-stable toxins generated by Gram-positive *Bacillus* are the common causes of sporadic point-source outbreaks of gastroenteritis (26). In 2013, nine EU member states reported 278 outbreaks in which *Bacillus* toxins were the causative agent, representing 5.4% of all outbreaks reported within the EU (17). *B. cereus* species have been identified as the cause of 27000 cases of food-borne illnesses in the US (27). 103 outbreak cases have been reported in the US in 2008 (28). Spores of this microbe were found in 88–100% of noodles, mashed potatoes and rice, in 50–83% of cooked vegetables and 25–75% of gravies sampled (29). Growth of vegetative *Bacillus* cells usually occurs within the temperature range of 5 to 50 °C and can easily be

inactivated by heating. Although spores are considerably more resistant and can cause food spoilage after subsequent germination (30).

Grey mould disease, caused by *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*), is one of the most serious diseases of a wide range of crops (more than 200 crop species) of worldwide importance (31). Infections caused by *B. cinerea* are of considerable economic importance in fruits and vegetables world-wide, especially when grown under protection (32). *B. cinerea* is difficult to control because it has a variety of modes of attack, diverse hosts as inoculum sources, and it can survive as mycelia and/or conidia or for extended periods as sclerotia in crop debris (31).

1.2. Non-thermal food safety technology

Traditional food processing relies on heat to kill foodborne pathogens, to make food safe to eat. Most of the currently employed preservation techniques act by inhibiting the growth of microorganisms (Table 1.1) and preventing their multiplication (chilling, freezing, drying, curing, adding acids, preservatives), and just few of them act primarily by inactivating micro-organisms in the food (14). The major inactivation technique is heating. However, there are many foods that pose a risk for bacterial foodborne disease for which heat is either undesirable or cannot be used (minimally processed food) (33). Most of the traditional food safety technologies invoke thermal or chemical effects, which usually induce undesirable changes in food flavour, color, texture and nutritional attributes such as protein and vitamin destruction (34). Thus, the development of modern, non-thermal, ecologically friendly and cost-effective antimicrobial technology is of importance. Researchers have been studying non-thermal processing methods (methods that do not use heat) that will destroy pathogens and keep foods safe to eat, while retaining the sensory attributes and nutrient content similar to raw or fresh products. These alternative processing methods are at various stages of development, and have the potential to destroy pathogens and retain desired food quality (33).

Table 1.1 Major existing technologies for food preservation (14).

Technique that slows or prevents the growth of microorganisms
Reduction in temperature (chilling, freezing)
Reduction in water activity (drying, curing, conserving with added sugar)
Reduction in pH (acidification, fermentation)
Removal of oxygen (vacuum or modified – atmosphere packaging)
Addition of preservatives (inorganic – nitrite; organic – propionate, sorbate, benzoate, etc.)
Control of microstructure (in water-in-oil emulsion foods)
Technique that inactivates microorganisms
Heating (pasteurization, sterilization)

On the other hand, non-thermal processing methods interest food scientists, manufacturers and consumers because they have a minimal impact on the nutritional and sensory properties of foods, and extend shelf life by inhibiting or killing microorganisms. Non-thermal food preservation processes are considered to be more energy efficient and to preserve better quality attributes than conventional processes.

Meanwhile, most of the developing novel technologies such as high hydrostatic pressure (HHP), pulsed electric field (PEF),ultraviolet light (UV), high-power pulsed light (HPPL), high-intensity focused ultrasound (HIFU), ionizing radiation (IR), atmospheric pressure plasma treatment (APPT) and biopreservatives are mostly still in the developmental stage.

HHP can be used for liquid and solid foods with or without packaging (100÷1000 M/l Pa, from milliseconds to 20 min duration) and can inactivate yeast, molds and most vegetative bacteria, leaving flavor compounds, nutrients and vitamins intact. HPP inactivated *Listeria* by 2.76 log in the liquid (35). The main disadvantage of this technique is that some types of spores (*C. botulinum*) are resistant to this treatment. Moreover, the implementation of HHP is expensive, thus, its use is limited to high-value products (36).

High-intensity PEF processing involves the application of high-voltage pulses (20÷80 kV/cm) for short period (less than a second) to fluid foods placed between two electrodes. PEF is an effective antimicrobial tool (but spores are resistant) reducing detrimental changes in sensory and physical

properties of food. However, PEF has a very limited effect on the reduction of *L. monocytogenes* (37). The main problems in the development of PEF technology deal with the generation of high electric field intensities and design of technique with optimal parameters (38).

UV has been used for decontamination of the food-related surfaces. However, high microbial resistance to this treatment, the long exposure time and enormous light intensities required and the degradation of exposed materials including food itself limit the wide application of this technique (39).

HPPL technique uses intense and short duration (μs) pulses of broad spectrum (from ultraviolet till infrared). This technology can be used to sterilize or reduce microbial population (vegetative cells and spores) on food surfaces, packaging materials or processing equipments. HPPL technology can inactivate *Listeria* by 6 log, but with some thermal effects (40,41). The main disadvantage of this technique is that light cannot penetrate deeper into the food matrix and can sterilize it just superficially (42, 43).

Ultrasound is well known to have a significant effect on the rate of various processes in the food industry. HIFU (10÷1000 W/cm^2, < 0.1 MHz) can sterilize liquid and solid foods. Meanwhile, spores are resistant to ultrasound treatment (39).

IR is known as one of the best non-thermal sterilization methods as can penetrate deeper in the material. IR reduced the amount of biofilm-associated *L. monocytogenes* cells by 6.4÷8.6 log, but the irradiation values ranged from 0.380 to 0.682 kGy, which were somewhat higher than usable in food industry (44). Treatment of food with IR is effective and legal in more than 40 countries. Nevertheless, this technology remains not approved in Europe mainly because of the suspicions of the consumers (45). EFSA's experts note that only a very limited quantity of food consumed in Europe is irradiated today(46).

APPT, non-thermal technology, is proposed as a potential alternative to traditional methods for decontamination of foods (47–49), meanwhile the type

of packaging material is crucial, and appropriate treatment conditions should be considered for achieving satisfactory inactivation level (50).

A plethora of natural antimicrobial compounds derived from plants are known as antimicrobials (phytoalexins, phenolic compounds). The main disadvantages of these antimicrobials are strong flavour, high working concentration and relatively low antimicrobial efficiency.

In spite of the intensive research efforts and investments, very few of these new preservation methods are implemented by the food industry. Further research is required to use these new preservation techniques on industrial scale with total warranties for consumer's health.

1.3. Basic concepts of antibacterial action of photosensitization-based treatment

The emergence of antibiotic resistance amongst pathogenic and harmful microorganisms is one of the most pressing worldwide health issues (51). The weakening efficacy of antibiotics has led to the development of alternative antimicrobial technologies (52). In this context, photosensitization treatment seems promising. Since the beginning of the 20^{th} century, when photosensitization-based destruction of highly proliferating living systems was discovered, many attempts have been made in order to understand the mechanism of action of this phenomenon (53,54).

Antibacterial photosensitization-based treatment (photodynamic therapy (PDT), when treating infectious diseases or cancer), as one of the modern biophotonic technologies, is based on the interaction of three factors: photoactive compound (photosensitizer (PS)), molecular oxygen and low doses of visible light of suitable wavelength to match the PS absorption peak (55). The illuminated ground state PS, located in the bacteria or at the bacterial surface, absorbs the light and is excited to its singlet state (Figure 1.1). The excited state electrons undergo intersystem crossing to a lower-energy but

longer-lived triplet state, from which reactive oxygen species (ROS) or reactive molecular transients are generated (56).

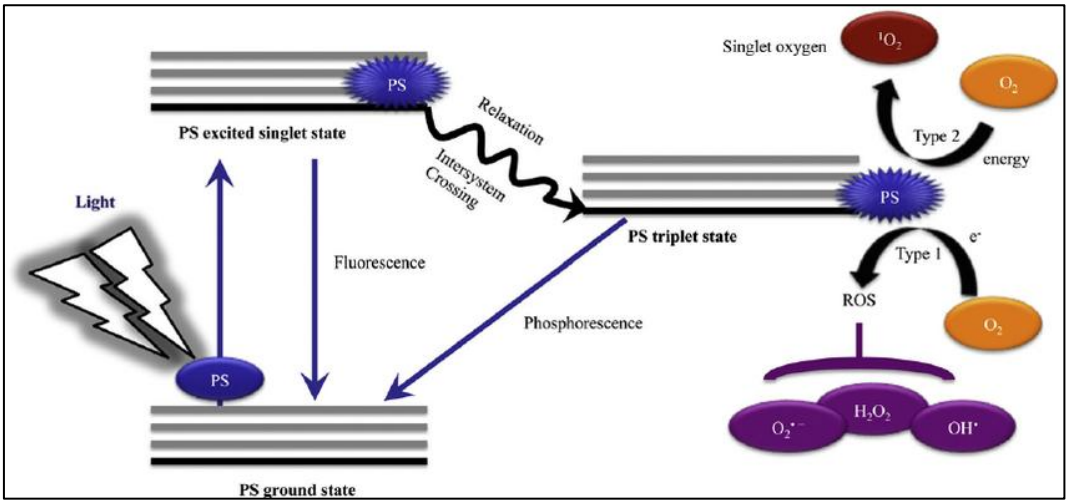

Fig. 1.1 Schematic illustration of photodynamic action (Jablonski diagram)(57)

The photochemical reactions proceed via a type I or type II mechanism and require close proximity between the PS triplet state and substrate (Figure 1.1). Type I reactions generate radicals following triplet state electron transfer from the PS triplet state to a substrate. A common terminal substrate for type I reactions is molecular oxygen, leading to the production of radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), carbonate radical anions ($CO_3^{\bullet -}$) that oxidize biomolecules and cause cell damage and ultimately death (56,58). In type II reactions, the excited PS reacts directly with molecular oxygen (O_2) and forms the highly reactive singlet oxygen ($^1O_2$) via PS triplet state → O_2 energy transfer (59). Both type reactions can occur simultaneously, and the ratio between these processes depends on the type of PS used and the microenvironment in which photosensitization is applied. It is also important that the initially produced $^1O_2$ can subsequently react with biological substrates (such as unsaturated fatty acids) to produce secondary radicals (such as lipid peroxide radicals) (60). The active products of both reactions are able to inactivate microorganisms by different cell damages (disruption of cell membrane, inactivation of enzymes, DNA damage, etc.) (12,61,62). One of the most important advantages of antibacterial photosensitization-based treatment

in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment (59,63,64), as the process is free radical-mediated (52,65). It allows us to achieve an extensive decrease in the population of pathogens with minimal damage or thermal effects on the surrounding matrix (62,66).

1.4. Photosensitizers

Table 1.2 The criteria of PS for photosensitization-based treatment (67).

High $^1O_2$ quantum yield
Photostable
Broad spectrum of antimicrobial action
Low binding affinity and low toxicity for mammalian cells (PDT)
No mutagenicity (DNA damage must be avoided)
No dark toxicity
Photodynamic inactivation parameters necessary where bacteria are killed without damage of eukaryotic cells

Numerous investigators have confirmed that different microorganisms including Gram-positive, Gram-negative bacteria, viruses, fungi, and protozoa, whether *in vitro* or *in vivo* can be killed after photosensitization treatment (13,54,68–70). The antibacterial efficiency of this treatment depends on many factors, including physiological state of bacteria, cellular structure and organization, physical-chemical properties of the PSs (Table 1.2) and their capacity to bind the cell.

One of the most important advantages of photosensitization in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment (63). According Liu et al. (59), this is due to several reasons:

1. The time between administration of the PS and photosensitization is too short for pathogen to develop resistance;
2. PSs exhibit no dark toxicity, as a result of which bacteria do not have to engage adaptive survival mechanisms against the PSs (any metabolic adaptations are directed elsewhere);

3. The cells are too damaged after photosensitization, disabling them to confer cross-generation additivity;
4. Photosensitization treatment does not target a single site in bacteria, so the ROS generated by photosensitization treatment target various pathogen cell structures and different metabolic pathways.

Most studied PSs are based on tetrapyrrolic macrocycles (porphyrins), chlorines, bacteriochlorin, phthalocyanines and texaphyrins. These molecules have low toxicity, can form long-lived triplet excited states and have high affinity to the life-essential molecules (71). It has been found that PS that fulfill these requirements are likely to have pronounced cationic charges (72). Moreover, neutral or anionic PSs are ineffective against Gram-negative bacteria (73). There are two main routes for PS- cell interaction. In the first case, it could form a tight conjugate with the surface of the cell wall. In way, the PS is transported inside the cell, where it associates with the key structures and irreversibly damages them after photosensitization (52).

Demidova and Hamblin (74) formed the hypothesis that there are three groups of antimicrobial PSs:

1. That are tightly bound and penetrate into microorganisms (such as poly-L-lysine chlorin (e6) conjugate (pL-ce6));
2. That are only loosely bound (such as Toluidine Blue O(TBO));
3. That does not demonstrate binding (such as Rose Bengal (RB)).

Various studies have demonstrated that some pathogenic microorganisms can produce endogenous PSs from exogenously applied precursor 5-aminolevulinic acid (ALA), also known as d-aminolevulinic acid, δ-aminolevulinate, 5-amino-4-oxo-pentanoic acid, and γ-keto-damino valerate (Figure 1.2a) (12,69,75–77).

It is clear from Figure 1.2b that ALA is an endogenous component in the heme biosynthesis pathway, and according to literature ALA is ubiquitous in nearly all cells (66). ALA itself is not photoactive, but when inside the bacteria cell (is transported to cytosol via different active transport mechanisms), it can

induce synthesis and accumulation of endogenous porphyrins (78). These endogenous porphyrins represent a mixture of coproporphyrin, uroporphyrin and endogenous PS protoporphyrin IX (PpIX) (Figure 1.2b) (54) and after excitation with λ=405 nm light can produce photocitotoxic effects in bacterial cells. Thus, photosensitization could be based on the endogenous synthesis of porphyrin-type PSs by ALA applied exogenously. It was postulated that the presence of endogenous porphyrins within the cell with no need to penetrate any cell barriers would result in effective photodestruction of the strains that can produce high amounts of endogenous porphyrins (71).

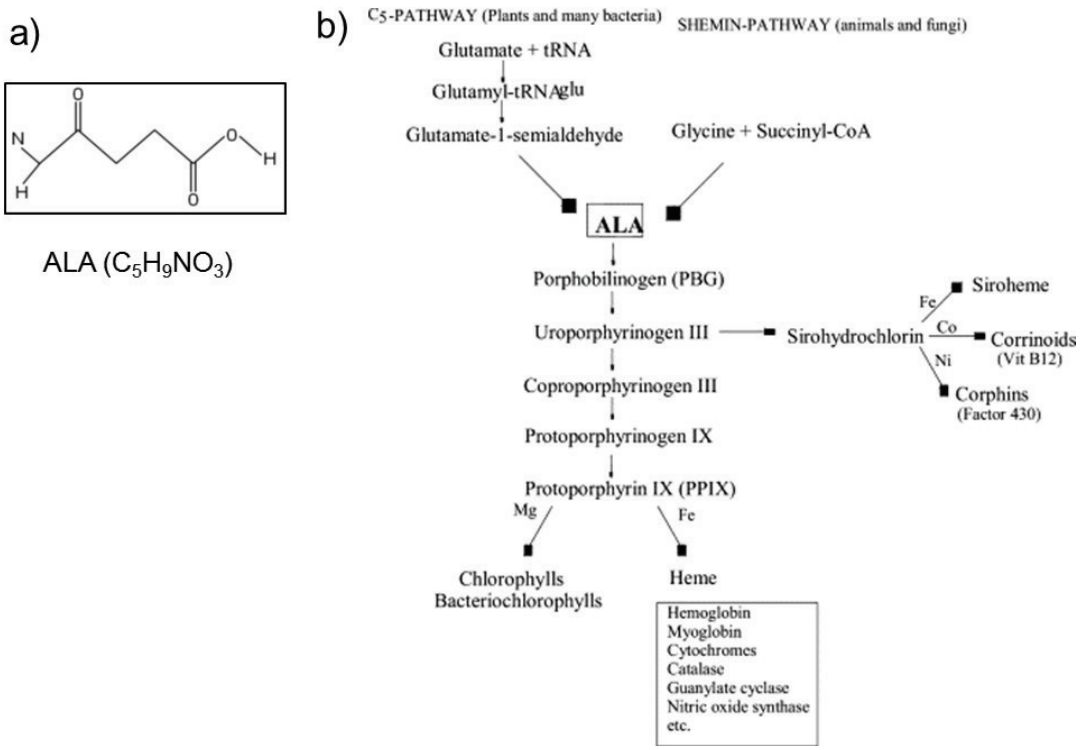

Fig. 1.2 Chemical formula (a) and biological role (b) of ALA (adapted from Fukuda et al. (76)).

Effective decontamination of packaging by ALA-based photosensitization from *B. cereus*, *S. enterica*, *L. monocytogenes*, their spores and biofilms indicate that this treatment is promising (14,71). However, ALA is a rather expensive compound and it requires a longer time (20–240 min) to produce endogenous porphyrins thus raising the cost of every ALA-based decontamination technology (14). As only living microorganisms can

metabolize colorless and odourless ALA to endogenous PSs, this appears to be a way of gaining selectivity.

Actually, as was described earlier, the optimal PS for decontamination of food or food-related surfaces should be endowed with specific features (in addition to the expected photophysical characteristics, for instance high yield of generation of $^1O_2$) such as high affinity for microbial cells, a broad spectrum of inactivation and the damage of bacteria without the development of mutagenic processes or resistant strains.

Chlorophylls are the most widespread phytochemical pigments in higher plants, algae and bacteria. Chlorophyll and its derivatives have been identified as potential antimutagens and may be chain-breaking antioxidants by acting as effective electron donors (79). The natural chlorophyll derivatives are widely known PSs in photodynamic therapy. Various chlorophyll preparations are used in the industry as food colorants to reinforce or give a green color to a manufactured product (80). Chlorophyll molecule contains a cyclic tetrapyrrole nucleus with coordinated magnesium atom at the center and a long chain hydrocarbon side chain attached through a carboxylic acid group (81). Chlorophyll is a chlorin pigment, which produced through the same metabolic pathway as other porphyrin pigments such as heme.

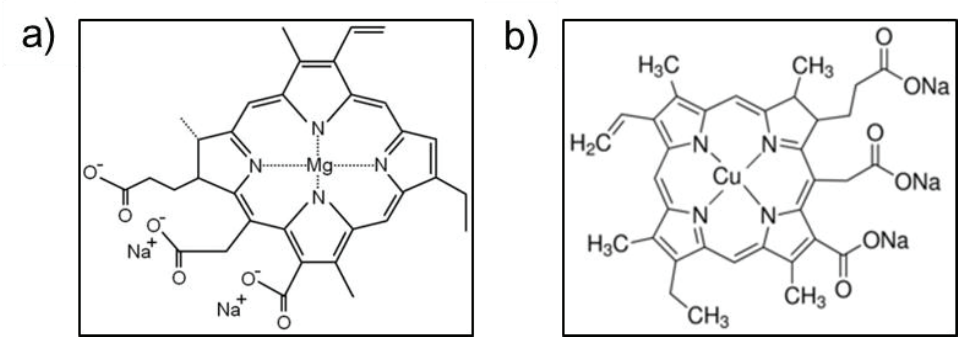

Fig. 1.3 Chemical formula of sodium chlorophyllin (not copperized form of chlorophyllin, E140ii) ($C_{32}H_2CN_4Na$) (a) and chlorophyllin sodium copper salt (E141ii) ($C_{34}H_{31}CuN_4Na_3O_6$) (b).

Natural chlorophylls are so unstable that in most research their semi-synthetic derivatives have been used as a model for several experimental designs (82). Chemical transformation of lipophilic chlorophylls into a freely

water-soluble sodium salt derivative involves removal of the phyttyl tail (Figure 1.3a) and additional replacement of the central coordinated Mg^{2+} with Cu^{2+} (Figure 3b) (83). The first type of these compounds is sample called sodium chlorophyllin, while the second one is known as chlorophyllin sodium copper salt.

Sodium chlorophyllin (Chl) (Figure 1.3a) is a water-soluble food additive (E140ii) and is widely used as food colorant in dietary supplements and in cosmetics (84).

Table 1.3 Technical information of Chl (Roth)

Appearance	grey-green powder
Dye content	≥ 97% Na chlorophyllin
Loss on drying (105 °C)	≤ 10%
pH value (10% H_2O solution)	8,5–10,0
Intensity of color E/1% 401 nm	6805 – 780 in buffer pH 9
Intensity of color E/1% 653 nm	145–165 in buffer pH 9
Arsenic (As)	≤ 0,0003 %
Lead (Pb)	≤ 0,0005 %
Average molecular weight	596 ± 9 Da

Chl is obtained by saponification of the solvent extracted products from edible plant material, grass, Lucerne, and nettle (80). It contains water soluble chlorophyll derivatives and is marketed as grey-green powder after dehydration of the chemical preparation (Table 1.3).

Although different classes of porphyrins have been tested against Gram-positive and Gram-negative bacteria, Chl-based photosensitization and the concomitant inactivation of food pathogens *in vitro* and *in vivo* is very new treatment.

1.5. Light sources for photosensitization

Various light sources can be used to activate the PS, ranging from lasers and narrow-band LED sources to high-intensity broadband light sources (14,85,86). Many factors such as the optical penetration depth of excitation light, wavelength, fluence, and drug-light interval have an impact on the photosensitization efficacy (87).

The wavelength of light necessary for the induction of lethal photobiological reaction in a microorganism depends on the structure and electron absorption spectrum of the PS (84). Porphyrin and chlorine derivatives that are commonly employed for photosensitization treatment have a characteristic absorption band between 405 and 430 nm (Soret band) and smaller absorption bands (Q-bands) above 600 nm. The wavelength also determines the penetration depth of light into tissue: 400÷500 nm light penetrates by about 300÷400 μm (surface treatment), whereas 600÷700 nm does by about 50÷200% more (deeper treatment).

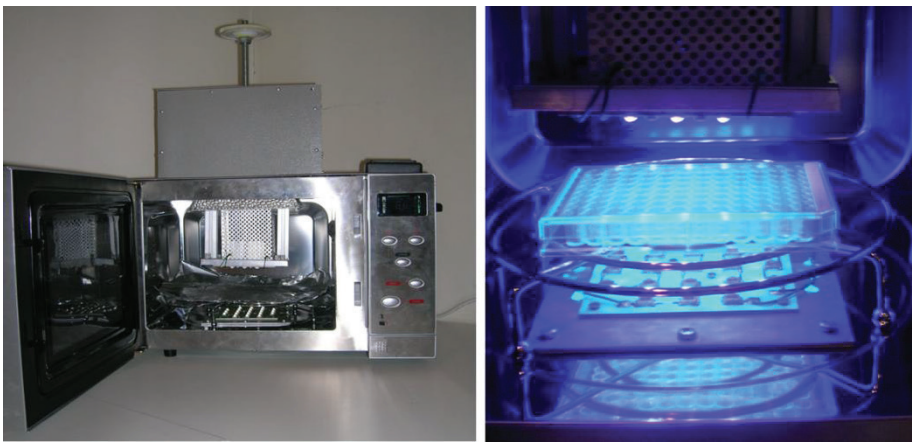

Fig. 1.4 LED-based light source prototype for decontamination of FV constructed in Vilnius University.

Initially, photosensitization was performed using conventional gas discharge and incandescent lamps equipped with color-glass filters for narrowing the spectrum. More recent applications involve incoherent sources of light specifically designed for photosensitization needs: metal halide lamp, which emits in the range of 600÷800 nm, short-arc xenon lamp (400÷1200 nm), as well as narrow-band UV free lamp (407÷420 nm) (69). Alternative light sources for activation of PSs are light emitting diodes (LEDs), which are in between lasers and conventional lamps in view of spectral properties and radiation pattern (88). With the rapid development of LEDs, these devices achieved maturity sufficient for their application in life sciences (89). The principle of operation of LEDs offers unsurpassed radiant efficiency. Besides, LEDs feature numerous advantages over conventional sources of light, such as

low driving voltage, robustness, shock and vibration resistance, the absence of hazardous agents (mercury), compactness, light-weightiness, flexibility in assembling into arrays of various forms, narrow-band emission, and the absence of unwanted spectral components. This makes them attractive for using in photosensitizing luminaries that can be safe, portable, battery driven, free of thermal side effect, and low-maintenance.

The present choice of the LED emission wavelengths covers a wide range from about 250 nm to about 7 μm. For surface treatment, deep blue and near UV InGaN LEDs, which emit at 380÷450 nm, can be used. UV LEDs have a line width in the range of 10÷15 nm. The output power of LEDs ranges from ~1 mW to 1 W. Differently from many lamps, LEDs usually feature no abrupt failure. Instead, the output flux gradually decreases with time. Advanced manufacturers provide LEDs with the output maintaining 70% of the initial value after 50,000 to 100,000 hours of exploitation (Figure 1.4).

Although various light sources can be used for photosensitization, the sufficient intensity of delivered light must be in the range 0.5÷100 mW/cm^2. Higher light intensities can induce thermal effects which actually are not desirable. The time of exposure depends on light intensity and varies from seconds to minutes. According to the World Meteorological Organization, the intensity of direct sunlight is > 12 mW/cm^2 (90). It means that the illumination required for food-processing areas (220÷540 lux) would be sufficient to photosensitize harmful and pathogenic microorganisms (52).

1.6. Antimicrobial efficiency of photosensitization

There are important differences in the efficiency of photosensitization with respect to photoinactivation of Gram-positive and Gram-negative bacteria. Gram-positive bacteria are rather more sensitive to photoinactivation as compared to Gram-negative species (65,68,73). It was found that neutral, anionic, or cationic PSs could effectively kill Gram-positive pathogen (73). Several studies have demonstrated that Gram-positive bacteria, as well yeasts,

micromycetes, bacteriophage and viruses could be inactivated by photosensitization *in vitro* (15,50,70,86,91,92). However, Gram-negative bacteria are more resistant to photosensitization. Only positively-charged (cationic) PSs or supplementation of photosensitization with permeabilizing agents are able to produce a significant killing of Gram-negative bacteria (52,73).

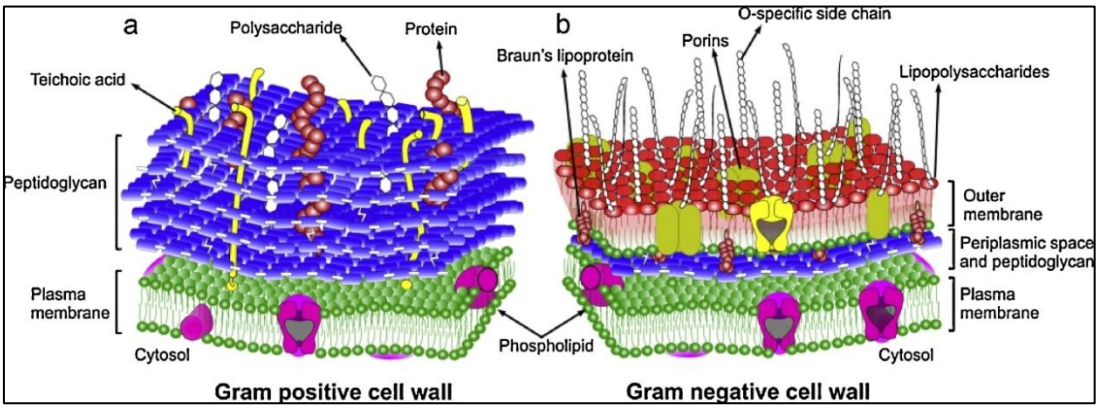

Fig. 1.5 Illustration of the cell envelope of Gram-positive (a) and Gram-negative (b) bacteria (93)

This phenomenon was further explained by the structural differences in the cell walls (Figure 1.5). Gram-positive bacteria are much more susceptible to anionic and neutral PS because they have a cytoplasmic membrane surrounded by a relatively porous layer of peptidoglycan (15÷18 nm) and lipoteichoic acid traversing this wall and allowing the PS to cross it (59,71). The cell envelope of Gram-negative bacteria consists of inner cytoplasmic and outer membranes which are separated by the very dense peptidoglycan-containing periplasm. The outer membrane contains phospholipids, proteins, lipopolysaccharide trimers and lipoproteins. Liposaccharides are the major component of the outer membrane of Gram-negative pathogens that protect the membrane (59). They have an additional negatively charged outer membrane that forms a protective barrier between the cells and the environment (94) and just positively-charged PS can bind the negatively-charged cell surface (71).

Preferably, photosensitization treatment should be performed with cationic PSs in Gram-positive and Gram-negative bacteria species, because they are generally more efficient and can act at lower concentrations than

neutral and negatively-charged PS molecules. Sometimes, negatively-charged or neutral PSs at high concentration were more effective than cationic PSs (95,96). In this case, generation of reactive intermediates in close vicinity of cell structures either causes direct oxidation of these components or allows transmembrane diffusion of reactive intermediates and oxidative damage to various intracellular targets (97). Generally, photosensitization treatment predominantly precedes via type II processes. However by comparing PSs that tend to undergo either type I or type II mechanism, Huang et al. reported that Gram-negative pathogen are more susceptible to ($^{\bullet}OH$) than ($^{1}O_2$) (56). A type I reaction is therefore favored when targeting Gram-negative species.

1.7. Decontamination of strawberries by photosensitization

Fruits and vegetables are essential for a nutritionally balanced diet since they contain a wide range of health promoting components. Strawberries (*Fragaria* × *ananassa* Duch.) are delicate fruits and a relevant source of bioactive compounds due to high levels of vitamin C, vitamin E, β-carotene, total phenolic acids and flavonoids (98). Moreover, they have been reported to contain highest antioxidant capacity among twelve fruits analyzed (99). The main contributors to antioxidant activity are phenolic compounds which prevent cancer, cardiovascular diseases, and age-induced oxidative stress (100). Anthocyanins are the most abundant flavonoids (phenolic acids) and are present in high levels in mature strawberries (101).

Strawberry is a major crop with 4÷5 millions in tons of production worldwide (9), whereas European production of strawberries reaches 1.1 Mt annually (102). However, short ripening and senescence periods of strawberries, susceptibility to mechanical injury, contamination during storage with fungi and bacteria reduce significantly their postharvest shelf life. Postharvest losses are typically more severe, especially when conditions are favorable for disease development; in some cases 80÷85% of a crop may be lost (103).

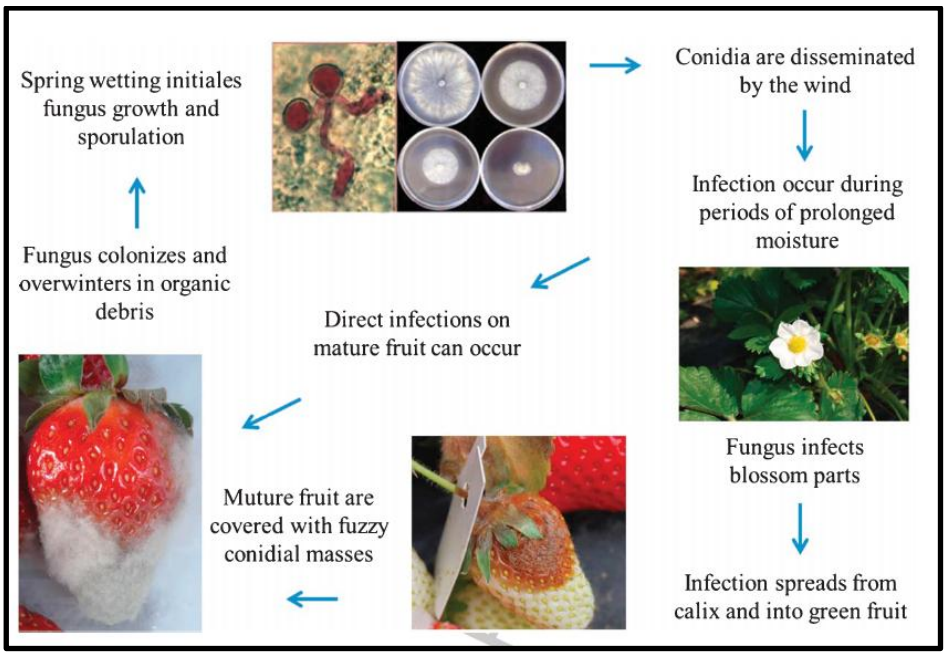

Fig. 1.6 Disease cycle of gray mold on strawberry (104)

Grey mould, caused by *Botrytis cinerea* Pers. (ex Fr.), is the most severe postharvest disease of strawberries. The disease can begin preharvest, remaining as latent infections, or begin postharvest (Figure 1.6). It can survive on organic debris in the field, as sclerotia, and under favorable conditions, it sporulates and releases quantities of spores that serve as a potential source for infection of flowers and fruits (105). This fungus continues to grow at 0 °C. However, growth is slow at this temperature. Thus, *B. cinerea* is one of the most economically important postharvest pathogens that causes 30÷40% losses of the harvest if no chemical control is applied (10).

As a role, strawberries are consumed in a raw, unprocessed form (9), thus no thermal treatment is possible for reduction of microbial contamination and delay of spoilage. Traditional methods used for extending the self-life of strawberries are low temperature and modified atmosphere packaging (106). Meanwhile the storage of strawberries at these conditions is not enough effective and induces detrimental effects on their nutritional quality (107). Natural antimicrobial compounds, such as methyl jasmonate or essential oils have been investigated as alternative methods to prolong their storage. Meanwhile, most of them reduce the activity of antioxidant enzymes (108).

Therefore it's necessary to find more effective and environmentally friendly techniques to meet consumer demands on safe, healthy and attractive fresh fruits. The development of edible coating based on natural biopolymers (lipids, proteins, polysaccharides and their derivatives) can be alternative to existing preservation technologies. Chitosan (CHS) is a polysaccharide derived from chitin, which is known as the second most abundant biopolymer in nature after cellulose. The cationic character of CHS offers good opportunities to take advantage of electron interactions with numerous compounds during processing and to incorporate specific properties into the material (109). The application of edible coating based on CHS on fresh fruits slightly extended their post-harvest life (110,111).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Reagents were obtained from company **Carl Roth GmbH&Co**: sodium chlorophyllin, KCl, KH_2PO_4, sterile 0.20 μm filter; **Fluka-Sigma-Aldrich**: low molecular weight chitosan, 5-aminolevulinic acid hydrochloride (ALA), methanol, acetate buffer, 2,4,6,-tripyridyl-s-triazine (TPTZ, $FeCl_3 \times 6H_2O$); **ACROS**: sodium azide, sodium hypochlorite (13%); **CHEMAPUR**: hydrochloric acid 35–38% (HCl), glycerol; **Liofilchem**: Luria-Bertani medium, agar, dichloran glycerol (DG18) agar , tryptone soya medium supplemented with 0.6% yeast extract, tryptone soya agar supplemented with 0.6% yeast extract; Chloramphenicol; **Difco**: Potato dextrose agar medium ; **OXOID**: NaCl; **REACHEM**: $Na_2HPO_4x12H_2O$; **Sigma**: 97% acetone; **MERCK**: Triton X-100; **Vilniaus degtinė**: 96% etanol; **B/Braun**: Meliseptol rapid disinfectant.

2.1.2. Media

The bacterial cultures were grown in Luria Bertani (LB) liquid and on agar medium (LBA), in Tryptone Soya supplemented with 0.6% yeast extract liquid (TSYE) and on agar (TSYEA) medium. Antifungal activity against molds was evaluated onto dichloran glycerol (DG18) agar and potato dextrose agar medium (PDA).

Composition of LB medium (1 l): 10 g tryptone, 5 g yeast extract, 10 g NaCl. Composition of TSYE medium (1 l): 30 g tryptone soya broth, 6 g yeast extract. Preparation of agar medium (LBA and TSYEA) was made by adding 15 g of agar per 1 l of the liquid medium. PDA and DG18 agar media was received from the manufacturer and prepared according to the manufacturer's

instructions. All media were sterilized by autoclaving. Antibacterial antibiotics chloramphenicol (0.1 g) was added in DG18 medium after autoclaving (medium temperature 50 °C).

2.1.3. Bacterial and fungal strains

Strains used in the study are listed in Table 2.1.

Table 2.1 Bacterial and fungal strains.

		Strain	**Source**
Bacteria	*Salmonella enterica* serovar Typhimurium	DS88 (SL5676 Sm^2 pLM32)	Prof. D. H. Bamford (University of Helsinki, Finland).
	Listeria monocytogenes	$ATC_{L3}C$ 7644	National Veterinary Laboratory (Vilnius, Lithuania)
	Bacillus cereus	ATCC 12826	National Centre of Public Health (Vilnius, Lithuania)
Fungus	*Botrytis cinerea*	Sclerotiniaceae family	Vilnius University collection.

Salmonella enterica is a Gram-negative, motile and non-sporeforming, facultative anaerobic rod (Figure 2.1a) that is 0.7÷1.5 by 2.0÷5.0 μm in size (112). *Salmonella* will grow in a broad pH range of 3.8÷9.5 and the minimum pH at which it can grow is dependent on temperature, presence of salt and nitrite and the type of acid present (113). *S. enterica* has been isolated from a wide range of food products: fresh produce, fruits, meat products and retail foods (114). Some *S. enterica* strains are resistant to at least five antimicrobial agents ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (18). *Salmonella* is susceptible to most disinfectants, moist heat and dry heat, but is resistant to nitrites (115).

Listeria monocytogenes is a Gram-positive, non-sporeforming, facultative anaerobic rod-shaped coccobacillus, typically measuring 0.5 to 2 μm in diameter (Figure 2.1b) (116). *L. monocytogenes* is highly pathogenic bacteria (20). The food-borne illness caused by these bacteria is known as listeriosis. *L. monocytogenes* has the ability to grow in a wide range of

temperatures (−0.4 to 45 ºC), pH (4.3 to 9.6) and salt concentrations (up to 10% NaCl) as well as at low water activity (*Aw* down to 0.90). Another threat is their extremely strong adherence to the different surfaces in food-processing industry (stainless steel, polypropylene, aluminum, glass). *L. monocytogenes* has been isolated from various range of food products: vegetables, milk, soft and farmhouse cheeses, fish and meat products as well as various ready-to-eat products (117). *L. monocytogenes* is rather susceptible to antibiotics (118) and at room temperature, is susceptible to sodium hypochlorite, iodophor compounds, and quaternary ammonium compounds (119). Meanwhile of *L. monocytogenes* inactivation was performed using several emerging antibacterial technologies: high hydrostatic pressure HHP(35,120), pulsed UV light (40,41,121), pulsed electric field (122), ionizing radiation (123).

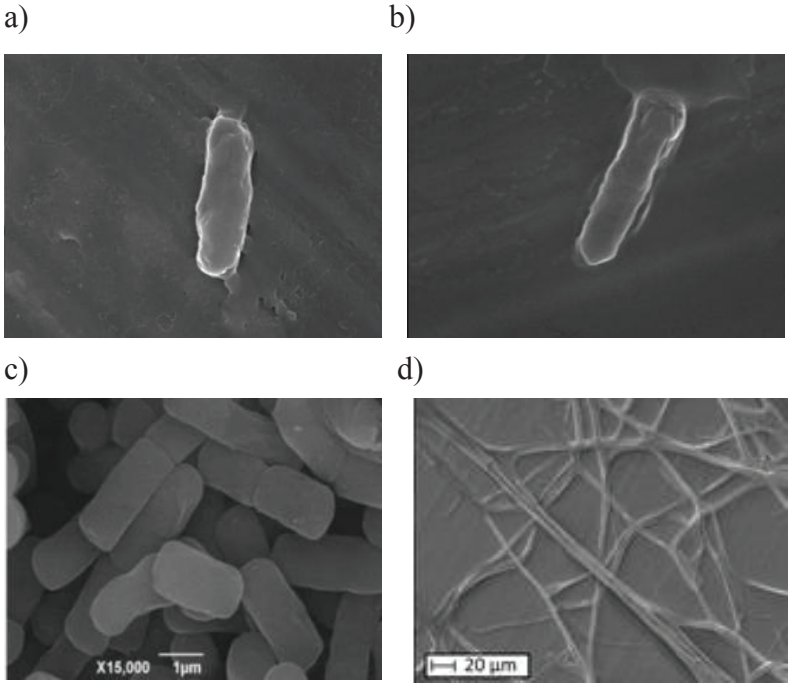

Fig. 2.1 Scanning electron microscopy image of *S. enterica* (a) (124), *L. monocytogenes* (b) (92), *B. cereus* (c) (125), *B. cinerea* (d) (92).

Bacillus cereus are 1.4 µm Gram-positive, facultative anaerobes rods usually appear as pairs and short chains that are motile and able to form endospores (Figure 2.1c). *B. cereus* is chemoorganotrophic and grows above 10÷20 ºC and below 35÷45 ºC with an optimum temperature of about 37 ºC (126). *B. cereus* can contaminate a wide range of foods including meat, cereals,

fresh vegetables, berries and fruits. The bacteria are able to produce six types of toxins: five enterotoxins and an emetic toxin, which can be heat-stable or heat-labile depending on the strain (127,128). *B. cereus* is resistant to penicillin, ampicillin, cephalosporins, trimethoprim (126). Gluteraldehyde, sodium hypochlorite, paracetic acid, activated hydrogen peroxide, chlorine dioxide, formaldehyde, iodine, acids, and alkali are chemical agents against *Bacillus* bacteria and spores. These chemical agents should be highly concentrated and require greatest contact time to kill spores (129).

Gray mold (*Botrytis cinerea*) is one of the most economically important pathogen of soft fruits worldwide (Figure 2.1d). *Botrytis* infects many host plants in all climate areas of the world, infecting mainly upper plant parts at pre and post-harvest stages (130). *B. cinerea* is a necrotrophic fungus which can live pathogenically and also saprophytically (105). This mold exists in the different habitats as chlamydospores, mycelia, sclerotia, ascospores, apothecia, micro and macro-conidia (130). *Botrytis* can survive in the field, as sclerotia, and under conducive conditions, it releases quantities of spores that serve as a potential source for infection (105). This fungus may cause 30÷40% losses of the harvest if no chemical control is applied (10).

2.1.4. Solutions

1. **ALA:** Molecular formula: $C_5H_9NO_3 \cdot HCl$. Stock solution of ALA was prepared by dissolving ALA in 0.01 M PBS buffer (pH 7.4). Na OH (0.2 M) 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 (131).
2. **CHS:** Aqueous stock solution of CHS (pH 2.4 at 20 ºC) containing 1 % of CHS and 0.18 % of HCl was prepared dissolving in water appropriate amounts of HCl and then CHS.
3. **Chl:** Aqueous stock solution of Chl was prepared by dissolution of Chl in autoclaved distilled water.

4. **Chl–CHS conjugate:** Aqueous stock solution of Chl–CHS conjugate (pH 2.4 at 20 ºC) containing 1% of CHS, 0.01% Chl and 0.18% of HCl was prepared by drop wise addition of aqueous 0.05% Chl solution into rapidly spinning aqueous solution containing 1.25% of CHS and 0.23% of HCl. After addition of Chl–CHS conjugate to bacterial suspension in NaCl, the pH of final bacterial suspension changed to 3.95. The 0.001–0.1% concentration of Chl–CHS conjugate corresponded to $1.5 \cdot 10^{-5}$ M concentration of Chl.
5. **0.01 M PBS buffer (pH 7.4):** 2.7 mM KCl, 1.8 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4.

2.1.5. Equipment

Environmental shaker - incubator ES–20 (Biosan, Latvia), Thermostat (Memmert, Germany), Magnetic stirrer C-MAG HS7 (IKA, Germany), Spectrophotometer Helios γ (ThermoSpectronic, Great Britain), spectrophotometer UV/Vis Lambda 25 (Perkin Elmer, Germany), centrifuge MPW-260R (MPW Med. instruments, Poland), PerkinElmer LS 55 fluorescence spectrophotometer (Beaconsfield, United Kingdom), Q150T ES sputter coater (Quorum Technologies, England), SEM microscope Apollo 300 (CamScan, UK), BagPage (Interscience,France), spectrometer E580 FT-EPR (Bruker Elexsys, JAV), 3 Sigma meter (Coherent, U.S.A.), autoclave, quartz cuvette (Hellma-analytics QS, Germany), capillaries (BLAUBRAND micropipettes, intraMark, Hinckley, Great Britain), Climacell 111, (MM Medcenter Einrichtungen GmbH, Germany), Raman Spectrometer "Bruker MultiRam" (Germany), Packing yellow trays (LINPAC, West Yorks, UK).

2.1.6. Light sources used for experiments

LED based light source was constructed at the Institute of Applied Research of Vilnius University. An InGaN light emitting diodes (LED) array

(LED Engine, San Jose, USA; Inc. LZ1-00UA00) were used for construction of light source to photoactivation of bacteria. It consisted of illumination chamber and supply unit (Figure 2.3).

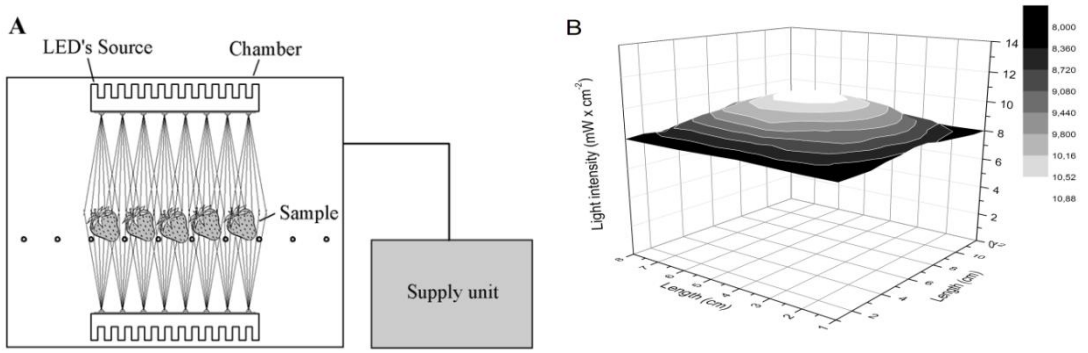

Fig. 2.3 Schematic presentation of LED-based light source prototype (A) and light intensity three-dimension distribution (B).

A cooling system was integrated in the light prototype to dissipate heat from the source and minimize any heat transfer to the sample. LED emission maximum was at 405 nm with a band width of 13 nm at full-width half maximum. Two rectangular 6 × 10 arrays (top and bottom), consisted of 60 LEDs, powered by a 20 V DC power supply were integrated in the chamber. The light intensity at the surface of samples from top and bottom LED reached approximately 10 mW/cm^2 (6 cm from the light source) and 11 mW/cm^2 (3.5 cm from the light source), respectively. Light dose was calculated as light intensity multiplied by irradiation time. The sample exposure time was adjusted according to the equation:

$$E = P \cdot t, \quad (1)$$

where E is the energy density (dose) in J/cm^2, P is the irradiance (light intensity) in W/cm^2, and t is the time in seconds. Three-dimensional model of distribution of power density of the emitted light from the top and bottom in the prototype is presented in Figure 2.3 B. Almost the same power density distribution was registered from LEDs in the bottom of prototype. However, distributions from the top and from the bottom cannot be placed in one picture

since it would overlap. The variation of light intensities on the illumination square was insignificant, since we use just central part of it (± 0.5 mW/cm^2).

2.1.7. Software and internet database

OriginPro 8 (OriginLab Corporation, JAV), Microsoft Excel 2010 (Microsoft, JAV), StatSoft Statistica 10 (JAV), Primer3 (v. 0.4.0) database (http://frodo.wi.mit.edu/Primer3).

2.2. Methods

2.2.1. Spectrophotometric and fluorimetric measurements

Absorption spectra were recorded by using Helios γ and UV/Vis Lambda 25 spectrophotometers, and fluorescence spectra were recorded by Perkin Elmer fluorescence spectrophotometer LS-55. Quartz cuvette was used for measurements.

1. Fluorescence and excitation spectrum measurements of intracellular porphyrins:

The cell suspensions for measurements of endogenous porphyrins from ALA were prepared as follows. Cells ($1 \cdot 10^7$ cfu/ml in 0.01 M PBS (pH 7.4)) were incubated in the dark at 37 °C with $3 \cdot 10^{-3}$ and $7.5 \cdot 10^{-3}$ M ALA concentration for the indicated time. Then 2 ml aliquots of bacterial suspension were withdrawn by centrifugation (10 min, 6 °C, 3574 g), resuspended in 0.01 M PBS and afterwards used for fluorescence measurements. Fluorescence spectrophotometer was used for the fluorescence detection. Scan range parameters for excitation spectrum are as follows: 1) emission wavelength: 610nm; 2) excitation wavelength: 250÷450nm; 3) ex slit: 2.5nm; 4) em slit: 15nm; 5) scan speed: 200 nm/min. Scan range parameters for fluorescence spectrum are as follows: 1) excitation wavelength: 390nm; 2) emission:

590÷750nm; 3) ex slit: 2.5nm; 4) em slit: 15nm; 5) scan speed: 200 nm/min. Evaluation of endogenous porphyrins produced by cells was performed according methodology described in literature (131).

2. Absorption and fluorescence measurements of Chl and Chl–CHS conjugate:

Chl diluted by 0.01 M PBS and Chl–CHS conjugate diluted by 0.9% NaCl were used for absorption and fluorescence measurements. The absorption spectra of Chl and Chl–CHS solutions were recorded between 300 and 710 nm by spectrophotometer. Fluorescence spectrophotometer was used for the fluorescence detection. Scan range parameters are as follows: 1) excitation wavelength: 405nm; 2) emission: 550÷750nm; 3) ex slit: 10nm; 4) em slit: 4nm; 5) scan speed: 200 nm/min.

To observe monomeric Chl forms of Chl–CHS conjugate 20 µl of Triton X-100 to 20 ml of suspension was added.

Cell-chlorophyllin interaction was evaluated fluorimetrically. Cells ($1 \cdot 10^7$ cfu/ml in 0.01 M PBS) (*S. enterica* and *L. monocytogenes*) with $1.5 \cdot 10^{-5}$ and $1.5 \cdot 10^{-7}$ M Chl were incubated in the dark at 37 ºC for the 2÷60 min. To evaluate the amount of cell-attached Chl, 3 ml aliquots of bacterial suspension after incubation were centrifugated (10 min, 6 °C, 3574 g) and resuspended in 0.01 M PBS. Supernatant and cells in PBS were used immediately for fluorescence measurements.

2.2.2. Cultivation of the microorganism

Table 2.2 Bacterial growth conditions.

Strain	Medium	OD_{540}	cfu/ml
Salmonella enterica serovar *Typhimurium* DS88 (SL5676 Sm^2 pLM32)	LB	1.3	$5 \cdot 10^8$
Listeria monocytogenes $ATC_{L3}C$ 7644	TSYE	0.9	$1.16 \cdot 10^9$
Bacillus cereus ATCC 12826	LB	1	$6 \cdot 10^7$

Conditions for bacterial growth are described in the Table 2.2. The bacterial cultures were grown overnight (~16 h) at 37 ºC in 20 ml of liquid

medium with aeration of 120 rev/min. The overnight bacterial cultures were 20-times diluted by the fresh medium (OD_{540} = 0.164) and grown at 37 °C to the mid-log phase (respective colony forming units (cfu)/ml and OD_{540}, Table 2.2) in a shaker (120 rev/min). Bacterial optical density was determined in a 1 cm glass cuvette at λ=540 nm (Heλios Gamma & Delta spectrophotometers). The cells were then harvested by centrifugation (10 min, 6 °C, 3574 g) and resuspended in a 1 ml of 0.01 M PBS buffer (pH=7.4) or normal saline 0.9% NaCl, to give ~$2.5 \cdot 10^9$ cfu/ml. These stock suspensions were diluted accordingly to ~$1 \cdot 10^7$ or $1 \cdot 10^8$ cfu/ml and immediately used for the experiments.

The culture of *B. cinerea* was isolated from strawberries. This fungus was sub-cultured on potato dextrose agar (PDA) (at 24 °C) for further experimentation.

2.2.3. ALA-based photosensitization *in vitro*

Aliquots (10 ml) of bacterial suspension (~$1 \cdot 10^7$ cfu/ml in 0.01 M PBS buffer (pH = 7.4)) with appropriate concentration of ALA ($3 \cdot 10^{-3}$ and $7.5 \cdot 10^{-3}$ M) were incubated in the dark in a 50 ml plastic bottle for cell culture cultivation at 37 °C. For the following experiments, the cells were incubated in the shaker (120 rev/min) 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 illuminated (0÷20 min). LED based light source for illumination was constructed in the Institute of Applied Sciences of Vilnius university and emitted light λ=405 nm (Figure 2.3).

2.2.4. Inactivation of bacteria by Chl-based photosensitization and photoactivated Chl – CHS conjugate *in vitro*

Aliquots of bacterial suspensions (~$1 \cdot 10^7$ cfu/ml) with appropriate concentration of Chl ($1.5 \cdot 10^{-5}$ M÷$7.5 \cdot 10^{-8}$ M) or Chl–CHS conjugate

(0.00001/0.001%÷0.001/0.1%) stock solutions were incubated for cell culture cultivation in the shaker (120 rev/min) in the dark at 37 °C for different periods (0–120 min). For the photoactivation treatment, 150 μl of the samples were withdrawn, placed into sterile flat bottom wells and then exposed to the light (λ=405 nm, illumination dose 0÷51 J/cm^2).

Also after appropriate incubation time (2 min for *Listeria* and 60 min for *Salmonella*) with various concentrations of Chl in the dark at 37 ºC the bacterial suspensions were centrifuged (10 min at 3574 g, + 6 °C) and the pellets resuspended in the same volume of autoclaved distilled water. Centrifugation of aliquots was used to remove the excess of PS that was not taken up by the microbial cells when experiments required it. Then 150 μl of the samples exposed to the light (λ=405 nm, illumination dose 0÷88.7 J/cm^2).

2.2.5. Inactivation of *Botrytis cinerea* by Chl-based photosensitization and photoactivated Chl–CHS conjugate

The culture of *B. cinerea* was isolated from strawberries and grown on potato dextrose agar (PDA) for further experimentation. The final $1.5 \cdot 10^{-4}$ M Chl and 0.001 Chl–0.1% CHS conjugate concentrations were obtained by adding 40 ml of stock solution to 400 ml autoclaved PDA medium. The control samples contained PDA only. Each Petri dish was inoculated in the center with an agar disk (diameter 5 mm) bearing mycelium growth from a 4-day-old *B. cinerea* culture. After 24 h incubation the tested plates containing Chl and Chl–CHS conjugate were exposed to light (λ=405 nm) for different fluence (illumination dose 35 and 76 J/cm^2 respectively). Afterwards all dishes were sealed with parafilm to avoid the evaporation of volatile compounds and incubated at 24 ºC until the control plates were completely covered with mycelium. Each treatment was repeated 3 times.

The radial growth of fungal mycelium was recorded and the radial inhibition was recorded, and the radial inhibition was calculated when growth

of mycelia in control plate reached the edge of the Petri dish. The following formula was used for calculation of the inhibition rate (%):

Inhibition rate (%) =R – r/R × 100,

where R is the radial growth of fungal mycelia on the control plate and r is the radial growth of fungal mycelia on the plate treated with Chl or Chl–CHS conjugate.

2.2.6. Evaluation of antibacterial activity of CHS and Chl–CHS conjugate (in the dark)

Aliquots of 20 ml of bacterial suspensions (~$1 \cdot 10^7$ cfu/ml in 0.9% NaCl) containing CHS (0.1%) and Chl–CHS (0.00001–0.001% and 0.001–0.1%) conjugate were incubated in 50 ml flasks for cell culture cultivation in the shaker (130 rev/min) at 37 ºC. The samples were removed after 1 min, 15 min, 30 min, 60 min and 120 min.

2.2.7. Scanning electron microscopy (SEM)

The effect of Chl-based photosensitization and photoactivated Chl–CHS conjugate treatment on the morphology of *S. enterica* and *L. monocytogenes* was examined by SEM (CamScan Apollo 300). After appropriate treatment, the samples consisting 20 µl of bacterial suspension were withdrawn, transferred on to copper foil, air-dried and coated with 15 nm gold layer using Q150T ES sputter coater. The scanning was performed using electron beam with an accelerating voltage of 20 kV.

2.2.8 Evaluation of cell membrane integrity of treated bacteria

The bacterial cell membrane integrity was examined spectrophotometrically evaluating the cell release material which has absorption at λ=260 nm (132) and λ=280 nm (133). The bacterial suspension

(~$1 \cdot 10^7$ cfu/ml *Salmonella* and ~$1 \cdot 10^8$ cfu/ml *Listeria*) after appropriate incubation time with $1.5 \cdot 10^{-5}$ M Chl or 0.001 Chl–0.1% CHS conjugate (at 37 ºC in the dark) was illuminated. Aliquots of 3 ml cell suspension were withdrawing at different illumination time points corresponding to illumination doses:

- $1.5 \cdot 10^{-5}$ M Chl *Salmonella*: 0; 5.8; 11.5; 17.3; 23; 40.3 and 46.1 J/cm^2;
- 0.001 Chl–0.1% CHS conjugate *Salmonella*: 25 and 38 J/cm^2;
- $1.5 \cdot 10^{-6}$ M Chl *Listeria*: 0; 0.29; 0.58; 1.15; 11.5 J/cm^2.

The samples (control and treated) were filtered (0.20 μm filter) to remove the bacteria(132). The UV absorbance of cell supernatant at 260 and 280 nm was determined using spectrophotometer (He*λ*ios Gamma *&* Delta).

2.2.9. Detection of $^1O_2$ in Chl-based photo treated bacteria

In order to identify the singlet oxygen ($^1O_2$) participation in Chl-based photosensitization induced cell damage and assess its impact on bacterial survival, bacteria cell suspensions (~$1 \cdot 10^8$ cfu/ml *Listeria* and ~$1 \cdot 10^7$ cfu/ml *Salmonella*) were amended with the exogenous scavenger sodium azide (NaN_3) (physical quencher of $^1O_2$) (60,134). Stock solutions of NaN_3 were prepared in autoclaved distilled water and used at a final concentration of 0÷40 mM. The bacterial cells were incubated in the dark at 37 ºC for 2 and 60 min with various concentrations of Chl with and without NaN_3 (with and without wash). After corresponding incubation time, 150 μl of the samples were withdrawn, placed into sterile flat bottom wells and then exposed to the light (λ=405 nm) for 0÷70 min (illumination dose 0÷88.7 J/cm^2).

2.2.10. Photoinactivation of bacteria attached to the packaging material

Packing yellow trays were provided by LINPAC (Figure 2.4). In order to simplify experiments, the packaging samples for photosensitization experiments were cut into 4 × 8 cm pieces and each immersed in 50 ml

bacteria suspension (~$1 \cdot 10^7$ cfu/ml). The immersed samples were kept in laminar for 30 min for further attachment of the bacterial cells. Then appropriate packing samples were incubated in the dark with $3 \cdot 10^{-3}$ M (experiments with *B. cereus*) and $7.5 \cdot 10^{-3}$ M (experiments with *S.enterica*) ALA, and $1.5 \cdot 10^{-5}$ M Chl for different periods (5, 10 and 20 min).

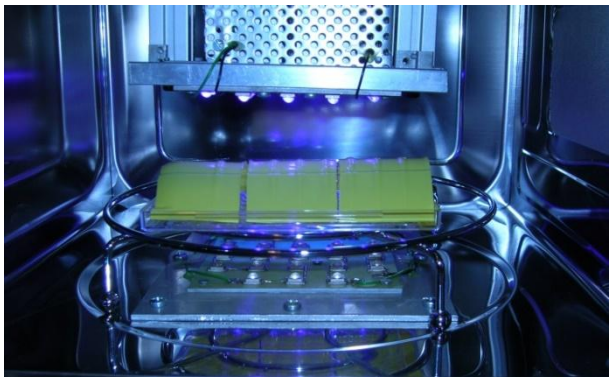

Fig. 2.4 Packaging samples for photosensitization experiments.

The control samples were incubated with PBS (7.4 pH) buffer. After incubation with PSs all packaging samples were dried at room temperature for 20 min, placed in the treatment chamber and exposed to light for different time (5, 10, 15 and 20min). The control sample was not illuminated.

2.2.11. Decontamination of strawberries by photoactyvated Chl–CHS

2.2.11.1. Comparative analysis of different antimicrobial treatments decontaminating strawberries

Strawberries (Fragaria × ananassa Duch.) in partially ripe stage were purchased in a local supermarket and used within 1 day. Some strawberries with natural microflora were soaked 30 min in 0.1% CHS (1, CHS coating), in 0.001 Chl–0.1% CHS (2, dark toxicity, Chl–CHS coating), in 0.001% Chl–0.1% CHS (3, photoactivated, Chl–CHS coating), in 0.001% and 0.1% Chl (4 and 5, photoactivated), and 1 min in 200 ppm sodium hypochlorite (NaOCl) (sample 6) solutions. The samples 3, 4 and 5 were placed in the treatment chamber in a sterile Petri dish (without cover) and exposed to 405 nm light for

60 min (light dose 76 J/cm^2). Control samples were unsoaked in 0.9% NaCl and were not illuminated in the chamber.

2.2.11.2. Evaluation of shelf-life of treated strawberries

For evaluation of shelf-life of treated berries the Kaplan-Meier survival curves were used (135). The samples after appropriate treatment were stored at 22 ± 2 ºC. The control samples were not irradiated and stored under the identical conditions. Berries were observed until visually detectable spoilage spots (visible fungi) occurred on the surface. In parallel the spoilage of treated berries was evaluated according to Kittemann et al. (136) The level of infected berries was scored on a 1÷6 scale. Results were expressed as a disease index between 0÷100 (0-no infection; 100-all berries are infected). The appearance of strawberries was examined according to the method described by Mahmoud et al. (137). The overall appearance of fruits was visually measured using a scale: 8÷9 = excellent, 6÷7 = very good, 5 = good, 1÷4 = not acceptable (visible mold grown).

2.2.11.3. Measurements of total antioxidant capacity in treated strawberries.

Total antioxidant capacity of strawberries was measured by ferric reducing ability of plasma (FRAP) method (138). Extract for measurement was prepared by homogenization of 1 g of fruit with 50 ml methanol. FRAP working solution included 0.3 M/l acetate buffer (pH 3.6), 0.01 M/l 2,4,6,-tripyridyl-s-triazine (TPTZ) in 0.04 M/l HCl and 0.02 M/l $FeCl_3 \times 6H_2O$ in distilled water. The FRAP reagent was prepared fresh daily by mixing 100 ml acetate buffer, 10 ml TPTZ, and 10 ml $FeCl_3 \times 6H_2O$, and it was warmed to 37 ºC in the dark prior to use. For measurement of antioxidant activity, 3 ml of FRAP reagent and 100 µl of sample solution were mixed, and the absorbance of the reaction mixture was then recorded at 593 nm (Heλios Gamma; ThermoSpectronic) after 4 min, 1 cm light path. The standart curve was constructed using Fe (II) sulfate solution (100÷1000 µM), and the results were

expressed as mM Fe^{2+}/kg dry weight of food material. All of the measurements were taken in triplicate and the mean values were calculated.

Possible changes of strawberry color after treatment were evaluated from absorption spectrum measuring OD of the sample in visible region of spectrum (139). Samples weighing 10 g of fresh berry were blended in a food processor for 2 min (speed 8) with 75 ml of a mixture of methanol, acetic acid, and distilled water (at a ratio of 25M:1A:24W). The mixture was then centrifuged at 13392 g for 20 min. OD (350÷650 nm) was measured using 1 cm path length quartz cuvette with UV/VIS spectrophotometer Lamda 25 (PerkinElmer). Each sample was extracted 3 times.

2.2.11.4. Measurements of strawberry weight losses

All strawberries were divided in 2 groups (every consisted of 20 berries). Control berries were not treated. Other group was coated with Chl–CHS, dried and afterwards illuminated with visible light (76 J/cm^2). Strawberries were kept in cooling incubator at 6 ± 0.5 °C with controlled humidity (75 ± 5%) (Climacell 111). Every 24 h the weight of berries was checked. Weight loss was determined in %.

2.2.11.5. FT-Raman spectroscopy for detection of water content in strawberries

FT-Raman spectra of control and Chl–CHS coated strawberries were recorded by FT-Raman Spectrometer "Bruker MultiRam" (Germany). Light for excitation was λ=1064 nm, power of excitation was - 100 mW. Wavenumber range of spectral investigation was 3500÷500 cm^{-1}.

2.2.11.6. Electron spin resonance (EPR)

EPR spectra were registered with Bruker Elexsys E580 FT-EPR spectrometer (Billerica, USA) working in X-band. EPR spectra of free radicals

in strawberries were recording after treatment by photoactivated 0.001 Chl–0.1% CHS conjugate (1 h incubation; light dose 76 J/cm^2). Before recording spectra the surface of treated and not treated strawberries was peeled, and the peelings were homogenized. The capillaries (BLAUBRAND micropipettes) were filled with 1 g of strawberry. After that capillaries were put into the standard EPR tube.

2.2.12. Evaluation of antimicrobial activity

The antibacterial effects of all treatments on bacteria and antifungal activity were evaluated by the spread plate method.

Antibacterial activity *in vitro*

Thus, 100 µl of appropriate dilutions of bacterial test culture after treatment *in vitro* was surface inoculated on the separate LBA plate. Afterwards, the bacteria were kept in the thermostat for 24 h at 37 °C. Bacterial counts were transformed from cfu/ml into $\log_{10}$/ml. Detection limit of spread plate method-one colony forming unit.

Antibacterial activity *in vivo*

In order to detach bacteria from the surface, all packaging samples were placed in a sterile BagPage with 30 ml of 0.01 M PBS and washed for 1 min with a Bagmixer. Then 100 µl of appropriate dilutions of bacterial test culture were surface inoculated on the separate plate. Afterwards, the plates were kept in the thermostat for 24 h at 37 °C. The surviving cell populations were enumerated and expressed as $\log_{10}$ cfu/cm^2.

Antifungal activity *in vivo*:

The 1 g of each strawberry and 9 ml of 0.9% NaCl solution placed to sterile BagPage and homogenized 2 min using BagMixer. Afterwards, 100 µl of appropriate dilutions (in 0.9% NaCl) of homogenized strawberries suspension placed onto TSYE agar for total mesophilic bacteria count and onto dichloran glycerol (DG18) agar for yeast/microfungi count. Plates were placed in the thermostat for 24 h at 37 and 30 °C respectively. The surviving cell

populations were enumerated and expressed as $\log_{10}$ cfu/g. Every sample consisted of 1 berry, and experiments were repeated 3–6 times.

2.2.13. Statistical analysis

The experiments were triplicated for each set of exposure. A standard error was calculated for every experimental point and marked in a figure as an error bar. Sometimes the bars were too small to be visible. The data were analyzed using Origin 7.5 software (*OriginLab Corporation*, Northampton, MA 01060, USA). The significance of the incubation time with Chl–CHS conjugate on survival of microorganisms *in vitro* and on the surface of strawberries was assessed by the analysis of variance (ANOVA) model with the Bonferroni post hoc test. A value of $P<0.005$ was considered as significant. For weight, amount of total antioxidants, viability of strawberries results a value of $P<0.05$ was considered as significant.

3. RESULTS

3.1. Detection of endogenous porphyrins synthesized from ALA in bacteria

In fact, not every bacterium can synthesize endogenous porphyrins from ALA in the amounts necessary for photosensitization-based inactivation (66,77). Thus, in the first stages of this work it was necessary to evaluate the potential of Gram-positive bacteria *B. cereus*, *L. monocytogenes* and Gram-negative *S. enterica* to produce endogenous porphyrins from extrinsically applied ALA. For this purpose, fluorescence spectrum in the region of 590–700 nm were analysed after incubation of cells with 3 and $7.5 \cdot 10^{-3}$ M ALA in the dark. According to Szocs (140), this spectral region with maximum at λ=612÷615 nm is attributed to the presence of endogenously synthesized porphyrins including uroporphyrins and coproporphyrins.

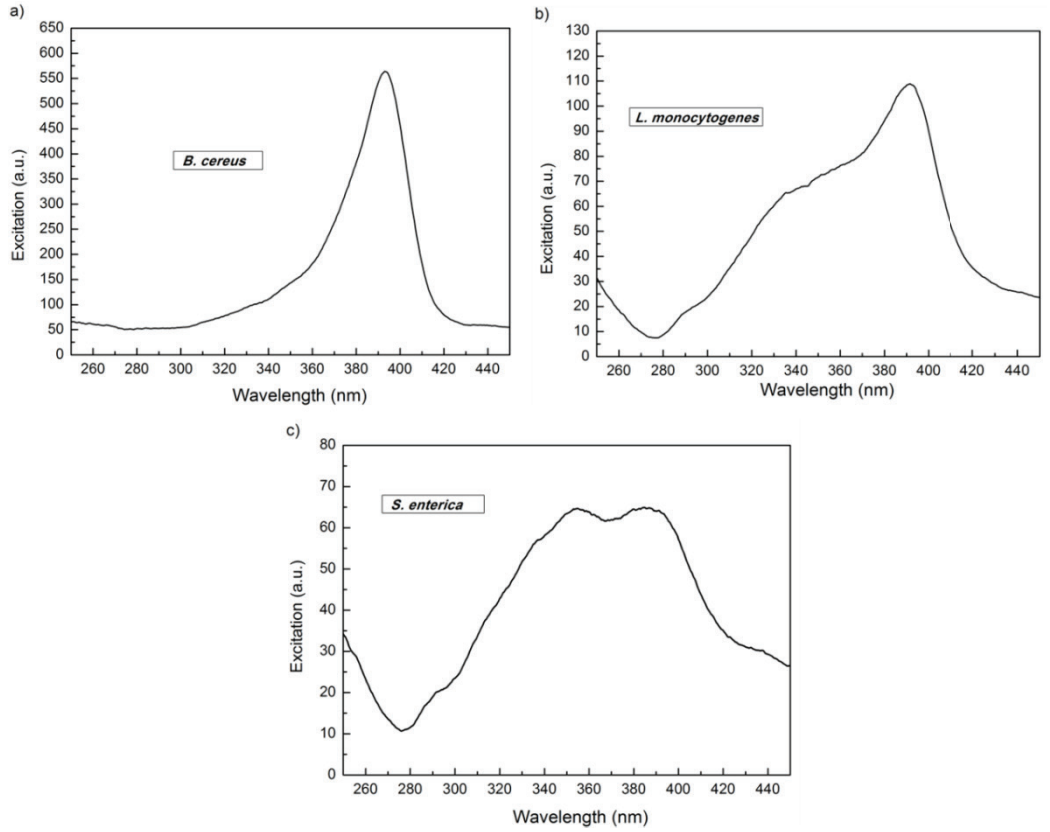

Fig. 3.1 Excitation spectrum of endogenous porphyrins produced by *B. cereus* after incubation with $3 \cdot 10^{-3}$ M ALA (a), by *L. monocytogenes* (b) and *S. enterica*) (c) after incubation with $7.5 \cdot 10^{-3}$ M ALA; emission at 610nm.

Listed ALA concentrations were taken from scientific literature (66,69,140).

Figures 3.1a and 3.1b show the excitation spectra of endogenous porphyrins, produced by Gram-positive bacteria *B. cereus* and *L. monocytogenes* after incubation with 3 and $7.5 \cdot 10^{-3}$ M ALA respectively. Also, excitation spectrum of Gram-negative *S. enterica* was analyzed after 60 min incubation of cells with $7.5 \cdot 10^{-3}$ M ALA in the dark (Figure 3.1c).

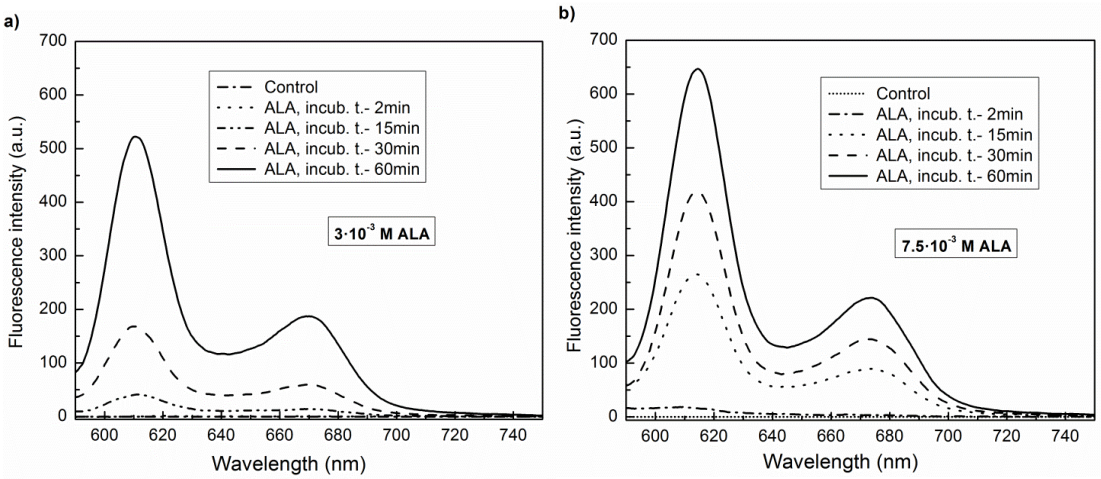

Fig. 3.2 Fluorescence spectra of endogenous porphyrins produced by *B. cereus* after incubation with $3 \cdot 10^{-3}$ M (a) and $7.5 \cdot 10^{-3}$ M (b) ALA for different time interval.

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 (131). Thus, the following fluorimetric analysis indicated that the intensity of fluorescence emission of endogenous porphyrins after incubation of *Bacillus* cells in the dark with $3 \cdot 10^{-3}$ M ALA for 0÷60 min was increasing in a time-dependent manner (excitation, λ=390 nm) (Figure 3.2a). Fluorescence intensity of endogenous porphyrins was insignificant after 2 min of incubation with $3 \cdot 10^{-3}$ M ALA but increased and reached more than 500 fluorescence intensity units after 60 min incubation. In order to understand if a suitable concentration of ALA solution was used, we increased ALA concentration to $7.5 \cdot 10^{-3}$ M. Analysis of the fluorescence emission spectra of endogenous PSs indicated that there was

some increase in fluorescence intensity of endogenous porphyrins (up to 650 units) when the same incubation time (60 min) was used (Figure 3.2b).

Using the same methodology we found, that Gram-positive bacterium *L. monocytogenes* did produce endogenous porphyrins from extrinsically applied ALA as well (Figure 3.3). Thus, the cells were incubated with $7.5 \cdot 10^{-3}$ M ALA in the dark for 0÷120 min. Afterwards, in order to detect the production of the endogenous porphyrins, the fluorescence emission spectra in the region of 590÷750 nm were analyzed (excitation, λ=390 nm). This spectral region is attributed to the presence of endogenously synthesized porphyrins. The data, presented in Figure 3.3 indicate that relative fluorescence intensity of endogenous porphyrins increases with increasing incubation time with ALA. For instance, fluorescence intensity is very low (11 a.u.) after 2 min of incubation with $7.5 \cdot 10^{-3}$ M ALA, but after 30 min of incubation it becomes more significant (39 a.u.). When the incubation time of *L.monocytogenes* cells with ALA was increased to 60 min, the fluorescence intensity has reached 60 a.u. Following the expansion of the incubation time to 120 min, fluorescence intensity was increased more than 10 times (105 a.u.).

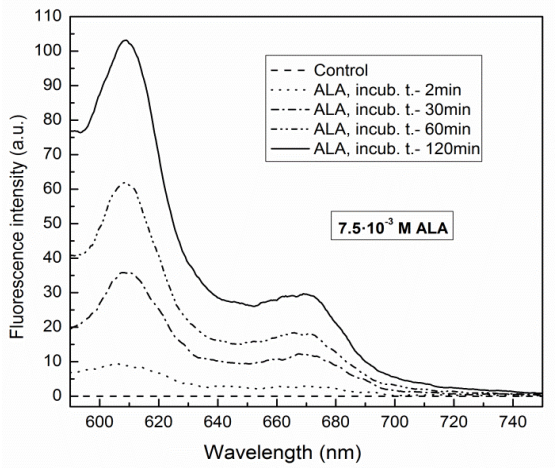

Fig. 3.3 Fluorescence spectra of endogenous porphyrins produced by *L. monocytogenes* after incubation with $7.5 \cdot 10^{-3}$ ALA for different time interval.

Also, we tried to find out if Gram-negative *S. enterica* is able to produce endogenous porphyrins from ALA. For this purpose, the cells were incubated in the dark with $7.5 \cdot 10^{-3}$ M ALA at 37 °C in 0.01M PBS for 0÷240 min. The

production of endogenous porphyrins was demonstrated by the fluorescence emission peaks in the region 590÷750 nm (excitation, λ=390 nm) (Figure 3.4). It is necessary to note that the time of incubation of bacteria with ALA is an important factor for the production of endogenous porphyrins. Figure 3.4 shows, that short incubation times with ALA (2÷30 min) enables bacteria to just start porphyrins synthesis, usually estimated by peak at 610÷630 nm, whereas 240 min incubation time can significantly increase relative production of endogenous porphyrins (246 a.u.).

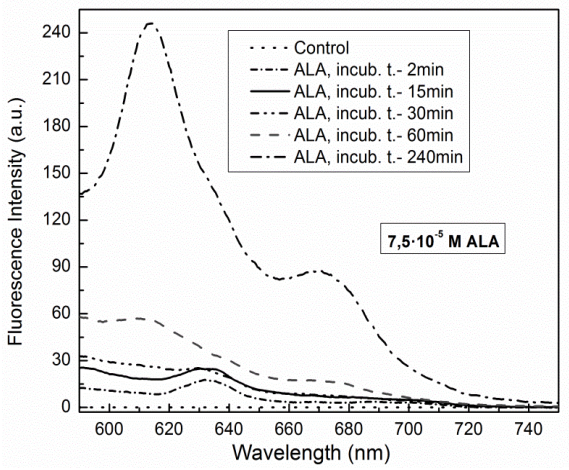

Fig. 3.4 Fluorescence spectra of endogenous porphyrins produced by *S. enterica* after incubation with $7.5 \cdot 10^{-3}$ M ALA for different time interval.

As our task was to investigate if Gram-positive *B. cereus* ATCC 12826, *L. monocytogenes* $ATC_{L3}C$ 7644 and Gram-negative *S. enterica* serovar Typhimurium DS88 (SL5676 Sm^2 pLM32) can produce endogenous porphyrins in general, we did not go deeper into spectral analysis of what type of endogenous porphyrins these bacteria were producing. In order to develop surface decontamination technology, our aim was to detect the synthesis of endogenous porphyrins in selected pathogens within shortest time without any deeper analysis of specific endogenous porphyrins produced by cells.

3.2. Susceptibility of bacteria to ALA-based photosensitization *in vitro*

Analysis of fluorescence emission spectra of endogenous porphyrins indicates that Gram-positive bacteria *B. cereus*, *L. monocytogenes* and Gram-

negative *S. enterica* can effectively synthesize endogenous PSs from exogenous ALA. It means that the main premise for the possibility to inactivate the troublesome food pathogen by ALA-based photosensitization was identified. Thus, the obtained results have prompted us to evaluate the applicability of ALA-based photosensitization for the inactivation of *B. cereus*, *L. monocytogenes* and *S. enterica*.

The data obtained revealed that toxicity of ALA alone (3 and $7.5 \cdot 10^{-3}$ M), without illumination, on viability of *Bacillus, Listeria* and *Salmonella* was, as usual, negligible. The following illumination of bacterial cells (light dose 0÷24 J/cm^2) decreased the survival fraction fairly sharply (Figure 3.5, 3.6, 3.7). It is important to note that a very small illumination dose (6 J/cm^2) was not effective due to the fact that not all endogenous porphyrins were excited by light to S_1 to produce cytotoxic reactions because of the lack of photons delivered.

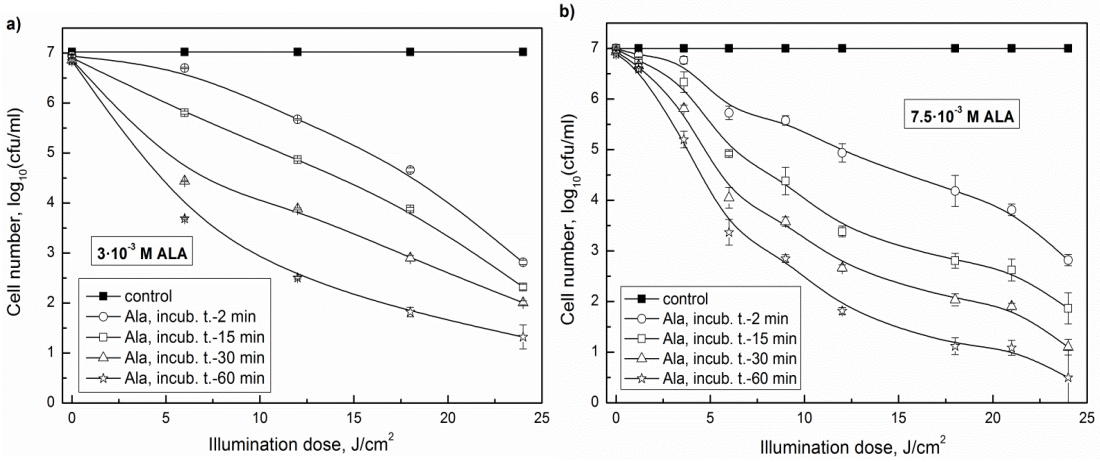

Fig. 3.5 Inactivation of *B. cereus* by $3 \cdot 10^{-3}$ M (a) and $7.5 \cdot 10^{-3}$ M (b) ALA-based photosensitization as function of illumination dose. Every point is average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

From the data presented in Figure 3.5a, it is clear that the prolongation of incubation time with $3 \cdot 10^{-5}$ M ALA from 2 min to 60 min increased the rate of *Bacillus* inactivation. The use of 60 min incubation time and 24 J/cm^2 illumination dose resulted in 5.75 log inactivation of *B. cereus*. As a matter of fact, photosensitization efficiency can be expressed as a function of accumulated PS multiplied by the total light energy delivered to the bacterial

cells. Taking this into account, we investigated the inactivation of *B. cereus* by ALA-based photosensitization as a function of time using higher ALA concentration ($7.5 \cdot 10^{-3}$ M). Data presented in Figure 3.5b reveal that higher ALA concentration ($7.5 \cdot 10^{-3}$ M) can enhance the inactivation of *B. cereus*. This inactivation of bacteria strongly depends on incubation with ALA time and illumination dose as well. A short incubation time (2 min) and low illumination dose (1.2÷9 J/cm^2) are not effective, whereas 15÷60 min incubation time and 12÷24 J/cm^2 illumination dose are much more suitable for the development of perspective antibacterial technology.

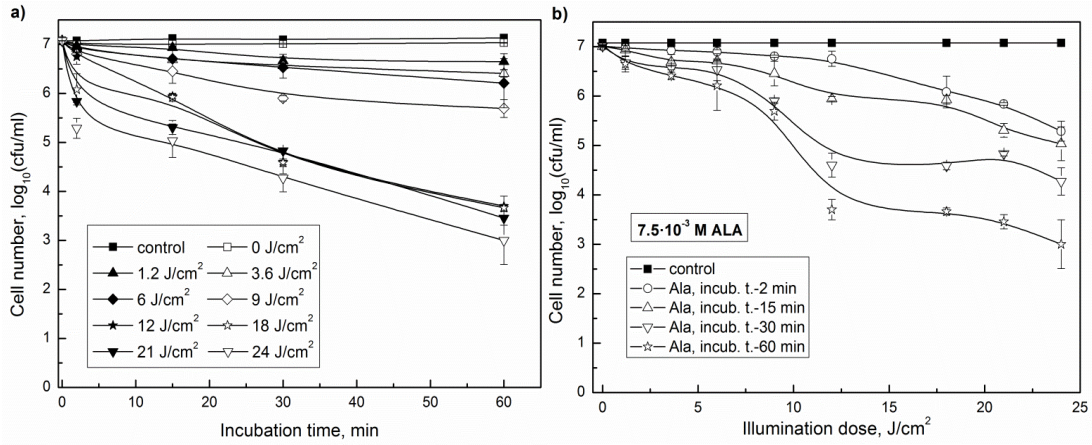

Fig. 3.6 Inactivation of *L. monocytogenes* by $7.5 \cdot 10^{-3}$ M ALA-based photosensitization when different incubation time (a) and illumination dose (b) was used. Every point is average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

Experimental data, presented in Figure 3.6a reveal clearly that the incubation of *L. monocytogenes* cells with $7.5 \cdot 10^{-3}$ M ALA (dark toxicity, light dose 0 J/cm^2) does not change the viability of *L. monocytogenes*. Light alone (without any incubation of cells with ALA) has no toxicity on bacteria (Figure 3.6b). Only the incubation of cells with ALA and subsequent illumination (λ=405nm) decreases the surviving fraction fairly sharply, especially when longer incubation times are used. Clear dependence of inactivation efficiency on the illumination dose as well as incubation with ALA time (or concentration of produced endogenous porphyrins) is observed (Figure 3.6b). The number of killed *L. monocytogenes* reaches even 4 orders of magnitude, when 24 J/cm^2 illumination dose and 60 min incubation time were used. It is evident that at a

given illumination dose the inactivation effect can be modified by ALA incubation time.

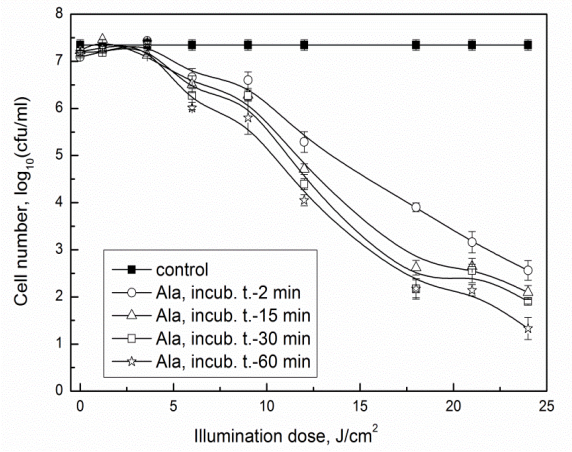

Fig. 3.7 Inactivation of *S. enterica* by $7.5 \cdot 10^{-3}$ M ALA-based photosensitization as function of illumination dose, when different incubation time was used. Every point is average of 3-6 experiments, and error bars sometimes are too small to be more visible.

The photosensitization of Gram-negative *S. enterica* was performed as follows. Bacteria at an exponential growth phase were incubated with $7.5 \cdot 10^{-3}$ M ALA for different times (0÷60 min) in the dark. In the next step, bacteria were exposed to light (λ=405nm) for different light dose (0÷24 J/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 (Figure 3.7; light dose 0 J/cm^2). Just following illumination of cells with increasing illumination doses (0÷24 J/cm^2) diminishes bacterial viability in dose-dependent manner (Figure 3.7). 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 illumination dose: 5 min illumination (6 J/cm^2) decreases viability of cells by 0.5÷1.5 log, whereas longer illumination dose (24 J/cm^2) by 4.5÷6 log. The increase of incubation time up to 60 min and the following illumination up to 20 min (illumination dose 24 J/cm^2) diminishes the viability of *S. enterica* up to six orders of magnitude.

3.3. Inactivation of food pathogens and harmful microfungi by Chl-based photosensitization *in vitro*

Data presented in Figure 3.8 show absorption and fluorescence spectra of Chl ($1.5 \cdot 10^{-5}$ M) in 0.01 M PBS. It is clear that the main absorption maximum of Chl molecule is at λ=405 nm and lower absorption at 655nm. Therefore LED-based light source of light wavelength λ=405 nm was used in experiments for the optimal excitation of Chl. In addition Chl is highly fluorescing compound and its fluorescence maximum in PBS is about 663 nm (Figure 3.8). Examination of fluorescence spectra, presented in Figure 3.8 indicate that $1.5 \cdot 10^{-5}$ M Chl molecules tend slightly to aggregate in PBS (640 a. u.), since fluorescence intensity slightly increases when 20µl 0.001% Triton-X100 was added to the solution (768 a. u.).

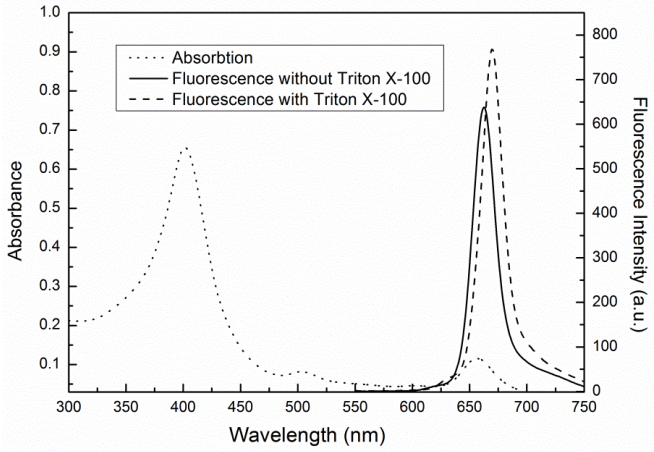

Fig. 3.8 Absorption and fluorescence spectra of $1.5 \cdot 10^{-5}$ M Chl (in 0.01 M PBS).

As no data exist on the photoinactivation of Gram-positive *B. cereus*, *L. monocytogenes* and Gram-negative *S. enterica* by Chl we examined the cytotoxic effect of wide range of Chl concentrations: from the highest $1.5 \cdot 10^{-5}$ M to the lowest $7.5 \cdot 10^{-8}$ M (Figures 3.9–3.11). The dark toxicity of the Chl was assessed from the survival fractions at 0 J/cm^2. Experimental data reveal clearly that light alone had negligible effects on viability of bacteria. The incubation of bacterial cells with different concentrations of Chl ($1.5 \cdot 10^{-5}$ M÷$7.5 \cdot 10^{-8}$ M) for 2÷120 min without following illumination had no impact on

their viability. Only subsequent illumination with visible light (λ=405 nm) decreased the number of bacterial cells more considerably.

Table 2.3 Dark toxicity of Chl to *B. cereus*.

Incubation time (min)	**Control (log_{10})**	**$7.5 \cdot 10^{-8}$ M Chl (log_{10})**	**$7.5 \cdot 10^{-7}$ M Chl (log_{10})**	**$7.5 \cdot 10^{-6}$ M Chl (log_{10})**	**$1.5 \cdot 10^{-5}$ M Chl (log_{10})**
2	7.1 ± 0.04	7.19 ± 0.09	7.18 ± 0.06	7.0 ± 0.06	6.95 ± 0.1
60	7.1 ± 0.13	7.1 ± 0.05	7.05 ± 0.06	7.0 ± 0.09	6.9 ± 0.2

Results are expressed as mean values of 3 replicate measurements ± standard deviation.

Data presented in Table 2.3 indicated that dark toxicity of Chl to *B. cereus* was negligible, since the cell number after 2 min incubation in the dark with maximum $1.5 \cdot 10^{-5}$ M concentration Chl was reduced only by 0.15 log. Also, prolongation of incubation time from 2 min to 60 min has negligible effect (0.26 log) on the inactivation of *Bacillus* (Table 2.3).

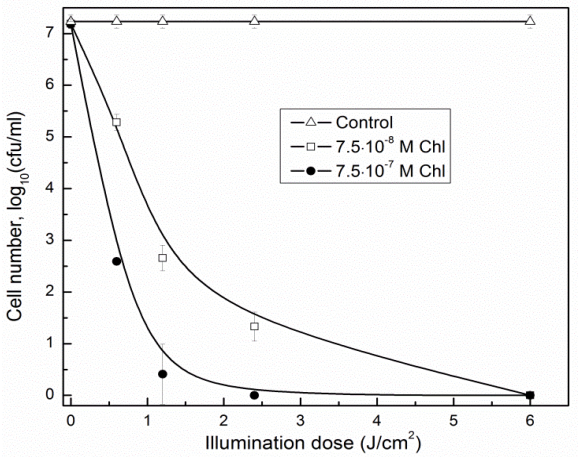

Fig. 3.9 Inactivation of *B. cereus* (~$1 \cdot 10^7$ cfu/ml) by $1.5 \cdot 10^{-8}$ and $1.5 \cdot 10^{-7}$ M Chl-based photosensitization as function of illumination dose. Incubation time (0.01 M PBS) - 2 min. Every point is average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

As depicted in Figure 3.9, incubation of *B. cereus* with the lowest $7.5 \cdot 10^{-8}$ M Chl concentration for shortest 2 min incubation time is enough effective as produce photoinactivation of bacteria by 7 log (illumination dose 6 J/cm^2). The incubation of bacteria with higher Chl concentration ($7.5 \cdot 10^{-7}$ M) requires lower illumination dose (2.4 J/cm^2) to inactivate them by 7 orders of magnitude. Clear dependence of inactivation efficiency on the illumination dose or used Chl concentration was observed (Figure 3.9).

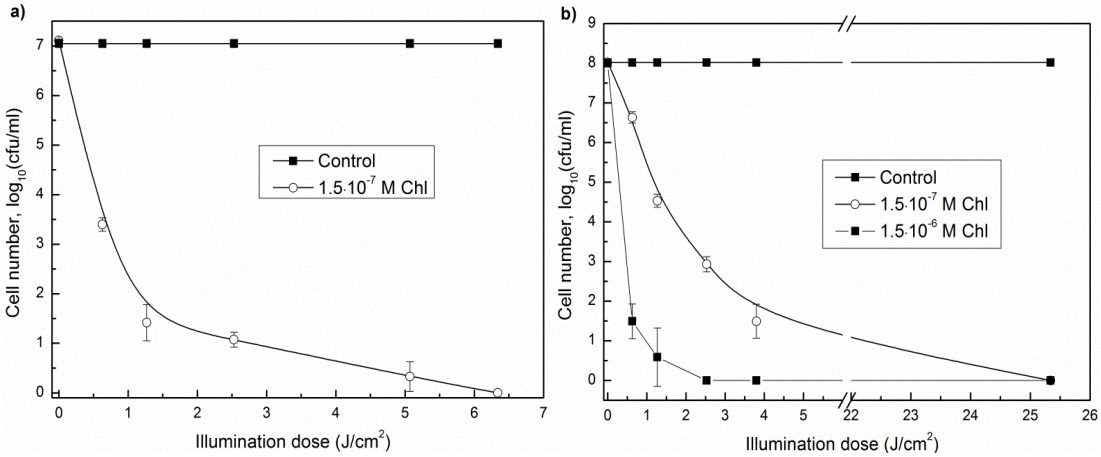

Fig. 3.10 Inactivation of *L. monocytogenes* at $1 \cdot 10^7$ (a) and $1 \cdot 10^8$ (b) cfu/ml cellular concentrations by Chl-based photosensitization as function of illumination dose. Every point is average of 3-6 experiments, and error bars sometimes are too small to be more visible.

Two 10-fold dilutions of Gram-positive *L. monocytogenes* cell concentration were used for photosensitization experiment: 10^7 and 10^8 cfu/ml (Figure 3.10). The effectiveness of Chl-based photosensitization increased with decrease of cell density. Experimental data, presented in Figure 3.10 reveal clearly that incubation of *L. monocytogenes* with $1.5 \cdot 10^{-7}$ and $1.5 \cdot 10^{-6}$ M Chl for 2 min had negligible effect on the survival. Bacterial population was drastically reduced after illumination with visible light (λ=405 nm). The data indicate that Chl-based photosensitization inactivation curve of *Listeria* strongly decreased as the illumination time increased. After 0.5 min of illumination (illumination dose 0.6 J/cm^2), the number of surviving *Listeria* cell count decreased by 1.4 orders if 10^8 cfu/ml was used (Figure 3.10 b). However, killing increased to 3.7 log when 10^7 cfu/ml was used (Figure 3.10 a). After 1 min of illumination (illumination dose 1.3 J/cm^2) the 10^8 and 10^7 cells count decreased by 3.5 and 5.6 orders of magnitude respectively. It is evident that incubation of 10^7 cfu/ml *L. monocytogenes* bacteria with 1.5 10^{-7} M Chl (Figure 3.10 a) concentration for 2 min and illumination for 5 min (illumination dose 6.3 J/cm^2) is effective enough to produce inactivation of bacteria by 7 log. However *Listeria* showed 8 log of killing just after 2 min incubation with $1.5 \cdot 10^{-7}$ M Chl and 20 min illumination (illumination dose 25.3

J/cm^2) if 10^8 cfu/ml was used. The incubation of bacteria with higher $1.5 \cdot 10^{-6}$ M Chl concentration requires lower illumination dose (2.5 J/cm^2) to inactivate them by 8 orders of magnitude (Figure 3.10 b).

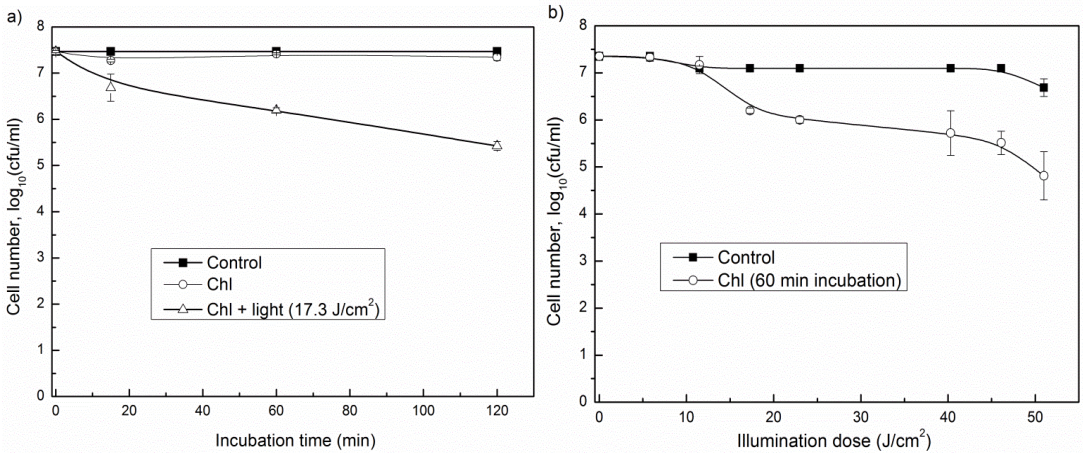

Fig. 3.11 Inactivation of *S. enterica* by $1.5 \cdot 10^{-5}$ M Chl-based photosensitization as function of incubation time (a) and illumination dose (b). Every point is average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

The susceptibility of Gram-negative *S. enterica* to Chl-based photosensitization is illustrated in Figure 3.11a and b. Experiments confirmed clearly that the light alone (51 J/cm^2) had insignificant effects on the viability of bacteria as well. The dark toxicity of $1.5 \cdot 10^{-5}$ M Chl to *Salmonella* was negligible, since the cell number after 120 min incubation in the dark with Chl was reduced only by 0.12 log. Just incubation of cells with Chl and subsequent illumination with visible light (λ=405 nm, 0÷51 J/cm^2 illumination dose) decreased the number of bacterial cells more considerably: in this case the photosensitization treatment (15 min incubation with Chl in 0.01 M PBS and following illumination, 17.3 J/cm^2) led to 0.79 log reduction of *S. enterica* (Figure 3.11a). An extension of incubation time from 15 to 120 min enhanced the inactivation of *Salmonella* to 2.05 log. Meanwhile prolongation of illumination time from 30 min (illumination dose 17.3 J/cm^2) to 80 min (illumination dose 46.1 J/cm^2) has negligible effect (0.7 log) on the inactivation of *Salmonella* (Figure 3.11b). However when illumination dose was increased to 51 J/cm^2, the number of surviving *Salmonella* cell count decreased by 2.5 orders.

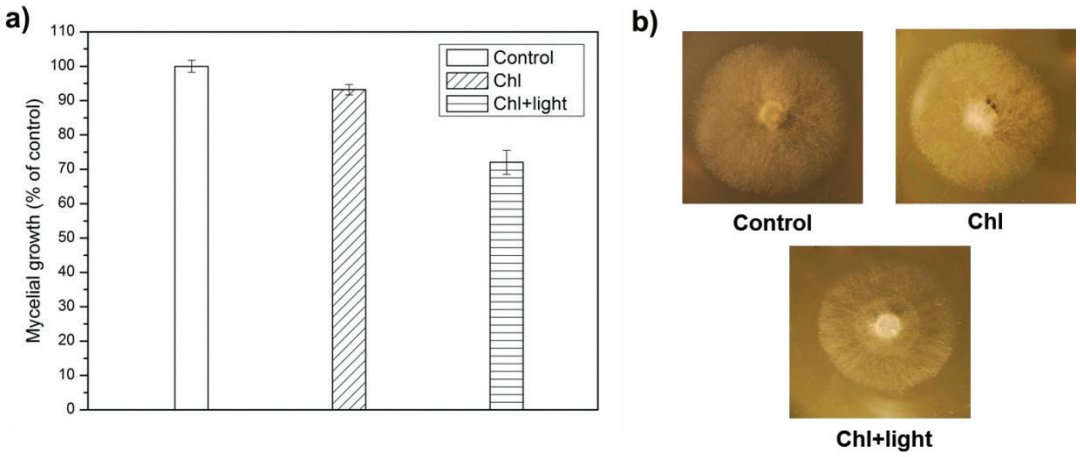

Fig. 3.12 Mycelial growth of *B. cinerea* 2 days after treatment by $1.5 \cdot 10^{-4}$ M Chl-based photosensitization (illumination dose 35 J/cm^2) (a); visualization of mould growth (b).

Micromycetes are more resistant to photosensitization than bacteria (12,13). Thus, for inactivation of *Botrytis* longer incubation time and higher Chl concentration have been used. Data presented in Figure 3.12 illustrated the inhibition of mycelial growth of *B. cinerea* after treatment with $1.5 \cdot 10^{-4}$ M Chl. The diameter of the *B. cinerea* in control sample was 100%. The obtained data indicated that Chl without light had no effect on *Botrytis* (Figure 3.12 a). After 2 days of growth the diameter of *Botrytis* in control reached 4.33 cm. When fungus was incubated with $1.5 \cdot 10^{-4}$ M Chl and illuminated with visible light (illumination dose 35 J/cm^2) it resulted in 3.35 cm diameter growth (28% inhibition) (Figure 3.12 b).

3.4. Evaluation of cell membrane integrity and structural damage in bacteria after Chl-based photosensitization

In order to understand the cell-PS interaction we measured fluorescence spectra of Chl when Gram-negative *S. enterica* and Gram-positive *L. monocytogenes* cells were incubated with Chl and compared with Chl spectrum in supernatant and Chl spectrum in cells alone (without supernatant) (Figure 3.13). Data in Figure 3.13a indicate that most of the Chl remained in the supernatant and just small part was bound to Gram-negative *Salmonella*. We proposed the idea that this small fraction of Chl-attached *Salmonella* cells was

damaged after photosensitization treatment. Data in Figure 3.13b indicated that ~ 50% of the Chl remained in the supernatant and ~ 20% was bound to Gram-positive *Listeria*.

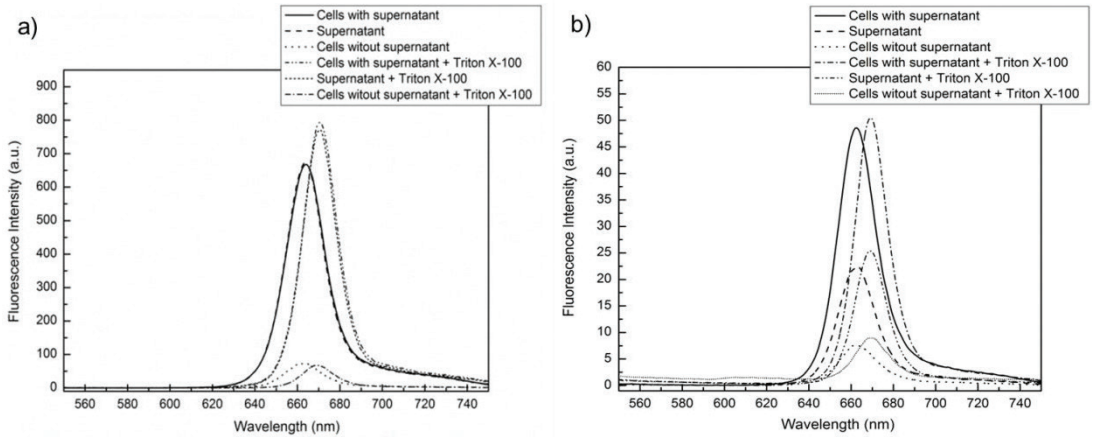

Fig. 3.13 Fluorescence spectra of $1.5 \cdot 10^{-5}$ M (a) and $1.5 \cdot 10^{-7}$ M (b) Chl (in 0.01 M PBS) after 60 min incubation with *S. enterica* and 2 min incubation with *L. monocytogenes* cells respectively.

Also, examination of fluorescence spectra of supernatant and separated cells alone, presented in Figure 3.13 propose that Chl molecules probably tend slightly to aggregate in PBS, since fluorescence intensity slightly increases when 20 µl 0.001% Triton-X100 was added to the solution.

In order to test whether the binding of Chl to the *Salmonella* and *Listeria* cells was important we carried out the studies both "without" and "with a wash", a centrifugation step after Chl incubation and before NaN_3 addition. For experiments using Chl, the effectiveness of photosensitization was dramatically reduced when Chl was washed out of the cell suspensions - centrifugation step after Chl incubation and before photosensitization treatment (Figure 3.14). This was applied to both microbial species - Gram-negative *Salmonella* and Gram-positive *Listeria*.

The inactivation of Gram-positive *L. monocytogenes* by Chl-based photosensitization ($1.5 \cdot 10^{-7}$ M Chl with 0÷25.3 J/cm^2 of 405 nm light) "with" (after centrifugation) and "without wash" (without centrifugation) is shown in Figure 3.14a. There was 7 log of inactivation obtained after Chl-based photosensitization treatment, and "no wash" killing was higher than the killing observed after a wash. As depicted in Figure 3.14a, after 2 min of incubation,

following centrifugation and illumination (illumination dose 1.3 J/cm^2) steps, the number of surviving *Listeria* cell count decreased by 2.2 orders. However, killing increased to 5.7 log when cells after incubation with Chl were illuminated "without a wash" (Figure 3.14a).

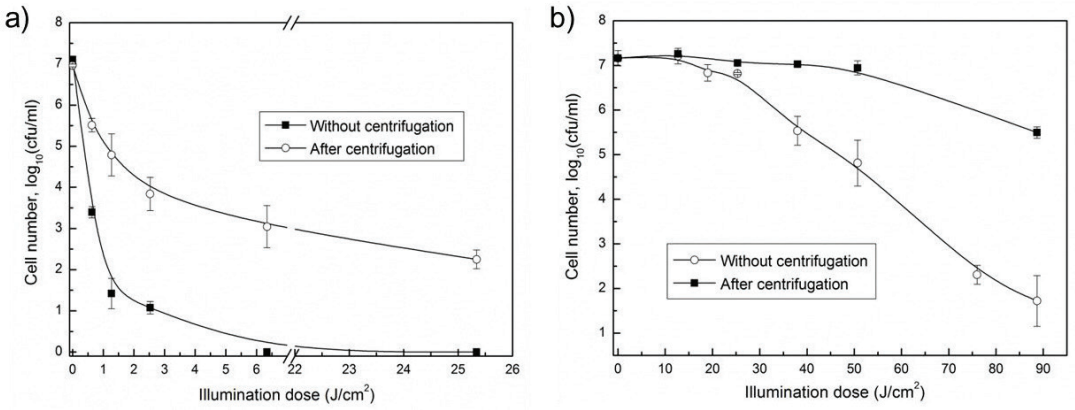

Fig. 3.14 Inactivation of bacteria by Chl-based photosensitization as function of illumination dose: a) *L. monocytogenes* - $1.5 \cdot 10^{-7}$ M Chl, incubation time 2 min b) *S.enterica* - $1.5 \cdot 10^{-5}$ M Chl, incubation time - 60 min. Every point is average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

The same set of experiments was done with the Gram-negative *S. enterica* by Chl-based photosensitization ($1.5 \cdot 10^{-5}$ M Chl with 0÷88.7 J/cm^2 of 405 nm light) as shown in Figure 3.14b. The killing was more effective with "without a wash" step than it was "with a wash". As depicted in Figure 3.14b, after centrifugation step and following 70 min of illumination (illumination dose 88.7 J/cm^2), the number of surviving *Salmonella* cell count decreased by 1.7 orders. However, killing increased to 5.4 log when cells after incubation with Chl were illuminated "without a wash" (Figure 3.14b).

So, in the next step it was interesting to assess whether singlet oxygen participate in the Chl-based photosensitization induced damage and asses their impact on *Listeria* and *Salmonella* survival. The bacteria cell suspensions were amended with the exogenous scavenger NaN_3 (physical quencher of $^1O_2$). We established that NaN_3 alone incubated with bacteria did not begin to show any toxicity until the NaN_3 concentration reached 40 mM (Table 2.4).

Table 2.4 Effect of NaN3 on *L. monocytogenes* cells viability without a centrifugation step.

	NaN_3 concentration (mM)			
	0	**5**	**10**	**40**
Control	8.00 ± 0.03	7.86 ± 0.07	7.80± 0.11	7.99 ± 0.03
$1.5 \cdot 10^{-6}$ M Chl*	7.92 ± 0.07	7.90 ± 0.07	7.94 ± 0.03	7.97 ± 0.08
$1.5 \cdot 10^{-6}$ M Chl + light**	ND*	ND	ND	ND

* Incubation time – 2 min. ** illumination dose 2.5 J/cm^2; Results are expressed as mean values of three replicate measurements ± standard deviation. * ND- not detectible level.

The effect of added NaN_3 on Chl-based photosensitization of *L. monocytogenes* "without a wash" step is shown in Table 2.4. Bacteria (~$1 \cdot 10^8$ cells/ml) were incubated with $1.5 \cdot 10^{-6}$ M Chl for 2 min followed or not by addition of NaN_3 (0÷40 mM) and illumination at 2.5 J/cm^2 of 405 nm light. There was 8 log (not detectible level) of inactivation obtained after Chl-based photosensitization treatment. The addition of NaN_3 (0÷40 mM) was no effect on the inactivation efficiency (Table 2.4).

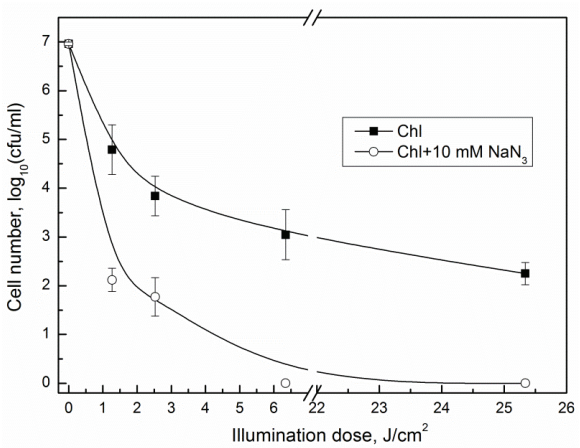

Fig. 3.15 Effect of NaN_3 on Chl-based photosensitization treatment of *L. monocytogenes* after centrifugation step. Bacteria were incubated with $1.5 \cdot 10^{-7}$ M Chl for 2 min followed or not by addition of NaN_3 and illumination up to 25.3 J/cm^2 of 405 nm light.

Figure 3.15 display the survival fraction curves obtained with $1.5 \cdot 10^{-7}$ M Chl for *L. monocytogenes* (~$1 \cdot 10^7$ cells/ml) with and without 10 mM NaN_3 after centrifugation step ("wash"). The addition of NaN_3 after centrifugation step to Chl-based photosensitization treatment, did not quench the *L. monocytogenes* killing, but potentiated it. As depicted in Figure 3.15, after centrifugation step (without 10 mM NaN_3) and following llumination (illumination dose 6.3 J/cm^2), the number of surviving *Listeria* cell count

decreased by 3.9 orders. However, killing was increased to 7 log (not detectible level), when bacterial cells after centrifugation and addition of 10 mM NaN_3 were illuminated with visible light (illumination dose 6.3 J/cm^2) (Figure 3.15).

It is important to note that the presence of widely accepted $^1O_2$ scavenger NaN_3 at nontoxic concentration (10mM) reduced the killing efficiency of Chl-based photosensitization in statistically significant way ($p < 0,05$) and most Gram-negative *Salmonella* cells survive ("without a wash") (Figure 3.16a). The lack of killing at 0 J/cm^2 shows that neither Chl in the dark or NaN_3 displayed any appreciable dark toxicity. The addition of NaN_3 inhibited the Chl-based treatment killing of *S. enterica* at 38 and 88,7 J/cm^2 by 1,3 and 2,4 log respectively (Figure 3.16a).

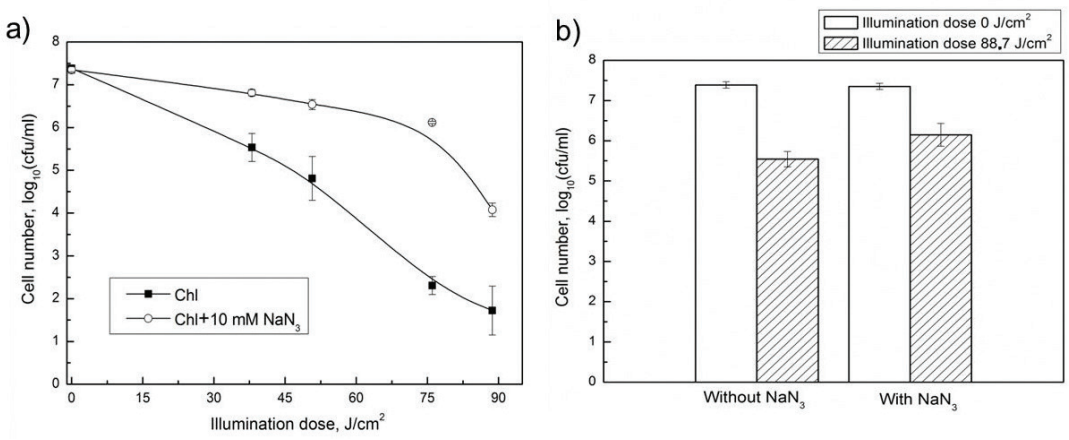

Fig. 3.16 Effect of NaN_3 on $1.5 \cdot 10^{-5}$ M Chl-based photosensitization treatment of *S. enterica*: a) without centrifugation step; b) after centrifugation step. Incubation time with Chl–60 min.

So far, very small effect of NaN_3 on survival of *Salmonella* was observed when cells were centrifuged after incubation with Chl and before NaN_3 (Figure 3.16b). Bacteria (~$1 \cdot 10^7$ cells/ml) were incubated with $1,5 \cdot 10^{-5}$ M Chl for 60 min followed by centrifugation step and addition of NaN_3 (10 mM). Then the cells suspensions were illuminated at 88,7 J/cm^2 of 405 nm light. As depicted in Figure 3.16b, the number of surviving *Salmonella* cell count decreased by 3.9 orders after addition of NaN_3.

In order to find new effective combination of antimicrobial treatments it is important to know cell injuries which are produced by each of them. For this

purpose, we exanimate the effects of Chl-based photosensitization treatment on main *L. monocytogenes* and *S. enterica* target membrane integrity. It was assessed by measuring the absorption at 260 nm (DNA absorption peak) and 280 nm (protein absorption peak) of cell free filtrates in control and treated samples (Figure 3.17 and 3.18).

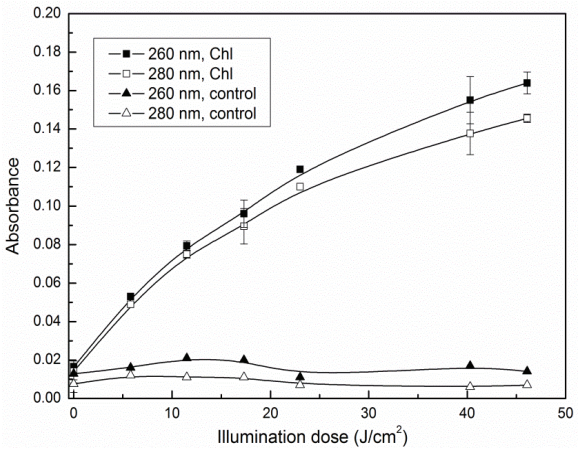

Fig. 3.17 Release of intracellular components (at 260 and 280 nm) from *S. enterica* after $1{,}5 \cdot 10^{-5}$ M Chl -based photosensitization treatment. Every point is the average of 3 experiments, and error bars sometimes are too small to be more visible.

The results indicated that the release of intracellular components absorbing at λ=260 nm and λ=280 nm in control *Salmonella* samples is insignificant and did not depend on illumination dose. On the contrary the release of intracellular components (both absorbing at λ=260 nm and λ=280 nm) in treated bacteria increased since absorption changed from 0.01 to 0.146÷0.164 when *S. enterica* was treated by photosensitization (60 min incubated with $1.5 \cdot 10^{-5}$ M Chl and afterwards illuminated, illumination dose 0÷46.8 J/cm^2). It means that some cell membrane injuries occur in *Salmonella* after Chl-based photosensitization (Figure 3.17).

The effects of Chl-based photosensitization treatment on Gram-positive *Listeria* cells membrane integrity were assessed by measuring the optical density at 260 nm (DNA absorption peak) and 280 nm (protein absorption peak) of cell free filtrates (supernatant) in control and treated samples (Figure 3.18). The results indicated that the release of intracellular material absorbing in control supernatant 0 and 60 min after photosensitization-based treatment

was insignificant: at λ=280 nm - 0.053 and 0.056;at λ=260 nm – 0.077 and 0,086 respectively.

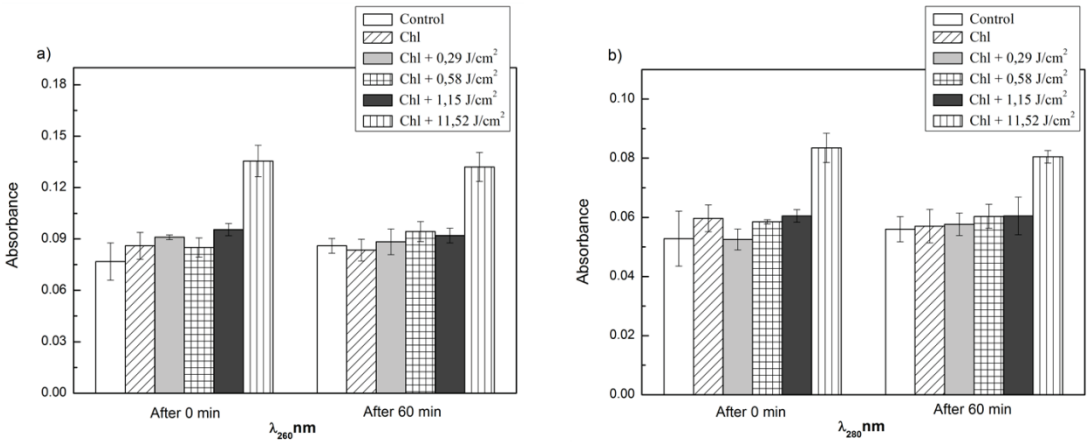

Fig. 3.18 Release of intracellular components at 260 (a) and 280 nm (b) from *L. monocytogenes* (~$1 \cdot 10^8$ cfu/ml) after $1.5 \cdot 10^{-6}$ Chl-based photosensitization. Measurement was made after 0 and 60 min after treatment.

The release of intracellular components (both absorbing at λ=260 nm and λ=280 nm) after Chl-based photosensitization (2 min incubation with $1.5 \cdot 10^{-6}$ M Chl, illumination dose 0÷11.52 J/cm^2) treatment increased just while increasing light dose. For instance, absorption at λ=260 nm increased from 0,086 OD (illumination dose 0 J/cm^2) to 0.136 OD and absorption at λ=280 nm increased from 0.06 OD to 0.084 OD, when *L. monocytogenes* was illuminated with light (11.52 J/cm^2 illumination dose) and the measurements were made immediately after treatment (Figure 3.18). No significant difference was detected 60 min after treatment.

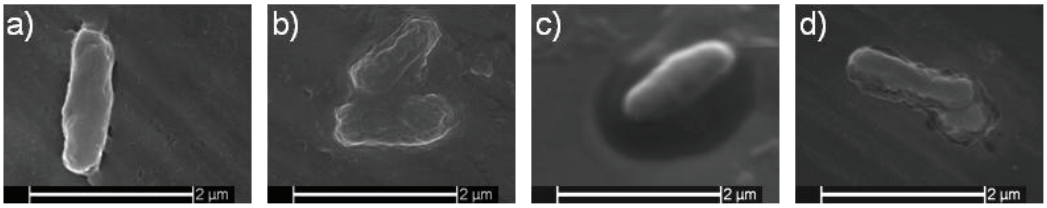

Fig. 3.19 SEM analysis of *S. enterica* and *L. monocytogenes* cells after Chl-based photosensitization treatment in comparison with control, not treated ones: a) and c) untreated *Salmonella* and *Listeria* cells respectively; b) *Salmonella* cells after $1.5 \cdot 10^{-5}$ M Chl-based photosensitization (incubation time 60 min; illumination dose 23 J/cm^2); d) *Listeria* cells after $1.5 \cdot 10^{-7}$ M Chl-based photosensitization (incubation time 2 min; illumination dose 1.3 J/cm^2).

Scanning electron microscopy (SEM) images presented in Figure 3.19 showed the multiple morphological changes in Gram-negative *S. enterica* serovar Typhimurium DS88 and Gram-positive *L. monocytogenes* $ATC_{L3}C$ 7644 cells induced immediately after photosensitization treatment (*Salmonella* – $1.5 \cdot 10^{-5}$ M Chl, 60 min. incubation, illumination dose 23 J/cm^2; *Listeria* – $1.5 \cdot 10^{-7}$ M Chl, 2 min. incubation, illumination dose 1.3 J/cm^2). It seems that intensive shrinkage of bacterial cells, leakage of intracellular components and eventually death took place in antimicrobial action of photosensitization. Thus, these images confirmed our previous data about membrane disintegration and leakage of some intracellular components in *Salmonella* and *Listeria* after photosensitization (Figure 3.17–3.18).

3.5. Antibacterial effect of photoactivated Chl–CHS conjugate against pathogens and harmful microfungi *in vitro*

To increase susceptibility of tested bacteria to photosensitization we used photoactive Chl–CHS conjugate. In order to confirm the structure of Chl–CHS conjugate the absorption and fluorescence spectra were analyzed. Figure 3.20a indicated that the absorption spectrum of Chl–CHS conjugate in 0.9% NaCl solution had peaks at λ=405 nm and at λ=652 nm.

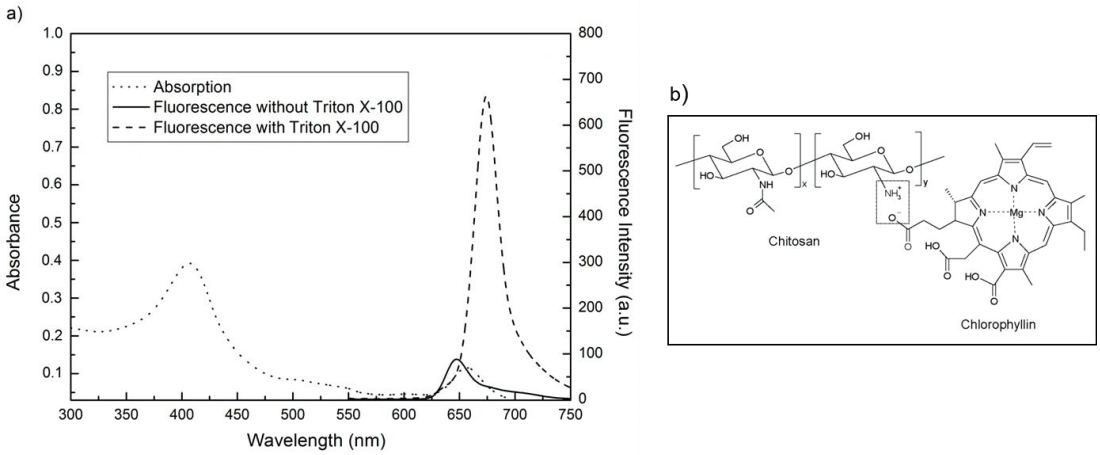

Fig. 3.20. Chemical formula (b), absorption and fluorescence spectra (a) of 0.001 Chl – 0.1 % CHS solutions in 0.9% NaCl (at 20 ± 2 ºC).

Examination of fluorescence spectrum of Chl solution indicated that Chl molecules tended slightly to aggregate in NaCl (468 a.u. at 662 nm), since fluorescence intensity slightly increased when 20 μL of Triton X-100 was added to the solution (732 a.u. at 671nm) (Table 3.5). The fluorescence spectra presented in the same table indicated very low (88 a. u.) fluorescence intensity (peak at 647 nm) of conjugate (Table 3.5). Just adding of 0.001% Triton X-100 to the conjugate solution monomerized Chl and increased the fluorescence intensity to 663 a.u. (peak at 674 nm).

Table 3.5 Fluorescence spectra peak of Chl and Chl–CHS conjugate in 0.9 % NaCl and 0.1 M PBS solutions.

	Peak wavelength (nm): fluorescence intensity (a. u.)			
	0.01 M PBS		**0.9% NaCl**	
	Without Triton	With Triton	Without Triton	With Triton
0.001% Chl	662.5: 638	670: 768	662: 468	670.5: 732
0.001 Chl–0.1% CHS	662.5: 256	670: 732	647: 88	674: 663

Taking into account the structure of both compounds, the interaction between positively charged chitosan NH_3^+ group and negatively charged Chl COO^- group is most probable (Figure 3.20b). In order to check the complexation of Chl with CHS, the absorption spectrum of conjugate was recorded every time before experiments.

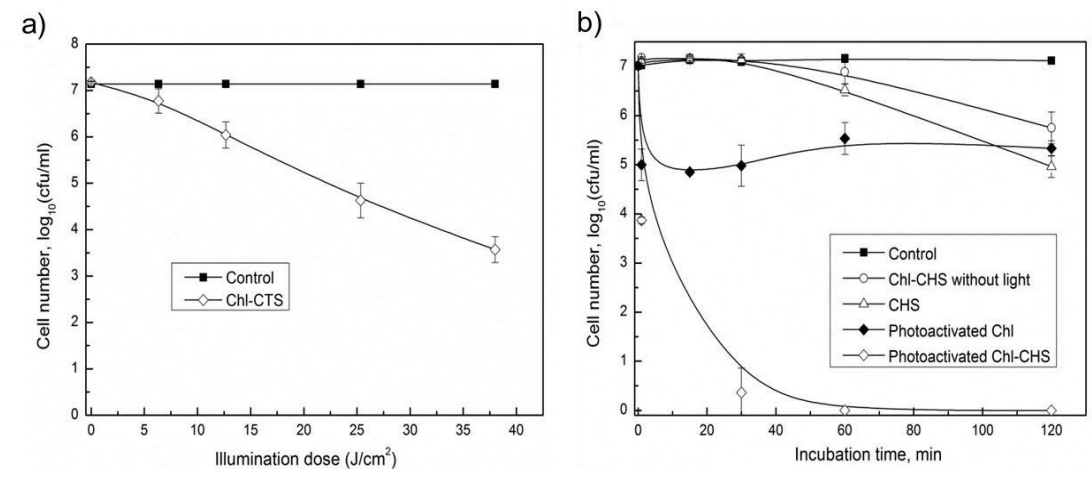

Fig. 3.21 Inactivation of *S. enterica* a) by photoactivated 0.001 Chl–0.1% CHS conjugate as function of illumination dose (incubation time 1 min); b) by 0.1% CHS (pH 3.46), photoactivated 0.001% Chl (pH 6,96) and 0.001 Chl–0.1% CHS conjugate (pH 3.95) as function of incubation time (illumination dose 38 J/cm^2). Every point is the average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

The antimicrobial activity of Chl–CHS conjugate on Gram-negative *S. enterica* and Gram-positive *L. monocytogenes* has been examined. The susceptibility of *S. enterica* to photoactivated Chl–CHS treatment is illustrated in Figure 3.21. Experiments confirmed clearly that the light alone (λ=405 nm, illumination dose 0÷38 J/cm^2) had insignificant effects on the viability of bacteria as well (Figure 3.21a). Previous experiments indicated that the activity of Chl is substantially higher against Gram-positive bacteria (*L. monocytogenes* and *B. cereus*) than against Gram-negative bacteria (*S. enterica*) and mold (*B. cinerea*) which meant we used a Chl–CHS conjugate concentration 100 times higher for *S. enterica* (0.001–0.1%) than for *L. monocytogenes* (0.00001–0.001%). The dark toxicity of Chl–CHS conjugate to *Salmonella* was negligible (illumination dose 0 J/cm^2). Just illumination with visible light (λ=405 nm, illumination dose 0÷38 J/cm^2) decreased the number of bacterial cells more considerably. As depicted in Figure 3.21a, 1 min of incubation and following illumination at 38 J/cm^2 led to 3.6 log reduction of *S. enterica*.

The dark toxicity of Chl to *S. enterica* was negligible (Figure 3.11 and 3.21), since the cell viability after 120 min incubation reduced only by 0.12 log. Incubation of cells with Chl (0÷120 min) and subsequent illumination with visible light (λ=405 nm, illumination dose 38 J/cm^2) decreased the viability of cells more considerably: in this case the photosensitization treatment led to 1.7 log reduction (Figure 3.21b).

The antimicrobial properties of Chl–CHS conjugate were assessed comparing its antimicrobial efficiency in the dark and with that of CHS alone. Thus, dark toxicity of Chl–CHS conjugate slightly depends on incubation time. As depicted in Figure 3.21b, viability of *Salmonella* incubated with CHS alone (1÷120 min) was diminished by 2.15 log. It indicated that Chl–CHS conjugate exhibited some antibacterial action (1.37 log) which was close to that of CHS alone. Just photoactivation of this conjugate drastically reduced the viability of *Salmonella* by 7 log at 2 times shorter incubation time (Figure 3.21b).

Since different experimental conditions (6 samples) may change the pH of bacterial suspension and hence affect the viability of bacteria it was necessary to measure pH values in all samples. It was determined that pH value of the bacterial suspension in 0.01 M PBS shifted from 7.4 to 6.8 when the cell suspension was mixed with Chl or Chl–CHS conjugate. However, when PBS was replaced by 0.9% NaCl, pH value after mixing with Chl–CHS conjugate decreased from 7.3 to 3.95. One might consider that antibacterial effect is related to relatively low pH value of the suspension in 0.9% NaCl. This was not the case, however, since it was found, that *Salmonella* cells kept their viability (7.2 log) incubated for 120 min in 0.9 % NaCl acidified by HCl to pH 4.4.

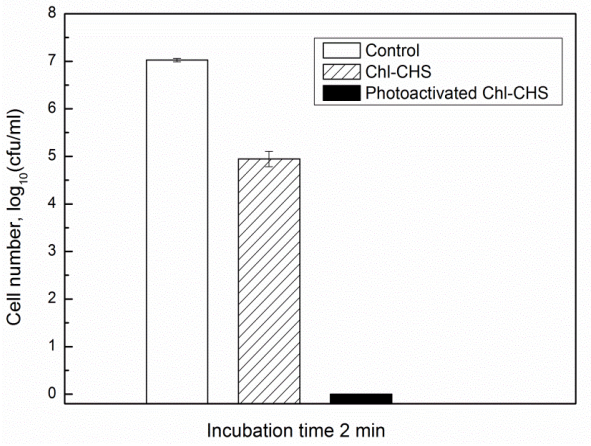

Fig. 3.22 Inactivation of *L. monocytogenes* by photoactivated Chl–CHS (0.00001–0.001%) conjugate (illumination dose·2.9 J/cm^2). Every point is the average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

As depicted in Figure 3.22, significant antimicrobial effects of Chl–CHS conjugate without light activation were observed on Gram-positive bacteria *Listeria.* Moreover, in the case of Gram-positive bacteria the antimicrobial activity of Chl–CHS was quite strong. Inactivation of *Listeria* by 2.1 log was achieved after 2 min of incubation. Photoactivated Chl–CHS reduced the viability of *Listeria* by 7 log within very short 2 min incubation time when 2.9 J/cm^2 illumination dose has been used (Figure 3.22). This means that Gram-positive food pathogen *L. monocytogenes* is more susceptible to photoactivated Chl–CHS conjugate treatment than Gram-negative *S. enterica*.

It was found in previous research (Figure 3.12), that *Botrytis* has low susceptibility to Chl-based photosensitization (28 % inhibition). It was important to test whether *Botrytis* can be effectively inactivated by photoactivated Chl–CHS conjugate. Thus, for inactivation of *Botryt*is with photoactivated Chl–CHS conjugate higher illumination dose have been used.

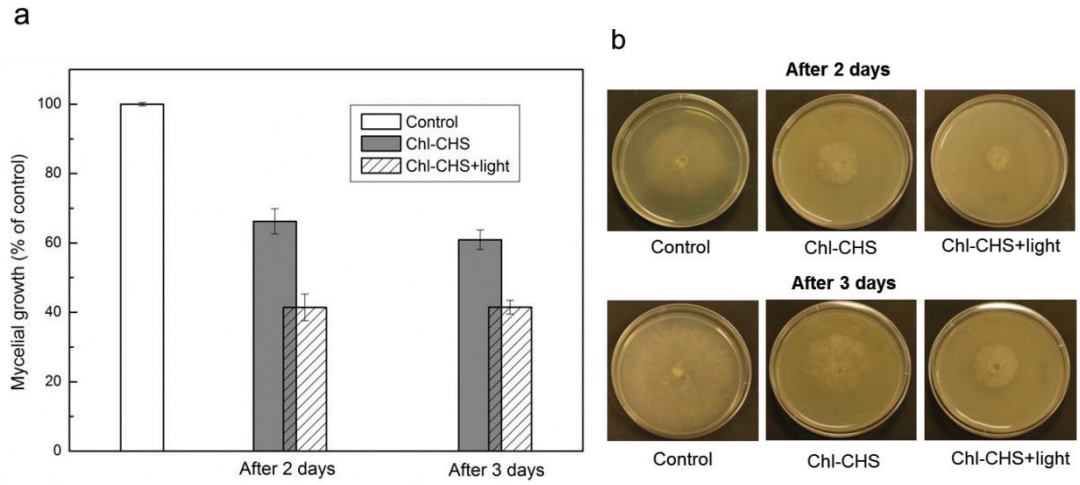

Fig. 3.23 Mycelial growth of *B. cinerea* 2-3 days after treatment by photoactivated 0.001 Chl–0.1 % CHS conjugate (illumination dose 76 J/cm^2) (a); visualization of mould growth (b).

The obtained data indicated that Chl–CHS conjugate without light had some little effect on *Botrytis*. Data presented in Figure 3.23 illustrated the inhibition of mycelial growth of *B. cinerea* after treatment with 0.001 Chl–0.1% CHS. The diameter of the *B. cinerea* in control sample was 100%. 2 days after the Chl – CHS treatment without light mycelial growth reduced by 39% compared to the control. Thus, photoactivated Chl–CHS conjugate in comparison with its dark toxicity was much more effective. After 24 h incubation of *B. cinera* with Chl–CHS conjugate and following illumination (illumination dose 76 J/cm^2) led to 59% reduction of diameter of fungal monocolony) (Figure 3.23). 3 days after treatment the mycelial growth of *Botrytis* remains the same (60% inhibition).

Also, we examined the effects of photoactivated Chl–CHS treatment on main *S. enterica* target membrane integrity. It was assessed by measuring the optical density at 260 nm (OD_{260}) and 280 nm (OD_{280}) of cell free filtrates (supernatant) in control and treated samples (Figure 3.24). The results indicate

that the release of intracellular material absorbing at λ=260 nm and λ=280 nm in control samples is insignificant. On the contrary the release of intracellular components in treated bacteria increases from 0.01 to 0.138 and 0.101 OD (OD_{260} and OD_{280} respectively) when *Salmonella* was treated by photoactivated Chl–CHS conjugate (60 min incubated with Chl–CHS and afterwards illuminated, light dose 38 J/cm^2). It means that some cell membrane injuries occur in *Salmonella* after photoactivation of Chl–CHS conjugate and depend on light dose (light dose 0÷38 J/cm^2).

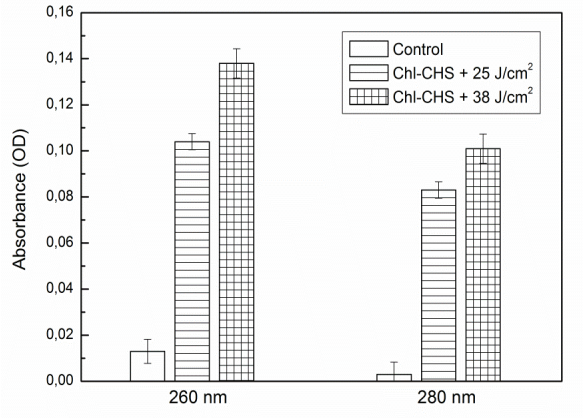

Fig. 3.24 Effect of photoactivated 0.001 Chl–0.1% CHS conjugate treatment on the leakage of UV-absorbing materials at 260 and 280 nm of *S. enterica* (illumination doses 25 J/cm^2 and 38 J/cm^2). Every point is the average of 3 experiments.

SEM images presented in Figure 3.25 show the multiple morphological changes in *Salmonella* cells, which were induced immediately after photoactivated 0.001 Chl–0.1% CHS conjugate treatment (incubation time 30 min; illumination dose 12.7 J/cm^2). SEM images, presented in Figure 3.31b indicate that Chl–CHS biopolymer covers all surface of bacterium and after interaction and photoactivation cells undergo shrinkage.

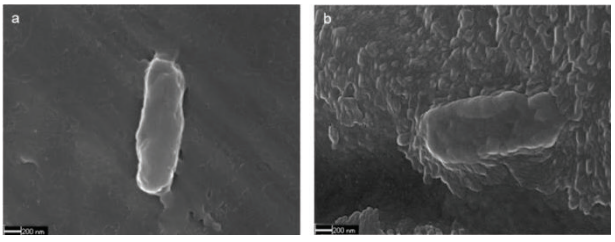

Fig. 3.25 SEM analysis of *S. enterica* cells after photoactivated Chl–CHS conjugate treatment in comparison with control, not treated ones: a) untreated cells; b) cells after photoactivated 0.001 Chl–0.1% CHS conjugate treatment (incubation time 30 min; illumination dose 12.7 J/cm^2).

3.6. Decontamination of food-related packaging from adhered pathogens by ALA and Chl-based photosensitizations

In order to evaluate antibacterial efficiency of ALA and Chl-based photosensitization for decontamination of surface, *B. cereus, L. monocytogenes, S. enterica* were attached onto food packaging material provided by LINPAC (Figure 2.4). Samples of the packaging material (4 × 8 cm) with bacteria attached to it were treated by photosensitization-based treatment. Afterwards treatment-survived bacteria were counted.

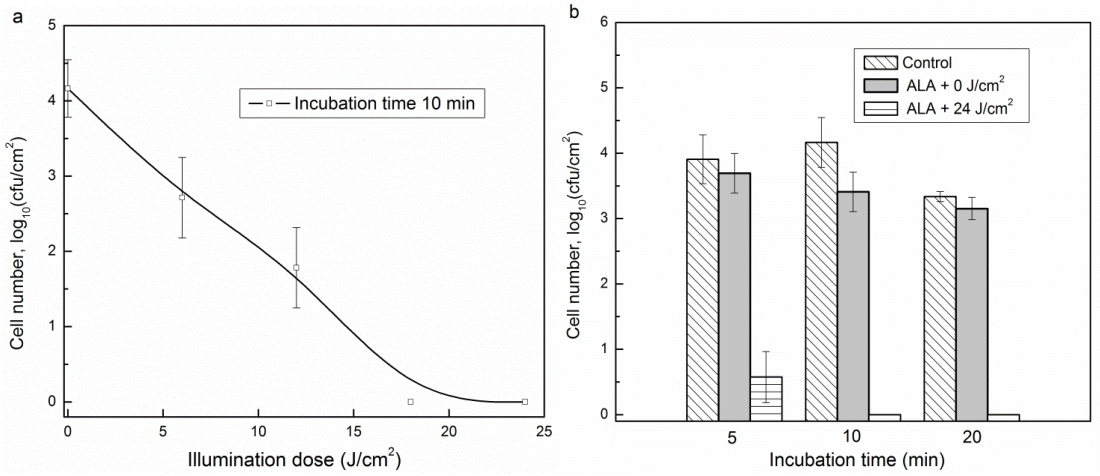

Fig. 3.26 Inactivation of *B. cereus*, attached on packaging samples by $3 \cdot 10^{-3}$ M ALA-based photosensitization as function of illumination dose (a) and of incubation time (b). Every point is the average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

Data depicted in Figure 3.26a describe the ALA-based photoinactivation of Gram-positive *B. cereus* as function of illumination dose. It is clear, that 18 J/cm^2 illumination dose is more than enough to inactivate *B. cereus* by 4 log, when incubation with ALA time is 10 min. Results, presented in Figure 3.26b indicate, that antibacterial efficiency of ALA-based photosensitization is a function of incubation with ALA time as well. When incubation with ALA time is short (5 min), the production of endogenous PSs in bacteria is insufficient and consequently inactivation of *Bacillus* reached just 3.3 log. Further prolongation of incubation with ALA time up to 10 or 20 min increases

antibacterial efficiency up to 4 log. Results, depicted in Figure 3.26 indicated that selected experimental parameters (incubation with ALA time, illumination dose) were optimal to decontaminate packaging materials from *B. cereus* at certain experimental conditions.

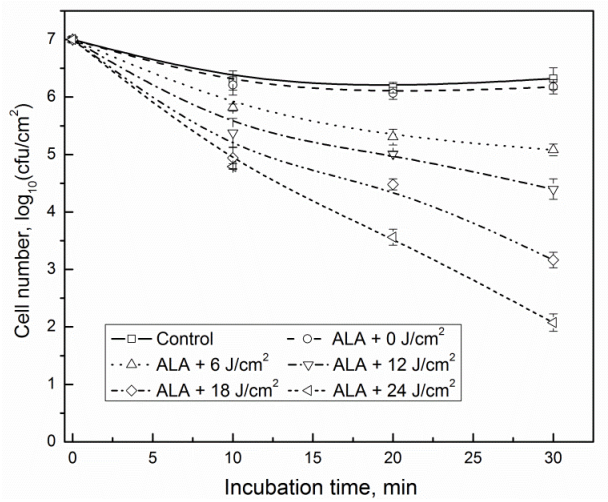

Fig. 3.27 Inactivation of *S. enterica*, attached on packaging samples by $7.5 \cdot 10^{-3}$ M ALA-based photosensitization as function of incubation time. Every point is the average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

The amount of *S. enterica* cells adhered onto one coupon of packaging material reached 7 log. Afterwards coupons of packaging material with the adhered bacteria were soaked in $7.5 \cdot 10^{-3}$ M ALA solution and illuminated with light λ=405nm (illumination dose 0÷24 J/cm^2). The data, depicted in Figure 3.27, clearly indicated that 24 J/cm^2 illumination dose can inactivated *Salmonella* cells just 4.2 log, when incubation with ALA time is 30 min. The obtained results indicated that ALA-based inactivation of *S. enterica* depends strongly on the incubation time and illumination dose (Figure 3.27). When incubation with ALA time is 10 min, the production of endogenous PSs in bacteria is insufficient and consequently inactivation of *Salmonella* after illumination (illumination dose 24 J/cm^2) reached just 1.5 log. Further prolongation of incubation with ALA time up to 10 or 30 min increases antibacterial efficiency up to 4.2 log. Results, depicted in Figure 3.27 indicated that Gram-negative *S. enterica*, adhered onto packaging material, incubated with ALA and then illuminated with light is rather resistant to photosensitization.

Data, depicted in Figure 3.28 describe the Chl-based photoinactivation of Gram-positive *B. cereus* and *L. monocytogenes*. Samples of the packaging material with *Bacillus* and *Listeria* attached to it were treated by $1.5 \cdot 10^{-7}$ M Chl-based photosensitization.

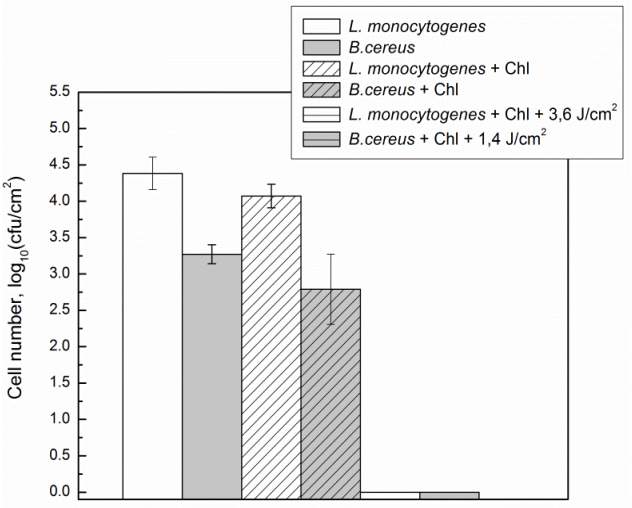

Fig. 3.28 Inactivation of *B. cereus* and *L. monocytogenes*, attached on packaging samples by $1.5 \cdot 10^{-7}$ M Chl-based photosensitization. Incubation time -2 min. Every point is the average of 3÷6 experiments, and error bars sometimes are too small to be more visible.

Gram-positive *L. monocytogenes* and *B. cereus* were attached to the surface of packaging material and samples were soaked in $1.5 \cdot 10^{-7}$ M Chl solution for 2 min. The dried packaging samples were illuminated with 405 nm (illumination dose 1.4÷3.6 J/cm^2). Following 24 hours treatment-survived bacteria were counted. It was determined that $1.5 \cdot 10^{-7}$ M Chl-based photosensitization was very effective and reduced the *Bacillus* and *Listeria* population by 3.3 and 4.4 log respectively (Figure 3.28).

3.7. Decontamination of strawberries by Chl–CHS conjugate: comparative analysis of different antimicrobial treatments

3.7.1. Microbial decontamination of strawberries by different treatments

As it was mentioned above strawberries are highly contaminated with molds and yeasts what is the main reason for their fast spoilage. Data presented in Figure 3.29 allowed us to compare antimicrobial efficiencies of different

surface decontamination techniques. Photosensitization-based treatment, CHS-based and photoactivated Chl–CHS-based treatments were compared with conventional surface treatments such as washing with NaOCl. It is obvious that washing with 200 ppm NaOCl diminishes population of mold and yeasts on the surface of strawberry by 0.55 log, Chl-based (0.001%) photosensitization reduces mold and yeasts in the same level, but increase of Chl concentration (0.1%) is able to enhance antimicrobial efficiency up to 0.9 log. CHS alone is less effective antimicrobial in comparison with that of Na- hypochlorite (0.4 log). The highest inactivation of yeasts and microfungi was found when strawberries were treated by photoactivated 0.001 Chl–0.1% CHS conjugate (1.4 log)(Figure 3.29).

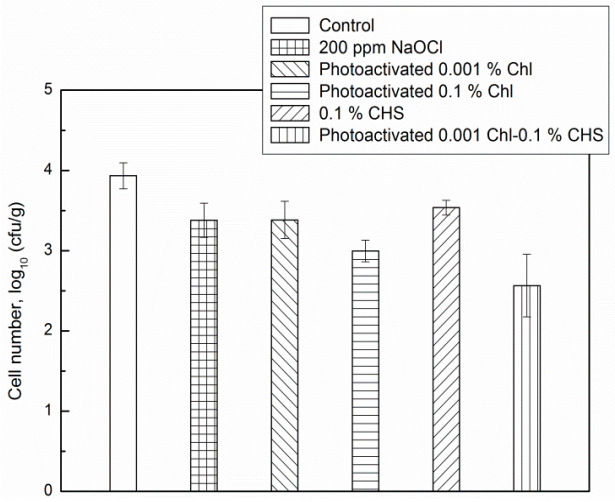

Fig. 3.29 Comparative analysis of different antimicrobial tools: efficiencies of inactivation of molds and yeasts on the surface of strawberries (200 ppm NaOCl–1 min treatment; 0.001 and 0.1% Chl, 0.001 Chl–0.1% CHS conjugate–30min incubation, illumination dose 76 J/cm^2; 0.1 % CTS – 30min incubation). Every point is the average of 3÷6 experiments.

Figure 3.30 illustrates the visual growth of molds and yeasts on the surface of strawberries after treatment with NaOCl (200 ppm, 1 min treatment), Chl-based photosensitization (0.001% and 0.1%, illumination dose 76 J/cm^2) and photoactivated Chl (0.001%)–CHS (0.1 %) (illumination dose 76 J/cm^2).

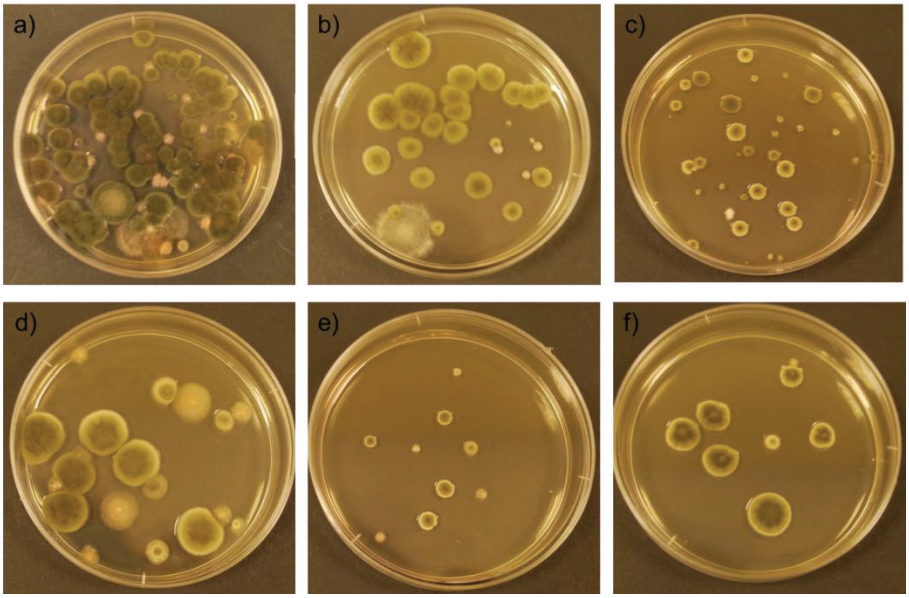

Fig. 3.30 Decontamination of strawberries by different antimicrobial treatments: a) untreated; b) 200 ppm NaOCl–1 min treatment; c) 0.1% CHS–30 min incubation ; d) 0.001% Chl-based photosensitization–30 min incubation; e) 0.1 % Chl-based photosensitization–30 min incubation; f) photoactivated 0.001 Chl–0.1% CHS conjugate – 30 min incubation. Illumination dose–76 J/cm^2.

3.7.2. Evaluation of visual quality and shelf-life of treated strawberries

Table 3.3 shows the effect of different treatments on the visual overall quality of treated strawberries during storage at 22 ± 2 °C for 10 days.

Table 3.3 Changes in the visual overall quality of strawberries treated with different antimicrobial tools during storage at (22 ± 2 ºC).

Storage time (day)	Control	Chl–CTS (0.1%)	Photoactivated Chl–CTS (0.001–0.1%)	Photoactivated Chl (0.001%)	NaOCl (200 ppm)
0	9.0 ± 0.0	9.0 ± 0·0	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
1	7.4 ± 1.0	7.7 ± 1,6	8.6 ± 0.5	8.4 ± 0.6	7.5 ± 0.5
2	6.0 ± 1.5	6.3 ± 2,1	8.2 ± 0.8	7.9 ± 0.9	7.1 ± 0.8
3	4.8 ± 1.8	4.8 ± 2,4	7.2 ± 1.5	6.8 ± 1.2	5.6 ± 1.1
4	3.2 ± 2.1	3.0 ± 2,3	5.3 ± 2.3	5.7 ± 1.9	4.8 ± 1.8
5	1.7 ± 1.4	1.9 ± 1,7	3.9 ± 2.5	4.7 ± 2.4	3.7 ± 2.3
6	1.1 ± 0.7	1.6 ± 1,4	2.8 ± 2.3	3.4 ± 2.4	2.7 ± 2.1
7	1.0 ± 0.0	1.3 ± 0,9	2.4 ± 2.0	2.5 ± 1.8	2.2 ± 2.0
8	1.0 ± 0.0	1.0 ± 0,0	1.9 ± 1.7	1.9 ± 1.7	1.9 ± 1.6
9	1.0 ± 0.0	1.0 ± 0,0	1.7 ± 1.5	1.6 ± 1.5	1.7 ± 1.6
10	1.0 ± 0.0	1.0 ± 0,0	1.4 ± 1.0	1.4 ± 1.0	1.6 ± 1.2

Results are expressed as mean values of three replicate measurements ± standard deviation

There were no significant difference between control and treated samples at day 0 of storage. The visual overall quality of strawberries gradually decreased over storage time. The score decreased from 9 to 4,8 after 3 days in control and from 9 to 5.6 in 200 ppm NaOCl treated strawberry group, from 9 to 4.8 in CHS treated strawberry group, from 9 to 6.8 in Chl-based photosensitization treated group, from 9 to 7.2 in Chl–CHS conjugate and light treated group. Also photoactivated Chl and Chl–CHS conjugate is most effective tool in delaying strawberry spoilage.

It is obvious that the most important advantage of any antimicrobial technology is ability to extend the self-life of treated berries. Shelf-life of berries was evaluated visually from surface color changes (visible fungi) (Figure 3.31). The overall appearance of treated strawberries during storage at 22 ± 2 ºC for 10 days was examined. Generally, the visual overall quality of strawberries gradually decreased over storage time.

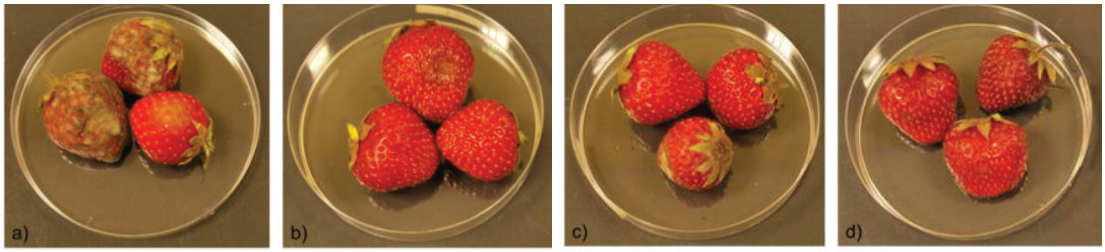

Fig. 3.31 Visual quality of strawberries after treatment (after 4 day storage at 22 ± 2 ºC): a) control group; b) 0.001 Chl–0.1% CHS conjugate; c) photoactivated 0.001% Chl (illumination dose 76 J/cm^2); d) photoactivated 0.001 Chl–0.1 % CHS conjugate (illumination dose 76 J/cm^2).

Our data on visual quality of control strawberries coated with Chl–CHS conjugate without illumination, and strawberries treated by photoactivated Chl – CHS conjugate (in every case 60 strawberries have been used) indicate that it is possible to achieve some delay of spoilage when berries are coated with Chl–CHS and illuminated. For instance, control strawberries 7 days after treatment were totally infected (visually detected spots of infection), whereas coating of strawberries with Chl–CHS (Figure 3.31c, dark toxicity) reduced the natural spoilage. But, it is obvious that Chl-based photosensitization (Figure 3.31a) and photoactivated Chl–CHS conjugate (Figure 3.31d) were most effective tool in

delaying strawberry spoilage. Thus, as depicted in Figure 3.31, the disease-free period of treated strawberries was prolonged about 3 days in comparison with control. This is a significant effect for strawberry producers.

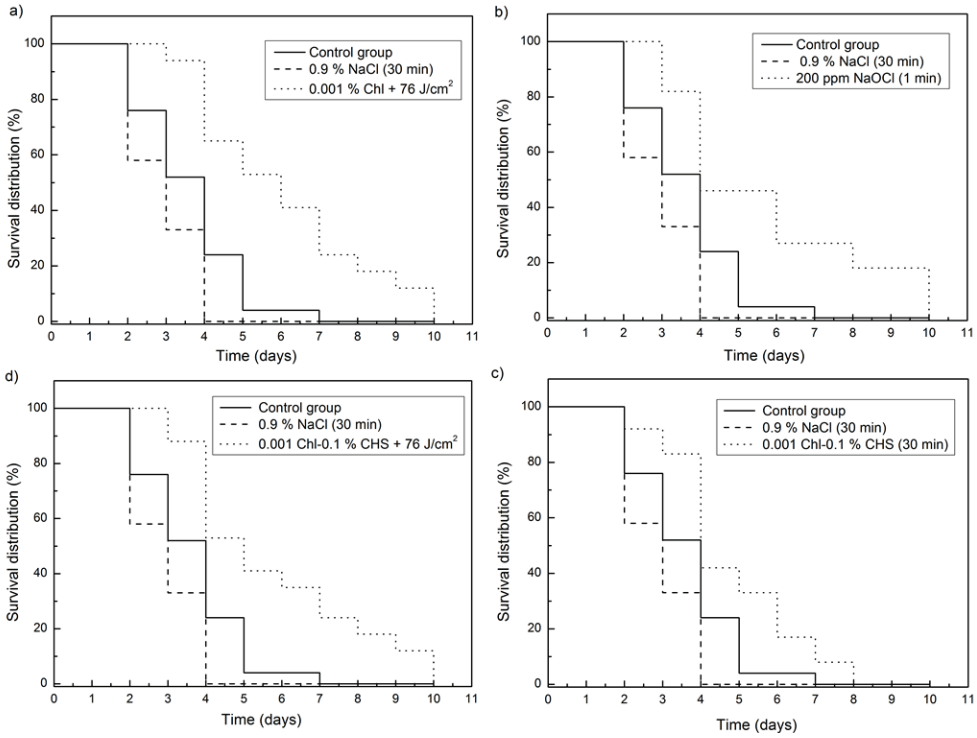

Fig. 3.32 Shelf-life (storage at 22 ± 2ºC) of strawberries treated by 0.001% Chl-based photosensitization –30min incubation, illumination dose 76 J/cm^2 (a), 200 ppm NaOCl –1 min treatment (b), 0.001 Chl–0.1% CHS conjugate – 30min incubation (c), photoactivated 0.001 Chl–0,1% CHS conjugate – 30min incubation, illumination dose 76 J/cm^2 (d).

Measuring extension of shelf-life of treated strawberry was evaluated according to Kittemann (136) system (1÷6 scale), 7 day storage makes all control beries (100%) infected and disease index (N) was the highest N=6, whereas just 76% of Chl–CHS treated berries were damaged by spoilage microorganisms, thus disease index was significantly lowered (N=4.6) (Figure 3.32d).

3.7.3. Total antioxidant activity

According to the obtained results depicted in Figure 3.33 the total antioxidant activity in control berries was 17,11 mmol Fe^{2+}/kg whereas in

treated berries it increased to more than 20,33 mM Fe^{2+}/kg. This statistically insignificant increase (19%) of total antioxidant activity was observed in the strawberry immediately after photosensitization and does not reduce during next 48 hours.

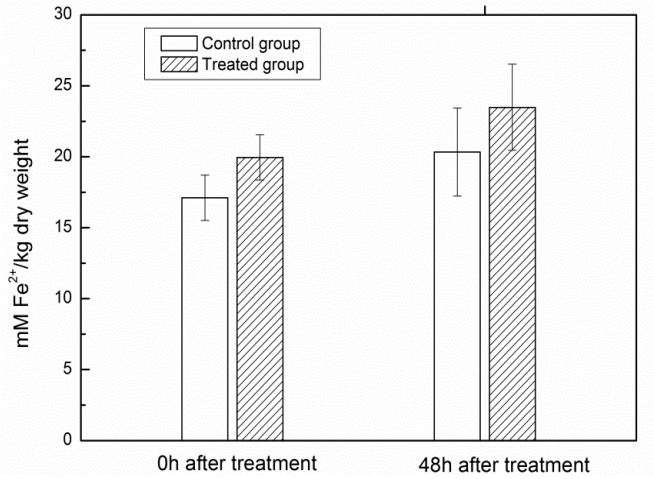

Fig. 3.33 Total antioxidant activity in strawberry after photoactivated 0.001 Chl–0.1% CHS coating of fruits in comparison with control during 0 and 48 h after treatment keeping them at 5 ± 0,5 ºC.

3.7.4. Measurements of strawberry color and weight losses

The other important characteristic which can be influenced by treatment is the appearance of berry, especially its color.

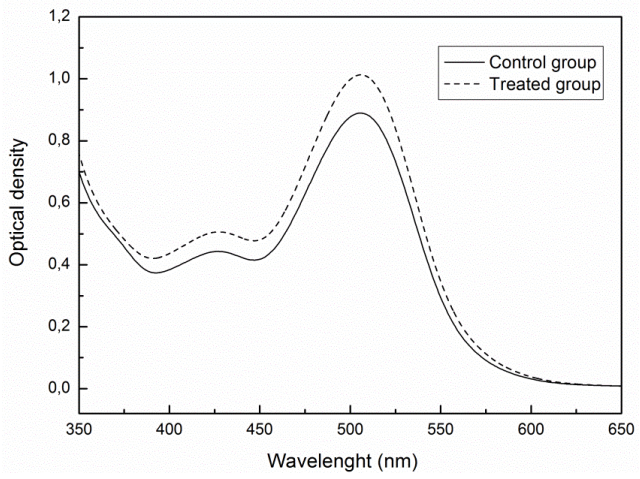

Fig.3.34 Strawberry color changes after photoactivated 0.001 Chl–0.1% CHS coating of fruits (illumination dose 76 J/cm^2): absorption spectrum of strawberry extract samples in visible region.

To determine whether photoactivated Chl–CHS coating of fruits had any negative effects on the color of the strawberry they were analyzed immediately after treatment. For this purpose absorption spectroscopy was used to analyze the spectrum of berry extract in visible region. It is evident from Figure 3.34 that no significant color changes are detected over all visible spectrum region (350÷650 nm) meaning that photosensitization has no significant impact on strawberry color.

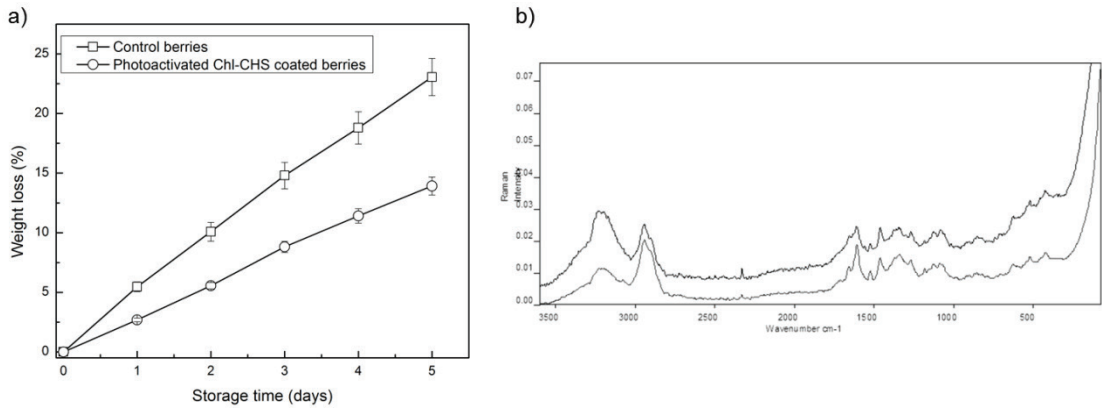

Fig. 3.35 Weight losses during storage in control and photoactivated Chl–CHS coated strawberry 5 days after treatment (a) and FT-Raman spectra of control (lower spectrum) and Chl–CHS coated berries (upper spectrum)-(b).

Figure 3.35a shows weight loss during 5 day storage of control and Chl – CHS coated strawberry. All samples (every consisted of 20 berries) demonstrated a gradual loss of weight during storage. Throughout storage, the loss of weight of control berries was significantly greater than that of coated ones. At the end of storage, uncoated strawberry showed 22% loss in weight, whereas the weight loss of coated strawberry was just 12%.

These data were confirmed by FT-Raman spectroscopy data of Chl–CHS coated strawberry (upper spectrum) in comparison with not coated control ones (lower spectrum) (Figure 3.35b). It is obvious that peak at 3200 cm^{-1} (typical water absorption) has much higher intensity for Chl–CHS coated strawberry in comparison with that of not-coated control ones.

3.7.5. Detection of free radicals in treated strawberry by electron paramagnetic resonance (EPR)

The data presented in Figure 3.36 show EPR spectra of control and photoactivated Chl–CHS coated strawberry in a wide field range (from 10 mT to 600 mT). The spectrum consists of typical 6 signals which were separated from each other by ~ 9 mT. According to Raffi and Stocker (141) these signals from 320 mT to 380 mT belong to Mn^{2+} which is normally in strawberry in measurable amounts. According to P. Leveque (142) the signal in lower field (170 mT) belongs to the Fe^{3+}. The number of lines from the hyperfine interaction was determined by the formula:

$n = 2NI + 1$,

where n is a number of spectral lines, N is the number of equivalent nuclei, I is the spin.

In our case, $N = 1$, $I = 5/2$ from the manganese nucleus, thus amount of spectral lines was 6.

Comparison of spectra of control and treated strawberry supposed that this treatment did not induce additional free radicals in the matrix of strawberry.

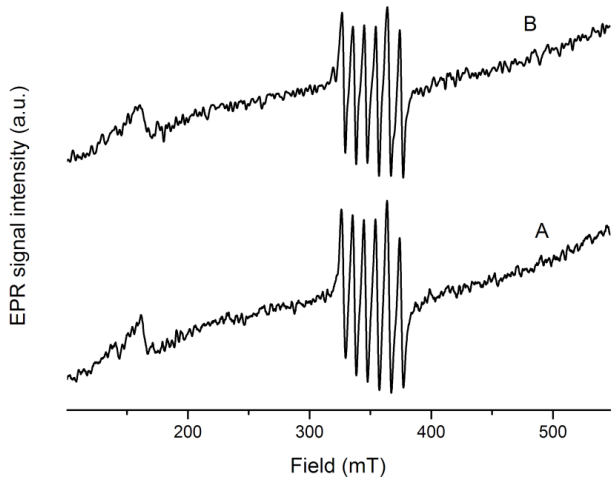

Fig.3.36 EPR spectra of strawberry: control (A) and (B) photoactivated Chl–CHS coated strawberry. Mn^{2+} lines (g=1.87608; 1.92801; 1.98124; 2.03521; 2.09085; 2.14451).

4. 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 (18). In this context, modern light-based technologies, including high power pulsed light or photosensitization might serve as promising antibacterial tools (42,74,143,144).

Since the beginning of the 20th century it is well known that certain microorganisms can be killed by the combination of dye and appropriate light (53,145). Antimicrobial photosensitization-based technique has been developed especially during the last 25 years (62). The main prerequisite for it were growing concerns about the emergence of antibiotic-resistant bacterial strains. One of the most important advantages of photosensitization-based treatment in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment (59,63,64). Also, it is important that all cytotoxic reactions are local; this fact allows us to achieve an extensive decrease in the population of pathogens with minimal damage effects on the surrounding matrix (62). It was confirmed by a series of experiments that photosensitization, in contrast to ionizing radiation, has no mutagenic or carcinogenic effects on living systems (146). Several infections, such as *Helicobacter pylori*, *Propionibacterium acnes*, *Candida albicans*, trauma-associated and oral infections have been treated by photosensitization using xanthene, acridine dyes and positively charged porphyrins (54). The photosensitizing properties of cationic phenothiazine dyes on Gram-positive and Gram-negative bacteria, yeast and viruses have been known since the 1960s and have been employed for sterilization of blood plasma in German and Swiss centers since 1992 (147).

The inactivation of pathogens on food or food-related surfaces by photosensitization-based treatment is a novel approach (52,148). Undoubtedly, the main premise in this case is the used PS, which besides its suitable photophysical and photochemical properties must be a pure and water soluble compound with a stable shelf-life, not bleaching and easy to produce. Moreover, it must be food constituent or food additive. We compared the antimicrobial efficiency of aminolevulinic acid-based (ALA) photosensitization and chlorophyllin-based (Chl) photosensitization treatments against food pathogens Gram-positive *L. monocytogenes* and *B. cereus*, and Gram-negative *S. enterica*. Our results indicate that *Salmonella* is more resistant to Chl-based photosensitization than to ALA-based, while *Listeria* was more sensitive to Chl-based than to ALA-based photosensitization treatment. Therefore Gram-positive *Bacillus* was sensitive to both photosensitization types. Based on the experimental results, we suggested an innovative approach to combat *L. monocytogenes*, *B. cereus* and *S. enterica* by photosensitization in an effective and uniform way.

It is worth emphasizing that the phototoxic action on pathogens is caused by the combined action of two factors – PS and visible light, which separately are devoid of any detectable toxicity at used doses (62). The selected PS must have suitable photophysical and photochemical properties and be easy to produce and effective against foodborne pathogens. It must be either a food constituent or at least be nontoxic at the concentrations required for microbial inactivation. It is know that a fundamental difference exists in the susceptibility to photosensitization between Gram-positive and Gram-negative bacteria. It is well documented that photosensitization-based inactivation is not enough effective to kill Gram-negative bacteria when neutral or anionic PSs have been used. Neutral, anionic, or cationic PSs can inactivate mostly Gram-positive bacteria (73), whereas Gram-negative bacteria due to more conjugate cell wall structure and additional negatively charged outer membrane are less susceptible to this treatment and need higher doses of PS and light (94). Several attempts have been made to achieve identical and equal inactivation of

naturally distributed microorganisms, consisting of both Gram-negative and Gram-positive bacteria.

There are myriads of Gram-positive bacteria (69) which might be destructed by the photosensitization-based technology. However, there are only a few reports on photosensitization-based treatment against *L. monocytogenes* (149–151). It is well known that neutral or anionic PSs 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 PS to cross (59,65,95,96).

The envelope of Gram-negative bacteria consists of 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. However, different approaches aim to overcome this problem by, for example, creating positively charged (cationic) PS or by coupling or combining the PS with positively charged entities such as poly-L-lysine(152), polyethylenimine (153) and polymyxin B nonapeptide (PMBN) (65). Nitzan and co-workers used membrane disintegrating agent polycationic PMBN to increase the permeability of the outer membrane of Gram-negative bacteria (65). After illumination viability of the bacteria was dramatically reduced. Chlorin e6 (ce6) has been used as a second-generation PS since it absorbs long-wavelength light and has a high photosensitizing efficacy (152). A second approach for inactivation of Gram-negative bacteria can be the usage of pre-treatment with toluene, ethylene diamine tetraacetic acid (EDTA) which then induced susceptibility of Gram-negative bacteria to photosensitization treatment (154,155).

One of the most ordinary approaches to kill Gram-negative bacteria is the use cationic PSs (56,156). George et al. (157) has found that the uptake of cationic PSs by the cell is mediated by electrostatic interactions and self-promoted uptake pathways. Usually, cationic sensitizers after accumulation in the cell tend to bind DNA molecule. The cationic blue dyes known as

phenothiazinium salts (toluidine blue O (TBO) and methylene blue (MB)) have been studied for 40 years (153). Ozkanca *et al.* (158) demonstrated photoinctivation of *Salmonella typhimurium* LT2 in filtered autoclaved seawater microcosm by TBO and light. A 1.5 log reduction in *Salmonella* cell viable count was observed after 8 h treatment. The photoinactivation of *S. enterica* using cationic dye malachite green resulted in 2.3 log reduction of bacterial population (15).

In our opinion, the main disadvantage of photosensitization-based treatment is high resistance of Gram-negative bacteria to this treatment. Photosensitization of pathogen by endogenously produced porphyrins is another effective approach to their eradication. It is well established that most bacteria use the heme biosynthetic pathway to produce porphyrins from precursor 5-aminolevulinic acid (ALA) (66). These porphyrins represent mixture of coproporphyrin, uroporphyrin and protoporphyrin (54) and after excitation with λ=405 nm light can produce photocitotoxic effects in bacterial cells. ALA-based photosensitization was found to induce a more pronounced antibacterial effect on Gram-positive bacteria than on Gram-negative ones. Nitzan *et al*. (69) reported that upon illumination of 3.8 10^{-3} M ALA induced bacterial strains with 407÷420 nm light (50 J/cm^2 illumination dose) resulted in 5 orders of decrease of Gram-positive *Staphylococcus* strains as opposed to 1 order of decrease in Gram-negative strains. The dates obtained in this study clearly indicate that Gram-positive *L. monocytogenes*, *B. cereus* and Gram-negative *S. enterica* produce endogenous porphyrins in sufficient amounts (Figure 3.2–3.4) and can be destructed by ALA-based photosensitization: *Bacillus* – 6.4 log, *Salmonella* –5.9 log, *Listeria* – just 4 log.

The first results obtained in this study on the inactivation of bacteria cells by ALA-based photosensitization *in vitro* look promising. It is important to note that Gram-negative multidrug resistant food pathogen *S. enterica,* which produced a smaller amount of endogenous PSs (Figure 3.4), was inactivated by $7.5 \cdot 10^{-3}$ M ALA-based photosensitization (60 min incubation time, 24 J/cm^2 illumination dose) in a slower but effective way (5.9 log

inactivation) without leaving resistant cells (Figure 3.7). In addition, Gram-positive biofilm forming food pathogen *L. monocytogenes* was most resistant and after 60 min incubation with 7.5 10^{-3} M ALA and 20 min illumination (24 J/cm^2 illumination dose) was inactivated only by 4 orders of magnitude (Figure 3.6). Other Gram-positive food pathogen *B. cereus* can effectively produce endogenous PSs from exogenously applied ALA at $3 \cdot 10^{-3}$ and $7.5 \cdot 10^{-3}$ M concentrations (Figure 3.2). Comparative analyses of fluorescence intensity of endogenous PSs of all tested bacteria reveal that *B. cereus,* during 60 min incubation with $7.5 \cdot 10^{-3}$ ALA, is producing endogenous porphyrins 10 times more efficient, than *L. monocytogenes* or *S. enterica.* As a consequence *B. cereus* is more susceptible to ALA-based photosensitization than *L. monocytogenes*. Really fast and significant inactivation (6.4 log inactivation) of this microorganism can be achieved after ALA-based photosensitization (Figure 3.5).

The data obtained in this study clearly indicate that the ALA-based photosensitization treatment efficiency of inactivation strongly depended on treated bacteria cells incubation with ALA time (produced endogenous porphyrins) and illumination dose delivered to the bacteria. Hence, there are enough ways to increase the inactivation efficiency of ALA-based photosensitization, if necessary. These data are in line with the results described by Fotinos et al. (66), who found that both Gram-positive (*Staphylococcus. aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) are able to produce endogenous porphyrins, and that inactivation efficiency mostly depends on the concentration of produced endogenous porphyrins.

Decontamination of packaging material from *Bacillus* and *Salmonella* adhered to the surface by ALA-based photosensitization treatment seems really promising. Even about 4 log inactivation was achieved after ALA-based photosensitization treatment (Figure 3.26 and 3.27). The data, depicted in Figure 3.27, clearly indicated that the inactivation of Gram-negative *Salmonella* cells attached to the surface of packaging after photosensitization-

based treatment reached just 4.5 log from initially attached 7 log. Meanwhile data, shown in Figure 3.26 indicated that amount of *Bacillus* cells adhered onto one coupon of packaging material was more lower (~4 log) and cells inactivation after ALA-based treatment reached undetectable level .

The application of photosensitization-based inactivation is considered for decontamination of food. Not every PS 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 will not change the organoleptic properties. According Luksiene et al. (70) decontamination of wheat sprouts from microfungi by ALA-based photosensitization revealed that besides significant antimicrobial properties of this treatment ALA could stimulate the growth of wheat seedlings and roots, increase photosynthesis rate and activity of antioxidant enzymes without impairing the vigor of germination and the viability of seeds. Also ALA increased the rate of photosynthesis and the activities of antioxidant enzymes, which could be associated with enhanced cellular capacity to detoxify reactive oxygen species (70). In addition, ALA is an essential precursor of such tetrapyrole compounds as vitamin B12 and hemes (159).

Meanwhile, ALA is comparatively expensive compound to use it in food industry. Moreover synthesis of endogenous porphyrins from ALA in the bacteria requires additional time (20÷60 min) what increases the duration and the costs of technology (14). Chlorophyll and its derivatives are widely known photosensitizer in PDT. Although different classes of porphyrins have been tested against Gram-positive and Gram-negative bacteria, chlorophyllin-based photosensitization and the concomitant inactivation of food pathogens has been never investigated.

Data obtained in this study reveal that sodium chlorophyllin (Chl) exhibits excellent photosensitizing properties. Chl is obtained by the saponification of oil soluble forms. Chl provides an olive green shade and has poor stability in both heat and light. Chl is a highly fluorescing food additive (E140ii) known for its anti- mutagenic and anticarcinogenic properties (160),

exhibiting high antioxidant capacity (161). The absorption and fluorescence spectra, presented in Figure 3.8 indicate that $1.5 \cdot 10^{-5}$ M Chl molecules tend slightly to aggregate in PBS. Fluorescence intensity increase from 640 to 768 a.u. when 20 μl 0.001% Triton-X100 was added to the solution.

The analysis of bacterial survival curves indicates that Gram-negative *Salmonella* is more resistant to Chl-based photosensitization than Gram-positive *Listeria* and *Bacillus* (Figures 3.9–3.11). The different susceptibilities of Gram-negative and Gram-positive bacteria to photosensitization-based treatment can be attributed to the particular structure of their cell wall (74). Theoretically, in Gram-positive bacteria the outer wall (15÷80 nm thick) contains up to 100 peptidoglycan layers which are associated with lipoteichoic and negatively charged teichuronic acids. This wall is not act as a permeability barrier for the most used PSs as porphyrins, whose molecular weight does not exceed 1500÷1800 Da (162,163). Thus, after accumulation of Chl (average molecular weight 596 ± 9 Da) by cell and ensuing photosensitization *B. cereus* and *L. monocytogenes* were diminished by 7 log (undetectable level) using very low PS concentration ($1.5 \div 7.5 \cdot 10^{-7}$ M) *in vitro* (Figures 3.9 and 3.10 a). As depicted in Figure 3.9, slower inactivation rate of *B. cereus* was observed when lower $7.5 \cdot 10^{-8}$ M Chl concentration was used. The incubation of bacteria with $7.5 \cdot 10^{-8}$ M Chl and following illumination (illumination dose 6 J/cm^2) decreased cell number by 7 log. The incubation of *Bacillus* with higher $7.5 \cdot 10^{-7}$ M Chl concentration requires lower 2.4 J/cm^2 illumination dose to inactivate them by 7 orders of magnitude. 10^7 and 10^8 cfu/ml cells concentrations of *L. monocytogenes* were used for Chl-based photosensitization experiments (Figure 3.10). The effectiveness of Chl-based photosensitization increased with decrease of *L. monocytogenes* cell density (Figure 3.11b). After 2 min incubation with $1.5 \cdot 10^{-7}$ M Chl and 1 min of illumination (illumination dose 1.3 J/cm^2) the 10^8 and 10^7 cells count decreased by 3.5 and 5.6 orders of magnitude respectively. However inactivation of *Listeria* cells to undetectable level requires higher illumination dose: 6.3 J/cm^2 for 10^7 cfu/ml cells concentration and 25.3 J/cm^2 for 10^8 cfu/ml cells concentration. The incubation

of 10^8 cells count bacteria with higher $1.5 \cdot 10^{-6}$ M Chl concentration requires lower illumination dose (2.5 J/cm^2) to inactivate them by 8 orders of magnitude (Figure 3.10 b). As a role, the efficiency of photoinactivation depends on the used PS concentration and illumination time, bacterial cells concentrations. In this case we found that variation of incubation time with Chl for 2÷60 min does not influence the photoinactivation efficiency when all other parameters were kept constant (Chl concentration, illumination time (dose)). These data are in line with previously published results (164) indicating that the extension of the incubation time from 2 min to 1÷2 hours has no effect on the amount of PS bound to the microbial cell. By no means, more detail quantitative evaluation of cell-bound PS would give additional information about kinetics of this process. Thus, summarizing our recent experience, all experiments with Gram-positive *B. cereus* and *L. monocytogenes* were performed using "shortest effective" Chl incubation time (2 min). The comparison of ALA- and Chl-based photoinactivation of *B. cereus* and *L. monocytogenes* indicates some advantages of last treatment. First, when we use Chl as PS we can shorten the incubation time from 20 min to 2 min. Second, we can reduce the illumination dose from 24 to 6 and 6.3 J/cm^2. Third, Chl is effective photosensitizing compound and "works" at extremely low concentrations ($1.5 \cdot 10^{-8}$ M) whereas ALA "working" concentration was much higher ($7.5 \cdot 10^{-3}$ M). Our data are in line with Kreitner et al. (165) who examined the photosensitivity of Gram-positive bacteria and yeasts to sodium chlorophyllin based photosensitization and found that S. aureus, B. subtilis, Saccharomyces cerevisiae, Rhodotorula mucilaginosa were susceptible to this treatment (3÷5 log inactivation) after incubation with 10^{-5} M concentration PS and illumination for 60 min with light.

In order to decrease microbial contamination, the fresh-cut industry commonly uses NaOCl and acids as disinfection agents but by-products such as trihalometanes and chloramines are potentially harmful for humans (14). Inactivation of *L. monocytogenes* and *B. cereus* on the surface of packaging material by Chl-based photosensitization indicates that this treatment can clean

surface from *Listeria* and *Bacillus* totally (Figure 3.28). Comparative analysis of efficiencies of different antimicrobial tools indicates that washing with sterile water or NaOCl is less effective than photosensitization in the case of *Listeria* and *Bacillus* strains.

It is well documented, that photosensitization-based inactivation is not enough effective to kill Gram-negative bacteria when neutral or anionic PS have been used. Gram-negative bacteria due to more complex cell wall structure and additional negatively charged outer membrane are less susceptible to this treatment and need higher doses of PS and light (94). Our results, obtained on inactivation of Gram-negative *S. enterica* by negatively-charged Chl-based photosensitization (Figure 3.11b) indicated that just 1.27 log reduction of microbial population can be achieved when cells were incubated with $1.5 \cdot 10^{-5}$ M Chl for 60 min and get 17.3 J/cm^2 illumination dose. An extension of incubation time from 60 to 120 min enhanced the inactivation of *Salmonella* to 2.05 log. However when illumination dose was increased to 51 J/cm^2, the number of surviving *Salmonella* cell count decreased by 2.5 orders (Figure 3.11b). This means that *Salmonella* incubated with Chl and then illuminated with light is rather resistant to photosensitization.

It is important to mention that we tested whether the binding of Chl to the *S. enterica* and *L. monocytogenes* cells was important. We carried out the studies both "without" and "with a wash", a centrifugation step after Chl incubation and before NaN_3 addition. Actually, there are three main ways for cell-PS interaction. In the first case, PS does not bind to the bacterium, remains outside and damages just cell wall. In the second way, it binds the bacterium externally and damages extracellular structures. In the third variant, PS accumulates inside the bacterium. In this case the PS is transported inside the cell, were it associates with the key structures and irreversibly damages them after photosensitization (52). It is important to note, that the increase of incubation time from 15 to 120 min with Chl change cell inactivation level slightly (Figure 3.11a) what means that probably Chl bound the cell from outside and did not need longer incubation time to accumulate inside the cell.

In order to understand cell-PS interaction intensity we measured fluorescence spectra of *Salmonela* and *Listeria* cells incubated with Chl and compared with that of supernatant and separated cells alone (Figure 3.13). Data in Figure 3.13a indicate that most of the $1.5 \cdot 10^{-5}$ M Chl remains in the supernatant and just small part is bound to Gram-negative *Salmonella*. So far, this small fraction of Chl-attached *Salmonella* is damaged after photosensitization-based treatment. Data in Figure 3.13b indicated that ~ 50% of the $1.5 \cdot 10^{-7}$ M Chl remained in the supernatant and ~ 20% was bound to Gram-positive *Listeria*.

Therefore the effectiveness of Chl-based photosensitization was dramatically reduced when Chl was washed out of the cell suspensions - centrifugation step after Chl incubation and before photosensitization-based inactivation (Figure 3.14). This was applied to both microbial species - Gram-negative *Salmonella* and Gram-positive *Listeria*. These data are in line with Demidova and Hamblin (74) published results indicating that cationic TBO was less effective after washing. As depicted in Figure 3.14a, after 2 min of incubation with $1.5 \cdot 10^{-7}$ M Chl, ensuing centrifugation step and illumination treatment (illumination dose 1.3 J/cm^2), the number of surviving *Listeria* cell count decreased by 2.2 log. However, killing increased to 5.7 log when Chl was not washed out of the cell suspension. The same decrease of cells photoinactivation was obtained when experiments were performed with Salmonella and $1.5 \cdot 10^{-5}$ M Chl (Figure 3.14b). After centrifugation step and ensuing illumination (illumination dose 88.7 J/cm^2), the number of surviving *Salmonella* cell count decreased by 1.7 orders. However, killing increased to 5.4 log when cells after incubation with Chl were illuminated without a wash.

In order to find new effective combination of antimicrobial treatments it is important to know cell injuries which are produced by each of them. The question arise what is the mechanism of cell damage after Chl-based photosensitization. Usually after light excitation the triplet-state PS interacts with molecular oxygen, electron donors or acceptors and can produce ROS. This interaction may proceed in two distinctly different ways: type 1 mechanism involves electron transfer and leads to free radicals, such as

superoxide anion, hydroxyl radical. The type 2 mechanism leads to the formation of $^1O_2$, which is reactive and directly destroy bacteria. Both reaction types can occur simultaneously and the ratio between them depends on the structure of PS and microenvironment (148). It is know that Gram-positive bacteria are more sensitive to $^1O_2$ and Gram-negative bacteria are more susceptible to $HO^{\cdot}$ (56). However the intracellular uptake of PS is not severally necessary to kill both type bacteria. ROS generated outside or at the cell wall by PS that are bound to bacterial cells, maybe cause lethal damage to the cell membrane by the process of diffusion (56,166,167).

NaN_3 is generally employed to quench $^1O_2$ during photodynamic therapy (PDT), especially when PDT is used to inactivate bacteria in suspension (60). Tavaras et al. (168) found that NaN_3 strongly inhibited the bactericidal photosensitization effects of porphyrin-based PS. Huang et al. (60) observed that addition of NaN_3 to Gram-positive and Gram-negative bacteria incubated with cationic MB and illuminated with light have significantly increased bacterial killing, unlike the conjugate PEI-ce6 showed reduced photosensitization-based killing after addition of 10 mM NaN_3. It should be noted that NaN_3 is toxic to all Gram-negative but not many Gram-positive bacteria (166,169). Initially, we checked that at 10 mM concentration for 60 min there was no NaN_3 toxicity to *S. enterica* and *L. monocytogenes*. We found that the addition of 10÷40 mM NaN_3 in $1.5 \cdot 10^{-7}$ M Chl and Gram-positive *Listeria* suspension without centrifugation step was not effect on the cells photoinactivation (Table 2.4). However data presented in Figure 3.16a indicate that $^1O_2$ quencher NaN_3 can protect Gram-negative *Salmonella* from Chl-based ($1.5 \cdot 10^{-5}$ M Chl) photosensitization induced killing (without a wash) what means that type II mechanism took place in photochemical reactions. The addition of NaN_3 inhibited the Chl-based treatment killing of *Salmonella* at 38 J/cm^2 by 1.3 log (Figure 3.16a). However after *Salmonella* cell centrifugation effect of NaN_3 was insignificant (Figure 3.16b). Also we demonstrated that 10 mM NaN_3 added after *Listeria* cell centrifugation in the presence of photosensitization treatment with $1.5 \cdot 10^{-7}$ M Chl potentiated photoantibacterial

activity by 3 log (Figure 3.15). Similar result was demonstrated by other authors. According to Kasimova et al. (166) there was a great variation in the effects of NaN_3 on the photosensitization-induced killing in between PS and also in between bacteria species. They showed that Gram-negative *E. coli* with a wash shows potentiation by NaN_3 with every single phenotiazinium dye. The differences in NaN_3 inhibition efficiency may depend on differences in the extent of FS binding to bacteria or differences in penetration of NaN_3 into cell walls of bacteria.

In addition, SEM images of treated bacteria indicate intensive shrinkage of bacterial cells which usually is result of leakage of intracellular components in the cell (Figure 3.19). To confirm membrane disintegration in *Salmonella* and *Listeria* after Chl-based photosensitization the leakage of some proteins (280nm) and DNA components (260nm) has been evaluated (Figures 3.17 and 3.18). Data indicate that after photosensitization extensive leakage of DNA and proteins was found in Gram-negative *Salmonella* what means that photosensitization treatment induced significant cell membrane disintegration what was confirmed by SEM images (Figure 3.19b). On the contrary, the release of intracellular components (both absorbing at λ=260 nm and λ=280 nm) in Gram-positive *Listeria* supernatant was insignificant when 1.15 J/cm^2 illumination dose was used. Only, when illumination dose was increased to 11.52 J/cm^2, the release of intracellular components in treated bacteria growth to 0.136 a.u.

The results obtained in this work indicate that inactivation of Gram-positive *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-negative bacteria. It was determined that Chl-based photosensitization was very effective and reduced the *Bacillus* and *Listeria* population on the surface of the polyolefine by 3.3 and 4.4 log respectively (Figure 3.28).

In order to increase the susceptibility of pathogens to Chl-based photosensitization the conjugate of Chl with chitosan (CHS) was performed. CHS (polyβ-(1, 4)-acetyl-D-glucosamine) is cationic linear polysaccharide, obtained from deacetylated derivative of chitin–most abundant polysaccharide in nature after cellulose (170). It is tasteless fiber, non-toxic and biodegradable. Antimicrobial activity of CHS was assessed for a wide variety of microorganisms(171–174). Moreover, CHS is nutritional supplement which exhibits film-forming properties. These features enable us to apply it as an edible coating for different types of food (175). To effectively extend the shelf life of postharvest fruit and vegetable, CHS-based coating is more and more concerned in food industry in recent years. However, single CHS coating sometimes demonstrates a certain defect, which includes limited inhibition to especial microorganism that leads fruit to decay (171,172). To effectively apply the CHS-based coating, the CHS was combined with organic compounds, inorganic compound, biological control agents (170) or with physical methods such as short heating, short gas fumigation, modified atmosphere packaging, and so on (176–178). The aim of this study is to improve antimicrobial efficiency of CHS coatings incorporating well known PS and food additive Chl into this biopolymer. Thus, first, the antimicrobial efficiency of photoactivated Chl–CHS conjugate against food pathogens and harmful microfungi were investigated. Second, the impact of coating of strawberry with Chl–CHS and illumination with visible light on microbial contamination, shelf life, nutritional and visual quality of strawberry were evaluated as well.

Data obtained from fluorescence spectra showed that remarkable decrease of Chl fluorescence intensity at λ=647 nm has been observed after conjugation with CHS (Figure 3.20a). It supported the idea that due to the Chl–CHS conjugate formation some aggregation of Chl and CHS in solution took place. Just addition of small amounts of Triton-X100 disaggregated Chl–CHS conjugates and recovered typical fluorescence intensity of monomeric Chl. Data obtained in the present study allow us to compare the susceptibility of

Gram-positive and Gram-negative food pathogen to photoactivated Chl–CHS treatment. It was found in our study that antimicrobial properties of CHS alone or dark toxicity of Chl–CHS conjugate are rather low (Figures 3.21–3.22). Chl–CHS conjugate in the dark exhibited some antimicrobial activity against *Salmonella* (1 log after 2 hours incubation) and it was comparable with that of CHS alone (Figure 3.21). On the contrary, significant antimicrobial effects of Chl–CHS conjugate without light activation were observed on Gram-positive bacteria *Listeria* after 2 min incubation (Figure 3.22). Inactivation of *L. monocytogenes* by this conjugate in the dark was rather significant and after 2 min incubation reached 2.1 log. It is important to note that antimicrobial efficiency of CHS depended on the incubation time, and these data are in line with results published in (179). As development of novel antimicrobial treatment mostly requires short treatment time we paid more attention to inactivation level at 0÷30 min incubation. It is clear that antimicrobial properties of CHS alone are insignificant at short incubation time (at 30 min 0 log). Just at longer incubation time (120 min) it reduced viability of *S. enterica* by 2 log. Chl-based photosensitization inactivated *Salmonella* by 2 log but not more (Figure 3.21b), and inactivation efficiency did not depended on incubation time (3.11a). It enables to presume that Chl interacts with the bacterium just superficially. Photoactivation of Chl–CHS conjugate enhanced drastically the killing efficiency and very sharp reduction of viable *Salmonella* cell population (6.7 log) was achieved even after 30 min incubation. Remarkable and very fast decrease of *Salmonella* viability (7 log) was observed when bacteria after 60 min incubation with Chl–CHS conjugate were illuminated by visible light (illumination dose 38 J/cm^2). Photoactivated Chl–CHS conjugate reduced the viability of *Listeria* by 7 log within very short incubation time (2 min) when low light dose (2.9 J/cm^2) has been used (Figure 3.22). This means that Gram-positive food pathogen *L. monocytogenes* is more susceptible to photoactivated Chl–CHS conjugate treatment than Gram-negative *S. enterica*.

By no means, question arises, whether low pH (in final Chl–CHS cell suspension in 0.9% NaCl, pH=3.95) or light alone (405 nm) can diminish *Salmonella* population. It must be noted, that lowered bacterial suspension pH (3.95) due to addition of Chl–CHS was not the factor, influencing viability of pathogens. Since different experimental conditions (6 samples) may change the pH of bacterial suspension and hence affect the viability of bacteria it was necessary to measure pH values in all samples. It was determined that pH value of the bacterial suspension in 0.01 M PBS shifted from 7.4 to 6.8 when the cell suspension was mixed with Chl or Chl–CHS conjugate. However, when PBS was replaced by 0.9% NaCl, pH value after mixing with Chl or Chl–CHS conjugate decreased from 7.3 to 3.95. Also we indicated that *Salmonella* cells preserved their viability 100% when being suspended and incubated for rather long time (120 min) in 0.9% NaCl acidified by HCl to pH 4.4. Thus, just minor impact of pH on viability of Chl–CHS treated *Salmonella* can be anticipated. These results have showed that an acidic environment (pH 3.95) promoted the effect of Chl–CHS photosensitization as opposed to an neutral pH conditions (pH 6.8 in 0.01 M PBS) in which inactivation of Gram-negative *Salmonella* decreased. It looks like Chl being negatively-charged has weak interaction with negatively charged bacterium. But immobilization of Chl into positively-charged CHS polymer enhances the Chl interaction with bacterium and after illumination triggers effective killing.

Murdoch et al. (180) published data about possibility to inactivate *Escherichia*, *Salmonella*, *Shigella*, *Listeria*, and *Mycobacterium* in suspension by LED-based light (405 nm). Meanwhile, statistically significant inactivation (3.5 log) of *Salmonella* was achieved at light dose 288 J/cm^2, whereas in our experiments just 38 J/cm^2 light dose has been used.

To understand whether Chl–CHS conjugate interacts with the bacterial surface SEM images analysis of *Salmonella* incubated with this conjugate was performed. The analysis of SEM images of *Salmonella* treated by photoactivated Chl–CHS conjugate indicates clear covering of bacteria by this biopolymer, shrinkage and reduction of bacterial volume and size (Figure

3.25). Taking into account that Chl–CHS conjugates have feature to form polymers, such “photoactive “antimicrobial films can serve as carrier of wide range of various antimicrobials in the future (181).

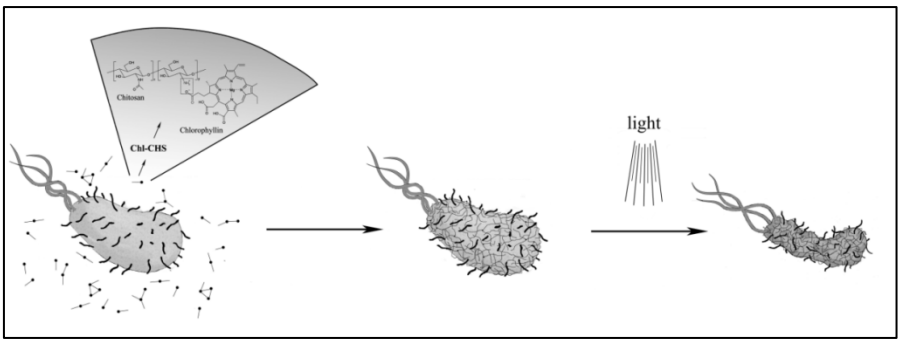

Fig. 4.1 Hypothetical model of Chl–CHS interaction with *Salmonella*.

It is important to note that antimicrobial properties of CHS alone or Chl-based photosensitization against *Salmonella* are rather low (no more than 2 log) (Figure 3.21). Meanwhile the treatment of *Salmonella* by photoactivated Chl–CHS conjugate and following illumination with visible light (dose 38 J/cm^2) reduced cell number by 7 log in very fast way.

What is the mechanism of this effective antimicrobial treatment? It is known that PSs that were covalently attached to polymers demonstrated high quantum yields of $^1O_2$ formation (182). $^1O_2$ is a reactive oxygen species, which has been shown to induce bactericidal effects. Data reveal that after light exposure (38 J/cm^2) intensive release of intracellular components (DNA and proteins) was detected in *Salmonella* what can be addressed to intensive membrane disintegration induced by photoactivated Chl–CHS conjugate (Figure 3.24). It looks like Chl being negatively-charged has weak interaction with negatively charged *Salmonella*. But immobilization of Chl into positively-charged CHS polymer enhances the Chl interaction with bacterium what triggers after illumination effective killing. Figure 4.1 presents hypothetical model of destructive *Salmonella*-Chl–CHS interaction.

Soft fruits, as well as strawberries, have high economic value but short postharvest life. Losses from *Botrytis cinerea* are several billion US dollars/year. The conventional treatment to reduce microbial load on the surface of fruits is based on disease control by fungicides (preharvest) and

subsequent postharvest storage at low temperature or modified atmosphere (106). However, it has been confirmed that due to increasing microbial resistance chemical fungicides are not enough effective (183). Moreover they leave residues that are harmful to human and environment (184). It is worth noting that multiple fungicide residues were found in more than 60% of strawberry (185). Traditional postharvest storage at low temperature has detrimental effects on nutritional properties as decreased the antioxidant capacity and flavonoids in strawberry (106). The carbon dioxide treatment reduced anthocyanin content and changed internal fruit color (107). Hydrogen peroxide (1%) reduced the microbial load on strawberry by 1.46 log, but parallel produced losses in color and content of total anthocyanins. Widely accepted hypochlorite (200 μg/ml, 2 min) reduced the microbial contamination of strawberry just 0.45 log (186). Moreover, hypochlorous acid, a substance present in NaOCl solution, interacts with organic tissue and releases chlorine (strong oxidant), which inhibits enzymes and chloramines that interfere in cell metabolism (187) and eventually causes formation of highly mutagenic compounds, trihalomethanes (188,189).

Thus, rapid development of alternative antimicrobial approach which would be more effective, safe to humans and harmless to environment has become imperative (190).

One of the innovative non-thermal techniques to extend shelf life of fresh fruits is high-intensity focused ultrasound (HIFU). HIFU takes short time (5–10 min), meanwhile, power higher than 60 W diminished significantly the quality of berries (191). Meanwhile, total soluble solid content, color, textural properties of treated strawberry diminished significantly at higher than 60 W powers. An emerging approach to control strawberry microbial contamination is atmospheric pressure plasma treatment (APPT). Misra et al. (192) observed that the total mesophiles and yeasts/ moulds of strawberry treated for 5 min with ACP were reduced by 2 log, meanwhile to control parameters of this treatment is rather difficult. Luksiene et al. (43) indicated that high power pulsed light (HPPL) inactivated naturally distributed mesophils on the surface

of strawberry by 2.2 log, inoculated *B. cereus* and *L. monocytogenes* by 1.5 log and 1.1 log respectively, yeasts/fungi - by 1 log what eventually extended the shelf-life of treated strawberry by 2 days. No significantly important changes were observed in nutritional quality and organoleptic properties of berries after treatment. Meanwhile, the limiting factor of this technology is significant increase of temperature at longer treatment time (43). International Consultative Group on Food Irradiation (193) allows irradiation of strawberry with maximum dose of 3 kGy. Yu et al. (194) approved that this dose extended the shelf-life of berries by a factor of 2, but induced significant changes in texture and color. Moreover, the irradiated fruits are not popular among consumers (195,196). Several attempts have been reported to extend shelf-life of strawberry with gaseous and aqueous forms of ozone. Nadas et al. (197) showed that gaseous ozone treatment (1.5 µl) reduced decay incidence, weight loss and softness but caused loss of aroma in strawberry. Aday et al (198) demonstrated that low (0.075 ppm) and middle (0.15 ppm) ozone concentrations can be applied to extend the shelf-life of strawberry by at least 3 weeks under refrigerated conditions.

Photosensitization seems to offer a promising alternative as effective non-thermal antimicrobial food preservation treatment which is environmental friendly, saves water and energy at very reasonable costs (148). After spraying of the PS on the surface of fruit most surface-distributed pathogens, harmful bacteria, viruses and molds bind to the PS (70,92,139,199,200). The following illumination of fruits with light induced photocytotoxic reactions and death in surface-attached microorganisms without any harmful effects on the environment (12,14,71). For instance, according to Aponiene et al. (199) hypericin-based photosensitization reduced the amount of *B. cereus* and surface-attached mesophilic microorganisms on FV (apricots, plumes, and cauliflowers) by 0.77÷1.3 and 0.6÷0.72 log respectively when 30 min incubation with 1.5 10^{-5} M Hyp and 6.9 J/cm^2 illumination dose was used. Data reveal that Hyp-based photosensitization treatment has no significant impact

on antioxidant activity and color of treated FV and was comparable with the effects of HPPL.

Over the last decade, interest has been rapidly growing in the development of bio-based packaging and coating. Edible coatings which can generate moisture and gas barriers have recently drawn considerable interest due to environmental concerns regarding not biodegradable plastic packaging materials (201). Importantly, edible films containing antimicrobial agents (such as green tea extract, essential oils, tocopherol, organic acid, and chitosan) have been used to preserve the quality of strawberry during storage(202). Chitosan has been used to maintain the quality of post-harvest fruits and vegetables (173,203,204). CHS possessed excellent film-forming properties, reduced moisture losses, controlled gas (CO_2, O^{-2}) and extended the shelf-life of strawberry, thus, can be applied as an edible surface coating to strawberry (203,205). Pre-harvest chitosan spraying have been noted to be effective in controlling post-harvest fungal infections in strawberry (206).

Thus, combining antimicrobial properties of photoactivated Chl with that of biopolymer CHS is possible to achieve not just higher antimicrobial efficiency but also to develop new generation photoactive edible antimicrobial coatings for preservation of strawberry. Data presented in Figure 3.29 reveal that conventional washing of strawberry with 200 ppm NaOCl (2 min) diminished population of yeasts/microfungi on the surface of berry by 0.55 log, Chl-based photosensitization (0.001%) reduced yeasts/microfungi in the same level, but increased Chl concentration (0.1%) enhanced antifungal efficiency up to 0.9 log (Figure 3.29). CHS alone was less effective (0.4 log) in comparison with that of NaOCl (0.55 log). The highest inactivation of yeasts/microfungi was found when strawberry were coated with Chl–CHS and illuminated with light (1.37 log). By no means, the irregularity and the different light reflecting properties of the illuminated strawberry surface can possibly account for the lower antimicrobial efficiency (1.37 log) treating food matrix in comparison with data *in vitro* (as shown our result inactivation reached up to 7 log). Nevertheless, taking into account that photosensitization

efficiency depends on illumination time and light intensity the obtained antibacterial efficiency can be significantly enhanced by usage of more powerful light sources or longer illumination time (144).

The most severe strawberry pathogen under Central European climatic conditions is *B. cinerea* (207). It was important to investigate whether this fungus is susceptible to Chl-based photosensitization and photoactivated Chl–CHS treatment. The obtained data indicated that Chl and Chl–CHS without light had no effect on *Botrytis* (Figures 3.12 and 3.23). Data presented in Figure 3.12 indicated that mycelial growth of *Botrytis* 2 days after Chl-based photosensitization treatment when fungus was incubated with $1.5 \cdot 10^{-4}$ M Chl and illuminated with visible light (illumination dose 35 J/cm^2) reached 3.35 cm diameter. However, photoactivation of Chl–CHS conjugate with higher illumination dose (76 J/cm^2) enhanced inactivation of fungus by 60 %. Mycelial growth of *Botrytis* 2 and 3 days after photoactivated Chl–CHS treatment reached 2.2 and 3.6 cm growth respectively.

It is obvious that the most important advantage of any antimicrobial technology is the ability to extend the shelf-life of treated berries. Shelf-life of strawberry was evaluated visually by observing fruits surface color changes (spots) (Figure 3.31). Thus, as depicted in Figure 3.32c, the disease-free period of Chl–CHS (dark toxicity) coated strawberry prolonged just 1 day in comparison with control (strawberry without any washing). Therefore, it is obvious that Chl-based photosensitization and photoactivated Chl–CHS (illumination dose 76 J/cm^2) was most effective tool in delaying strawberry spoilage. Thus, as depicted in Figure 3.32a and d, the disease-free period of treated strawberry was prolonged about 3 days in comparison with control. This is a significant effect as the Chl and Chl–CHS conjugate concentrations were not high (0.001 and 0.001–0.1 % respectively). These data are in line with the results of other authors. Luksiene and Paskeviciute (139) used a novel approach to obtain the microbial decontamination of strawberry fruits using $1 \cdot 10^{-3}$ M Chl-based photosensitization (illumination dose 14.4 J/cm^2), where they extended the shelf life by at 2 days under refrigerated conditions (+6 C).

Measuring extension of shelf-life of coated strawberry according Kittemann et al. (136) system (1÷6 scale) it was found that 7 - day storage made all control berries (100%) infected and disease index (N) was the highest N=6, whereas just 76% of Chl–CHS coated and illuminated berries were damaged by spoilage microorganisms, thus, in this case the disease index was significantly lower (N=4.6).

It is clear that visual overall quality of strawberry gradually decreases over storage time. Table 3.3 shows the effect of different treatments on the overall appearance of treated strawberry during storage (10 days). On 4 storage day the control berries were qualified as “not acceptable”, whereas coated with Chl–CHS berries were qualified as “good”. It is obvious that for delaying strawberry spoilage and for keeping visual quality Chl–CHS coating and illumination with light was more effective tool than 200 ppm NaOCl (2 min).

The main beneficial properties of strawberry have been partially attributed to the high content of antioxidant compounds (208). Antioxidants can scavenge free radicals and ROS which usually induce toxic processes in the living cell including oxidative damage to proteins and DNA, membrane lipid oxidation, enzyme inactivation and gene mutation that may finally lead to cancer genesis or other oxidative cardiovascular or inflammatory diseases (209). Li et al. (210) examined effects of UV(C) irradiation on antioxidant capacity of strawberry and found that this treatment during later period of storage reduced antioxidant capacity of strawberry. Therefore, it might be possible that photosensitization being an effective antimicrobial treatment modality can affect and result in some negative impact on the strawberry nutritional properties. Thus, it was necessary to investigate whether some changes of antioxidant activity took place after Chl – CHS photosensitization in strawberry. Data obtained in this study indicate that strawberry coated with Chl–CHS and illuminated with light preserved their total antioxidant activity (Figure 3.33). However, the mechanisms of these effects have not been thoroughly elucidated so far. In fact, plant cells usually keep the ROS level under tight control by production or activation of scavenging enzymes (211).

The visual quality of the treated strawberries is a key parameter for consumers. It is important to note that under this experimental set up no effect on the color of strawberries have been found. The red color of berry is due to the presence of anthocyanin pigments in the fruit epidermis and cortex (212). In addition, factors such as copigmentation, pH and anthocyanin metabolism may play a significant role in the expression of color in strawberry (213). The most effective copigments flavonols are located in external tissue (214,215). In order to find out whether photoactivated Chl–CHS coating has any effect on the color of strawberry, the fruits were analyzed immediately after treatment. According to the obtained data (Figure 3.34), the berry color was not affected by the Chl–CHS coating and illumination with light. Other studies performed on decontamination of strawberry using carbon dioxide treatment indicated that fruit surface color did not change, but remarkable changes were observed in internal fruit color (213).

Fruit weight loss is mainly associated with respiration and moisture evaporation through the skin. Edible coatings act as barriers, thereby restricting water transfer. Our data claimed that Chl–CHS coating preserved moisture in berries (Figure 3.35). The weight loss increased throughout cold storage period in photoactivated Chl–CHS coated and uncoated strawberry. Photoactivated Chl–CHS coating limited fruit weight loss compared to uncoated berry, and a better effect on delaying the weight loss of fruit during the five days of storage was observed for the photoactivated 0.001 Chl–0.1%CHS. At the end of the storage period, the uncoated strawberry showed 22% loss in weight, whereas the weight loss of coated strawberry was just 12% (Figure 3.35a). These data were confirmed by FT-Raman spectroscopy data of Chl–CHS coated strawberry in comparison with not coated control ones (Figure 3.35b). It is obvious that typical water absorption peak at 3200 cm^{-1} has much higher intensity for Chl–CHS coated strawberry in comparison with that of not-coated control ones. Strawberry fruits are highly susceptible to a rapid loss of water. These results are consistent with those of another authors studies demonstrating that CHS coating acts as a semipermeable barrier against

oxygen, carbon dioxide and moisture, thus counteracting the dehydration and shrinkage of the fruit (111,216). So far, the immobilized Chl into CHS polymer can serve in the future for the development of photoactive edible coating with more pronounced antimicrobial properties.

Leveque et al. (142) found out that EPR imaging could be applied for the monitoring of free radicals in various food samples. Other authors used EPR spectroscopy to evaluate antioxidant activity of spices and herbs (217–220). Moreover, EPR method was successfully applied to distinguish irradiated and not irradiated fruits and vegetables. Raffi and Stocker claimed that it is possible to detect irradiated berries (due to free radicals) as long as 25 days (stored at 4÷5 °C) (141). As photosensitization treatment involves radical reactions it was important to check whether photoactivated Chl–CHS coating induced additional long lasting ROS in strawberry. Data indicated that both registered spectra (control and treated strawberry) (Figure 3.42) exhibited strong signal due to the 6 lines of Mn^{2+}(which is a transition metal ion linked to enzymes in the strawberry) (141). Comparison of EPR spectra in control and treated strawberry revealed that no radical-based fundamental changes occurred 1 hour after treatment. It means that despite the high antioxidant activity of these berries this treatment does not induce long-lasting free radicals in the strawberry, as for instance do 2 kGy ionizing radiation (141). Hence, the obtained data indicate that photoactivated Chl–CHS coating has potential to combat harmful and pathogenic microorganisms distributed on the surface of strawberry and can serve in the future for the development of photoactive biodegradable edible coating with more pronounced antimicrobial properties.

CONCLUSIONS

1. Gram-negative food pathogen *Salmonella enterica* serovar Typhimurium (5.9 log), as well as Gram-positives *Listeria monocytogenes* (4 log) and *Bacillus cereus* (6.4 log) can be effectively inactivated by ALA-based photosensitization ($7.5 \cdot 10^{-3}$ M concentration, 60 min incubation, 24 J/cm^2 illumination dose) in the solution as well as adhered onto the surface of packaging material (to an undetectable level).
2. The present data clearly indicate that Gram-negative *S.enterica* and main strawberry pathogen *Botrytis cinerea* are more resistant to Chl-based photosensitization than Gram-positives *L. monocytogenes* and *B. cereus in vitro* as well as adhered onto the surface of packaging material. Chl-based photosensitization inactivated *Salmonella* by 2,5 log ($1.5 \cdot 10^{-5}$ M concentration, 60 min incubation, 51 J/cm^2 illumination dose), *Listeria* and *Bacillus* to an undetectable level ($1.5 \cdot 10^{-7}$ M concentration, 2 min incubation, 2.4 and 6 J/cm^2 illumination doses), and 2 days after treatment reduced *B. cinerea* mycelial growth by 28% compared to the control ($1.5 \cdot 10^{-4}$ M concentration, 24 h incubation, 35 J/cm^2 illumination dose).
3. Photoactive Chl–CHS conjugate exhibited high antimicrobial capacity against food pathogens Gram-positive *L. monocytogenes* (7 log), Gram-negative *S. enterica* (7 log), as well as against main strawberry pathogen *B. cinerea* (60% inhibition).
4. Data revealed extensive leakage of DNA and proteins after Chl-based photosensitization and photoactivated Chl–CHS conjugate treatments. Thus significant cell membrane disintegration occurred after the photosensitization treatment.
5. Overall reduction of yeasts/fungi on the surface of strawberry prolonged the disease-free period of strawberry by 3 days (40%) after treatment by photoactivated Chl–CHS conjugate without any negative impact on total antioxidant activity, visual appearance, color or weight.
6. Our date indicated that the Chl–CHS coating photoactivated with visible

light can be a useful tool for the preservation of strawberries according to the requirements of „clean green technology concept“.

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LIST OF PUBLICATION RELATED TO THE DISSERTATION

1. Paskeviciute, E, **Buchovec**, I, Luksiene Z. Control of food pathogens by chlorophyll-based photosensitization *in vitro*. J Processing Energy Agric. 2009; Vol 13(2), 181 – 184.
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SCIENIFIC PROJECTS

1. Lithuanian Science Council (SVE-12011): „Inovatyvios technologijos aviečių ir braškių uogų mikrobiologinei saugai ir kokybei“(“Uoga“), 2012-2015; coordinator habil.dr. Ž. Lukšienė; position – junior researcher.
2. COST FA 1104LT „Sustainable production of high-quality cherries for the European market“, 2012-2016, coordinator Dr Jose Quero Garcia; position – MC Substitute Member.
3. The World Federation of Scientists grant (http://www.federationofscientists.org/) 2009-2010: High-power pulsed light for decontamination of fruits and vegetables from food pathogens: a study on antimicrobial efficiency and organoleptic properties; supervisor Habil. Dr. Zivile Luksiene
4. FP6 "Innovative non-thermal processing technologies to improve the quality and safety of ready-to-eat (RTE) meals (HighQRTE)(Contract Nr 023140), 2005-2008, coordinator prof. E. Guerzoni, 2006-2008; position – junior researcher.
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SUMMARY

Despite tremendous progress in technology and biomedical science the number of reported food-borne infections continues to rise. Health experts estimate that food-borne illnesses in the US cost 77.7 billion US dollars in direct medical expenses and lost productivity. CDC estimates that each year 31 major pathogens acquired in the US caused 9.4 million episodes of foodborne illness, 55961 hospitalizations, and 1351 deaths. Thus, development of innovative approaches and technologies for more effective inactivation of food pathogens seems an urgent task. In this context, photosensitization treatment seems promising despite the fact that it was never evaluated as tool to combat food pathogens. Thus, the main task of this study was to evaluate prospects of photosensitization as antimicrobial treatment and its possible applications in food industry.

Data obtained indicate that main food pathogens (*Salmonella enterica*, *Listeria monocytogenes*, *Bacillus cereus*) can be effectively inactivated by ALA-based photosensitization (5.9 log, 4 log, 6.4 log respectively) due to formation of endogenous porphyrins from precursor ALA ($7.5 \cdot 10^{-3}$ M) and following illumination (λ=405 nm, 24 J/cm^2 illumination dose). It is important to note that all investigated pathogens attached to the surface of food packaging can be effectively inactivated as well (3.7÷4 log). Obtained data support the idea that photosensitization as non-thermal and effective antimicrobial treatment has potential to develop into environmentally safe, surface decontamination technique.

Antimicrobial efficiency of Chl-based photosensitization was tested against the same bacteria and gray mold (*Botrytis cinerea*). Data indicate that Gram-positive *L. monocytogenes* and *B. cereus* were significantly (to an undetectable level) inactivated by Chl-based photosensitization *in vitro* and on the surface of packaging. Data presented in the work indicated that incubation of *B. cinerea* with $1.5 \cdot 10^{-4}$ M Chl and illumination with visible light (illumination dose 35 J/cm^2) resulted in 3.35 cm diameter growth of fungus

mycelium 2 days after Chl-based photosensitization treatment compared to the control (4.33 cm) (28% inhibition). Due to the fact that Chl-based photosensitization inactivated Gram-negative *Salmonella* just 1.8 log the novel approaches to enhance the inactivation of Gram-negative food pathogens using Chl–CHS conjugate have been applied. CHS alone incubated for 2 h with *Salmonella* reduced viability by 2.15 log, whereas photoactivated Chl–CHS conjugate (0.001–0.1%, illumination dose 38 J/cm^2) diminished bacterial viability by 7 log. Moreover, it inhibited *B. cinerea* growth by 60%. SEM images indicated that Chl–CHS conjugate at these experimental conditions covered all bacterial surfaces. Significant cell membrane disintegration was the main lethal injury induced in Gram-negative bacteria by this treatment.

Analysis of strawberry decontamination from naturally distributed yeasts/molds revealed that CHS alone reduced their population just by 0.4 log, Chl-based photosensitization 0.9 log, whereas photoactivated Chl–CHS coatings caused 1.4 log reduction of yeast/molds on the surface of strawberry. Electron paramagnetic resonance spectroscopy confirmed that no additional photosensitization-induced long-lasting free radicals have been found in strawberry matrix. Visual quality (color, texture) of treated strawberries was not affected as well.

In conclusion, photoactive Chl–CHS conjugate exhibited strong antimicrobial action against more resistant to photosensitization Gram-negative *S. enterica* in comparison with Gram-positive bacteria *in vitro*. It reduced significantly the viability of strawberry surface–attached yeasts/molds without any negative impact on visual quality of berries. Experimental data support the idea that photoactivated Chl–CHS conjugate can be a useful tool for the future development of edible photoactive antimicrobial coatings which can preserve strawberries and prolong their shelf-life according to requirements of “clean green technology”.

SANTRAUKA

Nepaisant didžiulės pažangos technologijų ir biomedicinos mokslų srityje, sergamumas maisto patogenų sukeltomis ligomis vis didėja. Sveikatos ekspertai apskaičiavo, kad šių ligų sukelti ekonominiai nuostoliai JAV šalyje kasmet siekia 77 mlrd. dolerių. JAV ligų kontrolės ir prevencijos centro duomenimis, dėl 31 patogenų rūšies sukeliamų ligų kiekvienais metais JAV suserga 9,4 mln. žmonių, iš kurių 55961 hospitalizuojama, o 1351 miršta. Tai skatina ieškoti naujų efektyvesnių antimikrobinių maisto apsaugos metodų ir technologijų. Vienas iš naujausių ir niekada netaikytų neterminio apdorojimo metodų yra fotosensibilizacija. Pagrindinis šio tyrimo tikslas buvo įvertinti fotosensibilizacijos antimikrobinio poveikio perspektyvas ir galimybę pritaikyti ją maisto pramonėje.

Gauti duomenys rodo, kad pagrindinių maisto patogenų (*S. enterica*, *L. monocytogenes*, *B. cereus*) gebėjimas iš ALA rūgšties ($1,5 \cdot 10^{-3}$ M) sintetinti endogeninius porfirinus gali būti efektyviai naudojamas jų inaktyvacijai ALA indukuota fotosensibilizacija (atitinkamai pasiekiama 5,9 log, 4 log, 6,4 log bakterijų inaktyvacija). Svarbu pažymėti, kad taip pat efektyviai visos trys bakterijos (*S. enterica*, *L. monocytogenes*, *B. cereus*) inaktyvuojamos ant maisto pakuočių (poliolefino) (3,7÷4 log). Gauti rezultatai dar kartą patvirtina tai, kad fotosensibilizacija, pasižyminti neterminiu ir efektyviu antimikrobiniu poveikiu, turi puikias galimybes tapti nauja ekologiška ir saugia paviršių sterilizavimo technologija.

Antimikrobinis Chl-indukuotos fotosensibilizacijos poveikis buvo tiriamas prieš anksčiau minėtus bakterijų kamienus ir pilkąjį kekerą (*Botrytis cinerea* Pers. ex. Fr.). Darbo rezultatai parodė, kad Chl indukuota fotosensibilizacija visiškai inaktyvuoja Gram-teigiamas bakterijas *L. monocytogenes* ir *B. cereus in vitro* ir ant pakuočių paviršiaus. Tyrimų metu buvo nustatyta, kad *B. cinerea* inkubavimas su $1,5 \cdot 10^{-4}$ M Chl ir po jo atliktas švitinimas (λ=405 nm; šviesos dozė 35 J/cm^2), stabdo grybo radialinį augimą.

Micelio diametras antrą dieną po Chl indukuotos fotosensibilizacijos buvo 3,35 cm, kontrolės – 4,33 cm (26% augimo slopinimas). Po Chl indukuotos fotosensibilizacijos poveikio Gram-neigiama *Salmonella* inaktyvuojama tik 1,8 log. Po 2 val. inkubacijos su CHS *Salmonella* ląstelių skaičius sumažėja 2,15 log. Tuo tarpu fotoaktyvuoto Chl–CHS konjugato (0,001–0,1%, šviesos dozė 35 J/cm^2) bandymu pasiekta 7 log bakterijų inaktyvacija ir 60% *B. cinerea* augimo slopinimas. SEM nuotraukose matyti, kad šiomis eksperimentinėmis sąlygomis Chl – CHS konjugatas padengia visą bakterijų paviršių. Pagrindinė žūtį lemianti Gram-neigiamų bakterijų pažaidos priežastis yra žymus ląstelių membranos suirimas šio poveikio metu.

Ištyrus natūraliai braškių paviršiuje esančių mielių/mikromicetų inaktyvaciją buvo nustatyta, kad CHS sumažina jų populiaciją 0,4 log, Chl indukuota fotosensibilizacija –0,9 log, o fotoaktyvuotas Chl–CHS konjugatas – 1,4 log. Elektronų paramagnetinio rezonanso spektrai rodo kad po poveikio fotosensibilizacija braškėse neatsiranda ilgalaikių radikalų. Vizualinė apdorotų braškių kokybė (spalva, tekstūra) taip pat nepakinta.

Apibendrinant, galima teigti, kad fotoaktyvuotas Chl–CHS konjugatas pasižymi stipriu antimikrobiniu poveikiu prieš Gram-neigiamą *S. enterica in vitro*, kuri yra labiau atspari fotosensibilizacijai nei Gram-teigiamos bakterijos. Jis sumažina braškių paviršiuje esančių mielių ir mikromicetų gyvybingumą, nepaveikdamas vaisių kokybės. Mūsų tyrimų rezultatai patvirtina, kad fotoaktyvuotas Chl–CHS konjugatas ateityje gali būti naudojamas sintetinant valgomus fotoaktyvius antimikrobinius apvalkalus, kurie apsaugo braškes nuo greito gedimo ir prailgina jų saugojimo laiką, remiantis švarios ekologiškos technologijos (“clean green technology”) reikalavimais.

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CONTROL OF FOOD PATHOGENS BY CHLOROPHYLL-BASED PHOTOSENSITIZATION *IN VITRO*

KONTROLA PATOGENA HRANE HLOROFILOM NA BAZI FOTOSENZITIVNOSTI

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SUMMARY

Biophotonic technology based on photosensitization might serve as an effective, environmentally friendly and promising antibacterial tool. Data obtained indicate, that most important food pathogens Listeria monocytogenes, Bacillus cereus can be inactivated by chlorophyll derivative-based photosensitization. The inactivation of these microorganisms was very significant and reached 6 orders of magnitude in vitro. Salmonella Typhimurium was more resistant to this treatment. When pathogen Bacillus cereus was inoculated on the food matrix the inactivation was lower (3-4 orders of magnitude). It is important to note, that Bacillus spores are susceptible to this treatment as well.

Summarizing, chlorophyll-based photosensitization has potential to be effective antimicrobial tool and can be used to inactivate harmful and pathogenic microorganisms in different environments.

Keywords: *Listeria, Bacillus, decontamination, food safety.*

INTRODUCTION

Foodborne diseases have been estimated to cause millions of hospitalizations and cost billions of dollars each year. It means that existing food safety technologies can not guarantee safe food. Thus, development of novel, more effective non-thermal technologies to improve microbial control and to reduce the risk of foodborne diseases outbreaks becomes issue with global dimension. In this context, biophotonic technology based on photosensitization might serve as an effective and promising antibacterial tool. This treatment involves the administration of photosensitizer that selectively binds microorganism. After illumination with UV/visible light, plethora of photochemical reactions induces selectively death of microorganism without any harmful effects on surrounding.

One of the most important advantages of photosensitization in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment (Nitzan and Ashkenazi 2001), as process is free radical-mediated (Polo et al. 2002). Moreover, due to the fact that a photoactive compound is localized inside microorganisms, all cytotoxic reactions are just local. It allows us to achieve an extensive decrease in the population of pathogens with minimal damage effects on the surrounding matrix (Jori 2006). Eventually, it was confirmed that photosensitization, in contrast to ionizing radiation, has no mutagenic or carcinogenic effects on living systems (Luksiene 2005).

Thus, in our previous studies we have been focused on the possibility to inactivate series of harmful and pathogenic microorganisms exploiting photosensitization. Our previous data (Luksiene et al. 1989; 2004a, b) indicate that yeast *Saccharomyces cerevisiae* as well as micromycetes *Ulocladium oudemansii,Trichotecium roseum* and *Aspergillus flavus* might be inactivated by photosensitization. Moreover, inhibition of spore germination was further observed in *Aureobasidium* sp., *Rhodotorula* sp., *Penicillium stoloniferum, Aspergillus fumigatus, Aureobasidium pullulans, Ulocladium chartarum, Alternaria alternata, Rhizopus oryzea, Fusarium avenaceum, Acremonium strictum* (Luksiene et al. 2005). Moreover, food pathogens *Salmonella enterica, Bacillus cereus and Listeria monocytogenes* can be effectively inactivated by aminolevulinic-based photosensitization. According to the obtained data, fast and significant inactivation (6.5 log) of *Bacillus cereus, Listeria monocytogenes* (4 log*), Salmonella* Typhimurium (6 log) were obtained after this treatment (Buchovec et al. 2009; Le Marc et al. 2009; Luksiene et al. 2009) (Lithuanian patent Nr 2008060). It seems that plethora of harmful micromycetes and pathogens might be inactivated by photosensitization, a method that is completely safe, reproducible, nonmutagenic, noncarcinogenic, environmentally and human friendly (Luksiene 2005).

This study was focused on the possibility to inactivate some Gram-positive and Gram-negative bacteria, main food pathogens by chlorophyll sodium salt–based photosensitization *in vitro.*

MATERIALS AND METHODS

B. cereus ATCC 12826, *Listeria monocytogenes* ATCL3C 7644 and *Salmonella enterica* Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2) were grown at 37 ^{0}C in Luria-Bertani (LB) medium to the mid-log phase (~ 6x10^7 colony forming units (cfu)/ml, OD_{540}=1) and then harvested by centrifugation (10 min, 5000g), resuspended and accordingly PBS-diluted to ~1 x 10^7 cfu/ml final concentration. Aliquots (10 ml) of bacterial suspension (~1x10^7 cfu/ml in 100 M PBS buffer (pH 7.2) with appropriate concentration of chlorophyll sodium salt (7.5 x 10^{-5}, 7.5 x 10^{-6}, 7.5 x 10^{-7}, 7.5 x 10^{-8} M) were incubated in the dark in plastic 50 ml test tubes at 37 ^{0}C. Immediately 150 µl aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and exposed to light for different time (0–20 min). Light emitting diodes (LED) based light source (constructed in the Institute of Applied Sciences of Vilnius university) emitting light λ = 400 nm with intensity 20mW/cm^2 at the surface of samples was used for treatment. Light dose was calculated as light intensity multiplied on time. Light power density measurements were performed with a light energy measure by 3 Sigma meter (Coherent, Santa Clara, U.S.A.) equipped with piro-electrical detector J25LP04. No thermal effects were detected at the exploited experimental conditions.

The antibacterial effect of photosensitization on *B. cereus, L. monocytogenes and S.* Typhimurium was evaluated by the spread plate method. Thus, 100 µl of appropriate dilutions of bacterial test culture after photosensitization *in vitro*, using the spread plate method, was surface inoculated on the separate LBA plate. Afterwards the bacteria were kept in the incubator for 24 h at 37 ^{0}C. The surviving cell populations were enumerated and expressed as $\log_{10}$ (cfu/ml)

RESULTS AND DISCUSSION

It has been shown that at a given illumination dose the effect of Chl-based photosensitization depends on the bacterial cell strain used, the Chl-derivative concentration and incubation time. We investigated susceptibility to photosensitization of several food pathogens. Data obtained indicate, that Salmonella is more resistant to this treatment, than Bacillus and Listeria. For instance, the increase of photosensitizer concentration from 10-7 to 10-5 M reduces the survival fraction of Salmonella just by 1.6 log(Fig.1).

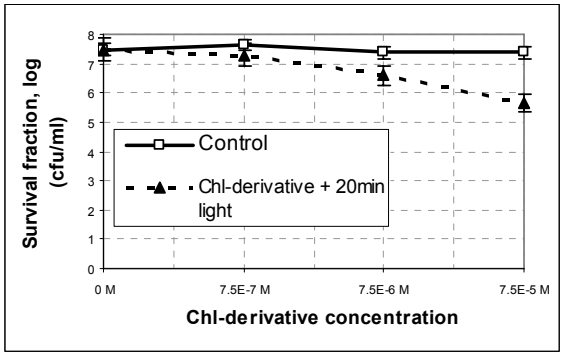

Fig. 1. Inactivation of Salmonella enterica Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2) by 7.5E-7 M, 7.5E-6 M and 7.5E-5 M chl-derivative based photosensitization
Sl. 1. Inaktivacija Salmonella enterica Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2)
7.5E-7 M, 7.5E-6 M i 7.5E-5 M Chl derivatom zasnovan na fotosenzitivnošću

The following investigation on susceptibility of *Bacillus* to photosensitization indicate, that this bacterium can be inactivated even at very low Chl-derivative concentration (10^{-8}-10^{-7} M) (Fig.2). The inactivation efficiency strongly depends on used photosensitizer concentration and illumination time. Meanwhile, pre-incubation of cells with Chl derivative (10^{-7} M) and following 5 min illumination is enough to reduce the surviving fraction from 7 to 0 log. Data obtained revealed that toxicity of Chl-derivative alone, without illumination was, as usual, negligible (not shown). Just following illumination (photosensitization) of bacterial cells (0–5 min) decreased the survival fraction fairly sharply.

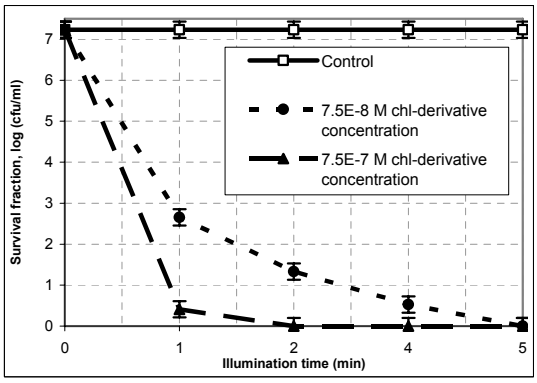

Fig. 2 Inactivation of Bacillus cereus ATCC 12826 by 7.5E-7 M and 7.5E-8 M chl-derivative based photosensitization
Sl. 2. Inaktivacija Bacillus cereus ATCC 12826
7.5E-7 M and 7.5E-8 M Chl derivatom zasnovan na fotosenzitivnošću

From the data presented in Fig. 3 it is clear that *Listeria* is susceptible to this treatment as well. In this case we used the lowest working Chl concentration (10^{-7} M) and lowest working illumination time (5 min), which were extrapolated from Fig. 2. Results indicate the surviving fraction of *Listeria* can be reduced by Chl-derivative-based photosensitization by 7 log as well.

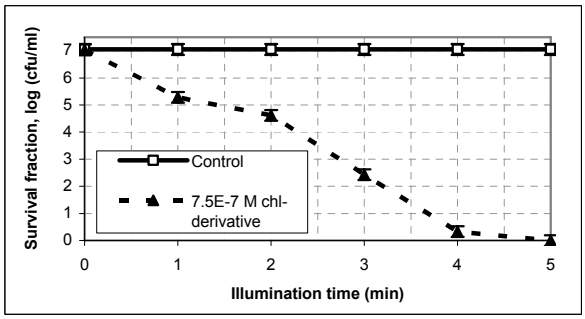

Fig. 3 Inactivation of Listeria monocytogenes $ATC_{L3}C$ 7644 by 7.5E-7 M chl-derivative based photosensitization
Sl. 3. Inaktivacija Listeria monocytogenes $ATC_{L3}C$ 7644
7.5E-7 M Chl derivatom zasnovan na fotosenzitivnošću

In the next step sensitivity of B. cereus spores to Chl derivative-based photosensitization was tested. Data, presented in Fig. 4 indicated that the incubation of spores with Chl-derivative without illumination did not induce significant changes in their number.

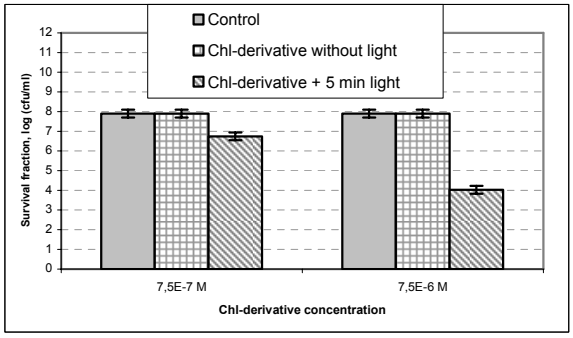

Fig. 4 Inactivation of Bacillus cereus ATCC 12826 spores by 7.5E-7 M and 7.5E-6 M chl-derivative based photosensitization
Sl. 4. Inaktivacija Bacillus cereus ATCC 12826 spora
7.5E-7 M and 7.5E-6 M Chl derivatom zasnovan na fotosenzitivnošću

Following illumination of spores can drastically reduce their population from 7.9 log in the control to 6.7-4 log in treated sample. The inactivation rate of spores, as usually, strongly depended on used photosensitizer concentration. From our previous work it is clear that for instance, the use of 3 mmol l-1 ALA concentration, at given experimental conditions can inactivate spores about 2 log, whereas 7.5 mmol l-1 ALA concentration inactivate them by 3.7 log (Buchovec et al. 2009; Luksiene et al. 2009).

It is worthy to note that the viability of bacteria and spores after illumination with visible light in the absence of photosensitizer remains unchanged.

CONCLUSION

Data obtained in this study clearly indicate, that Gram-positive bacteria *Bacillus cereus and Listeria monocytogenes* are susceptible to chlorophyll natrium salt-based photosensitization, and can be reduced by 7 log *in vitro*. Maybe, due to more com-

plicated cell wall structure and less cell-photosensitizer interaction Gram-negative bacteria *Salmonella enterica* is more resistant to this treatment and can be inactivated even by 1.6 log respectively. It is important to note, that *Bacillus* spores being important target for food safety technologies can be inactivated by photosensitization without any resistance. Meanwhile, as photosensitization efficiency depends on light dose delivered to the sample and cell-bound photosensitizer concentration, there are many ways to increase the interaction of Gram-negative bacteria with photosensitizer and optimize photosensitization efficiency in the future.

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Original scientific paper
Originalni naučni rad

DECONTAMINATION OF FOOD-RELATED SURFACES BY PHOTOSENSITIZATION
DEZINFEKCIJA POVRŠINA KOJE SU U KONTAKTU SA HRANOM METODOM FOTOSENZITIVNOSTI

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SUMMARY

The use of effective decontamination technique on surface-attached microbes minimizes risks of foodborne diseases, enhances microbiological safety of product and expands its shelf-life. This study deals with the development of novel approach to decontaminate packaging from food pathogens by photosensitization.

For this purpose packaging samples with adhered pathogen were submerged Chlorophyll derivative solution (1.5 x 10^{-5} M) for 5 min. and afterwards illuminated with 20 mW/cm^2 (λ=400nm) light for 20 min up to the total dose 24 J/cm^2. Gram-positive Bacillus cereus as well as Listeria monocytogenes was inactivated by 3-4 log, depending on experimental conditions. Inactivation of Listeria biofilms by 1.7-3.1 log indicate that this treatment has potential to combat biofilms. Moreover, obtained data indicated that the bacillus spores are susceptible to this treatment as well. The spore population on the surface of packaging material was reduced by 3.8 log after photosensitization.

In conclusion, our data support the idea, that photosensitization is effective non-thermal and not-chemical antibacterial treatment, which inactivates food pathogens Bacillus cereus and Listeria monotytogenes as well as spores and biofilms on the surface of packaging material and has potential to be useful in the development of novel food safety technologies.

Key words: *Decontamination of packaging, photosensitization.*

INTRODUCTION

Contamination of food and food-related surfaces by various aerobic mesophils and food-borne pathogens is one of the main problems in food industry. Microbial contamination may begin in food post-processing, or in the packaging and distribution processes. Packaging material irradiation with γ rays is prevalent in U.S. (U.S. Food and Drug Administration, 2007). Some working groups are creating packaging materials with well known antimicrobial additives – nisin, lysozyme in combination with EDTA, bacteriocins (Limjaroen et al. 2003). Washing food-related surfaces with chemical sanitizers are commonly-used. Supposedly, it is one of the cheapest sanitation; do not require special equipment and skills and are conditionally effective against microbes. Disinfectants approved for use in the food industry are alcohols, chlorine-based compounds, quaternary ammonium compounds, oxidants (peracetic acid, hydrogen peroxide, ozone) persulphates, surfactants and iodophors (Wirtanen and Salo 2003). But, generally, microorganisms attached to the surfaces are concerned to build-up biofilms witch are more resistant to any treatment as compared with their planctonic counterparts (Pan et al. 2006). Moreover microbes have been found in disinfectant solutions, which is due to their ability to form resistant strains. This means, that microbial contamination can be spread on the surface to be cleaned instead of being cleansed (Wirtanen and Salo 2003)

One of the possible alternatives to chemical packaging decontamination treatments can be photosensitization. This treatment involves the administration of a photosensitizer that selectively accumulates in the target microorganism. After illumination of this microorganism with visible light, plethora of photochemical reactions induces selectively death of microorganism without any harmful effects on surrounding (Luksiene, 2005).

One of the most important advantages of photosensitization in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment (Nitzan and Ashkenazi 2001). It allows us to inactivate pathogens with minimal damage of the surrounding matrix (Jori 2006) and has no mutagenic or carcinogenic effects on living systems (Luksiene 2005; Luksiene et al. 2005).

Our previous data (Luksiene et al. 1989; 2005; 2007) indicate that yeasts as well as micromycetes might be inactivated by photosensitization *in vitro* as well as on the surface of food matrix. Moreover, food pathogens *Salmonella enterica, Bacillus cereus and Listeria monocytogenes* are susceptible to aminolevulinic-based photosensitization (Buchovec et al. 2009; Le Marc et al., 2009). It is important to note, that *Bacillus* spores being important target for food safety technologies can be inactivated by aminolevulinic acid-based photosensitization (Luksiene et al. 2009). Moreover, *Listeria* biofilms which spread on different food-contact surfaces and are resistant to hypochlorite can be destroyed by aminolevulinic acid-based photosensitization as well. It seems that plethora of harmful micromycetes and pathogenic bacteria might be inactivated by photosensitization, a method that is completely safe, reproducible, not-mutagenic, not-carcinogenic, environmentally and human friendly (Luksiene, 2005; Lithuanian patent, Nr 2008060).

However, there are no reports on the evaluation of applicability of chlorophyll sodium salt-based photosensitization to decontaminate packaging, as this food additive is much cheeper and more effective than aminolevulinic acid. This study was focused on the key issue - effectiveness of the chlorophyll sodium salt-based photosensitization for decontamination of packaging from several food pathogens (*Bacillus cereus, Listeria monocytogenes*). Moreover, it was important to evaluate the susceptibility of pathogenic spores and biofilms to this treatment.

MATERIALS AND METHOD

B. cereus ATCC 12826 and *Listeria monocytogenes* $ATC_{L3}C$ 7644 grown at 37 ^{0}C in Luria-Bertani (LB) medium to the mid-log phase (~ 6x10^7 colony forming units (cfu)/ml, OD_{540}=1) were harvested by centrifugation (10 min, 5000g), resuspended

and accordingly PBS-diluted to ~1 x 10^7 cfu/ml final concentration. For *B. cereus* ATCC 12826 spores preparation, culture was grown for 3 days at 37 ^{0}C in brain heart infusion (BHI) broth (Liofilchem) containing (per liter) 0.05 mg manganese until 80-90% sporulation was obtained. Spore suspension was prepared by washing with sterile distilled water, centrifuging (20 min, 6000g) and heating to 80 ^{0}C. *L. monocytogenes* biofilms were prepared according to the method of Pan et al. (2006). Packing yellow trays, cut into 4 cm × 8 cm pieces were soaked in 50 ml *B. cereus* ATCC 12826 and *L. monocytogenes* $ATC_{L3}C$ 7644 suspension separately for better cell adhesion. Afterwards the packaging samples were kept in laminar 30 min for further bacterial adhesion. Then appropriate packing samples were incubated in the dark with the 7.5 x 10^{-7} M and 1.5 x 10^{-4} M chlorophyll sodium salt for 5 min. The control samples were incubated with PBS (7.2 pH) buffer.

After incubation with chlorophyll sodium salt all packing samples (plastic packing yellow trays provided by LINPAC) were dried at room temperature for 20 min, placed in the treatment chamber and exposed to light for different time (2 min or 5 min) at λ= 400nm. The control sample was not illuminated. Then samples were mixed with 30 ml PBS and homogenized with a BagMixer separately. 100 µl of appropriate dilutions (0.9 % NaCl) of suspension were placed on LBA plates. The colonies were counted after 24 h incubation at 37˚C. The surviving cell populations were enumerated and expressed as log (cfu/ml).

RESULTS AND DISCUSSION

In order to estimate the decontamination efficiency of photosensitization, food packaging material was submerged in *B. cereus* spore solution. Concentrations of Chl sodium salt solution used during these experiments was 7.5 x 10^{-7} M. Data, depicted in Fig. 1 describe the chlorophyll derivative-based photoinactivation of *B. cereus* on the surface of packaging. It is obvious, that photosensitizer alone without following illumination has no significant antibacterial effect. Moreover, even 20 min illumination of bacteria adhered on the surface has no effect on their viability. Just photosensitization treatment inactivated them to 0 log and cleaned the surface of packaging.

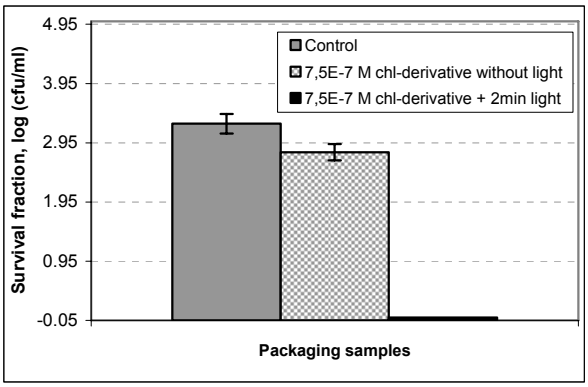

Fig. 1. Inactivation of Bacillus cereus ATCC 12826 by 7.5E-7 M chl-derivative based photosensitization onto packaging samples.
Sl. 1. Aktivacija Bacillus cereus ATCC 12826 by 7.5E-7 M chl-derivatom zasnovan fotosenzitivnošću na upakovan uzorak

The data, depicted in Fig. 2, clearly indicate that the inactivation of *Listeria* cells attached to the surface of packaging after photosensitization treatment reached 4.5 log from initially attached 4.5 log. Very low (7.5 x10^{-7} M) photosensitizer concentration was enough effective and cleaned the surface to 0 log.

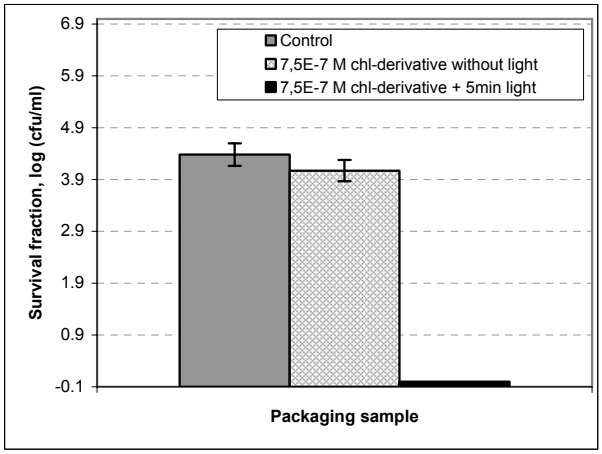

Fig. 2. Inactivation of Listeria monocytogenes $ATC_{L3}C$ 7644 by 7.5E-7 M chl-derivative based photosensitization onto packaging samples.
Sl. 2. Aktivacija Listeria monocytogenes $ATC_{L3}C$ 7644 by 7.5E-7 M chl derivatom zasnovan fotosenzitivnošću na upakovan uzorak

Data, shown in Fig. 3 indicate that *B. cereus* spores are able to attach on plastic food-related packaging material and can be inactivated by chlorophyll sodium salt-based photosensitization (3.8 log).

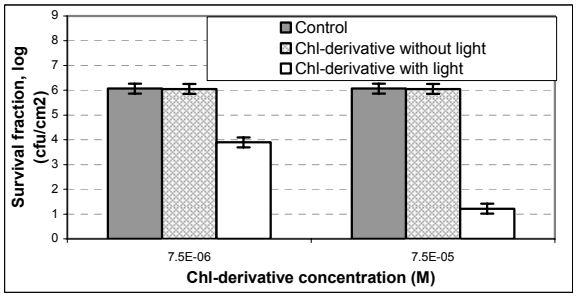

Fig. 3. Inactivation of Bacillus cereus ATCC 12826 spores by 7.5E-6 M and 7.5E-5 M chl-derivative based photosensitization onto packaging samples.
Sl. 3. Aktivacija Bacillus cereus ATCC 12826 spore 7.5E-7 M chl- derivatom zasnovan fotosenzitivnošću na upakovan uzorak

Our task was to evaluate susceptibility of *Listeria* biofilms to chlorophyll derivative-based photosensitization treatment. For this purpose, bacterial biofilms were adhered on the surface of packaging material. The treatment of biofilm-associated cells by 1.5 x 10^{-5} - 1.5 x 10^{-4} M chlorophyll sodium salt destructed them by 4.5 log from 4.5 log of attached cells (Fig. 4).

Nowadays, several technologies are practicing to reduce the risk of surface contamination. Meanwhile all of them, as described in Introduction, have specific disadvantages. First results obtained on packaging decontamination by chlorophyll sodium salt-based photosensitization seems promising, as antibacterial efficiency of this method seems high in comparison with chemical disinfectants. Wirtanen et al. (2001) evaluated efficacy of common commercial disinfectants (based on hypochlorite, hydrogen peroxide, alcohol and peracetic acid) against some foodborne pathogens and spoilage microbes in biofilm-constructs. It came clear, that Gram-positive bacteria tested in poloxamer hydrogels was reduced from ~0.1- to ~2-log and Gram-negative

bacteria were even more resistant (Wirtanen et al. 2001). Furthermore, survived cells are able to grow and recontaminate surfaces. Also harmful chemical compounds commonly are unstable, corrosive and toxic (Wirtanen and Salo, 2003).

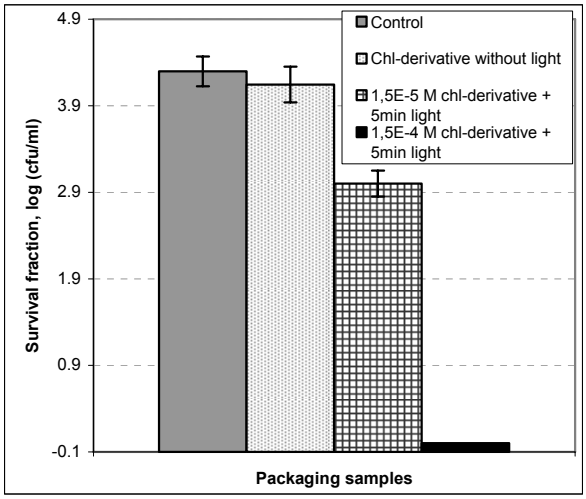

Fig. 4. Inactivation of Listeria monocytogenes $ATC_{L3}C$ 7644 biofilms by 1.5E-5 M and 1.5E-4 M chl-derivative based photosensitization onto packaging samples.
Sl. 4. Aktivacija Listeria monocytogenes $ATC_{L3}C$ 7644 biofilmovi pomoću 1.5E-5 M and 1.5E-4 M chl-- derivatom zasnovan fotosenzitivnošću na upakovan uzorak

Destruction of biofilms can be a problem not even in food packaging, but in food processing as well. Due to insufficient efficiency of cleaning the remaining cells start to grow and contaminate the product (Hood and Zottola 1995). According to our data, chlorophyll sodium salt-based photosensitization can inactivate biofilms on the surface of packaging by 4.5 log from 4.5 log of attached cells.

CONCLUSION

In conclusion, obtained results reveal, that chlorophyll sodium salt–based photosensitization treatment is effective antibacterial tool against such food pathogens as *Bacillus cereus and Listeria monocytogenes* and can totally decontaminate packaging from these bacteria adhered on the surface of packaging. It is important to note, that chlorophyll sodium salt–based photosensitization treatment can combat resistant *Listeri*a biofilms and *Bacillus* spores on the surface of packaging.

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ORIGINAL ARTICLE

Modelling the photosensitization-based inactivation of *Bacillus cereus*

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Keywords
Bacillus cereus, inactivation, photosensitization, predictive model, Weibull.

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Abstract

Aims: To study and to develop a model for the photo-destruction of the food-borne pathogen *Bacillus cereus*, initially treated with a precursor of endogenous photosensitizers (5-aminolevulinic acid, ALA).
Materials and methods: The cells were incubated in the presence of ALA (3 or 7·5 mmol l^{-1}) for incubation times ranging from 2 to 60 min, inoculated onto the surface of LB Agar plates and submitted to light irradiation. The Weibull model was used to describe the survival curves of *B. cereus*. Quadratic equations were used to describe the effects of ALA concentration and incubation time on the Weibull model parameters.
Results: ALA-based photosensitization proved to be an effective tool for inactivation of *B. cereus*. The decrease in viable counts observed after 20 min of irradiation, ranged from 4 to 6 log CFU g^{-1}.
Conclusions: The developed model proved to be a parsimonious and robust solution to describe the observed data.
Significance and Impact of the Study: The study demonstrates the effectiveness of photosensitization on *B. cereus* on agar plates. The model developed may be useful to optimize inactivation treatments by photosensitization.

Introduction

Bacillus cereus is a widespread foodborne pathogen which can cause illness by producing emetic and diarrhoeal enterotoxins. Strains producing diarrhoeal toxin can be found in a variety of foods from rice and vegetables to meat. Because of the ability of *B. cereus* to survive mild heat treatments and dried storage, the use of alternative nonthermal technologies for its inactivation are of great interest. Among these emerging technologies, photosensitization is a pioneering approach for surface decontamination of foods and materials along the food chain (Luksiene *et al.* 2004; Luksiene 2005). The method is based on the principle of activation of photosensitizers by visible light. After accumulation of photosensitizers inside the cell and following irradiation, the singlet oxygen generated interacts with bacterial structures, which lead to series of cytotoxic reactions and microbial death. This technique has already proved to be effective against some highly proliferating cells and is a promising treatment for cancer. It has also been found that photosensitization is effective against various micro-organisms such as *Escherichia coli* O157 : H7 and *Listeria monocytogenes* (Romanova *et al.* 2003; Luksiene 2005). Some photosensitizers available are food additives or food constituents which do not induce any toxic effects in foods (Buchovec *et al.* 2009). One considerable advantage of photosensitization is that, according to Nitzan and Ashkenazi (2001), bacteria do not develop resistance after this treatment.

From the beginning of the twentieth-century, microbial inactivation has drawn a major interest from food researchers. The first models were developed on the assumption that bacterial inactivation follows a first order kinetics. However, deviations from log-linear curves have been observed by numerous authors (e.g. Cerf 1977; Peleg and Cole 1998; Xiong *et al.* 1999; Geeraerd *et al.* 2000). In a survey of 120 inactivation curves, van Boekel (2002) found that only 5% of the inactivation curves actually followed a first order kinetics. Three kinds of deviation have been observed: sigmoid, concave and convex shapes. Since the shape of sigmoid survival curves are similar to those of growth curves, some authors adapted growth

models such as the logistic, the Gompertz or the Baranyi equations to model bacterial inactivation (Chen and Hoover 2003). However, these models are unsuitable for nonsigmoid survival curves.

In recent years, the Weibull model has received much attention as an alternative. Due to its flexibility, it can describe concave, convex and linear shapes. This model has been successfully used in a number of studies to model thermal (Fernandez *et al.* 2002; Mafart *et al.* 2002; Albert and Mafart 2005; Couvert *et al.* 2005) or nonthermal bacterial inactivation (Chen and Hoover 2003).

While a considerable amount of work has been carried out on nonthermal inactivation (e.g. dried storage, high pressure) only a few studies have focused on modelling the effect of photosensitization. Luskiene *et al.* (2009), however, showed that *B. cereus* can effectively synthesize endogenous porphyrins from 5-aminolevulinic acid (ALA) at low concentrations (3–7·5 mmol l^{-1}), confirming the possibility of this pathogen to be inactivated by ALA-based photosensitization (Luksiene 2005). Previous studies (Luskiene *et al.* 2009) indicated that concentrations of ALA lower than 3 mmol l^{-1} would be ineffective. In this work, we studied the antibacterial effectiveness of ALA-based photosensitization against *B. cereus in vitro* at two levels of ALA concentrations, three and 7·5 mmol l^{-1}.

Materials and methods

ALA preparation

A stock solution of 5-aminolevulinic acid hydrochloride (ALA) (Fluka, Rehobot, Israel) was prepared by dissolving ALA in phosphate-buffered saline (0·1 mol l^{-1} PBS, pH 7·2) up to the concentration 200 mmol l^{-1} NaOH was used to adjust pH of the solution to 7·2. ALA stock solutions were made immediately before use and sterilized by filtration through 0·20 μm filter (Roth, Karlsruhe, Germany) (Luksiene *et al.* 1999).

Micro-organism and culture conditions

The target bacterium, *B. cereus* ATCC 12826, was kindly provided by Prof. D.H. Bamford (University of Helsinki, Finland). The bacterial culture was grown at 37°C and maintained onto Luria Bertani Agar (LBA) (Liofilchem, Roseto degli Abruzzi, Italy).

The bacterial culture was grown overnight (~14 h) at 37°C in 20 ml of Luria-Bertani medium (LB) (Liofilchem), with aeration of 120 rev min^{-1} (Environmental Shaker–Incubator ES-20; Biosan, Riga, Latvia). The overnight bacterial culture grown in LB medium was diluted 20-fold with fresh LB medium (OD_{540} = 0·164) and grown at 37°C to the mid-log phase (~6×10^7 CFU ml^{-1}, OD_{540} = 1) in a shaker (120 rev min^{-1}). Bacterial optical density was determined in a 10·01 glass cuvette at λ = 540 nm (Helios Gamma & Delta spectrophotometers; ThermoSpectronic, Cambridge, UK). Cells were then harvested by centrifugation (6000 rev min^{-1}, 20 min) and resuspended in a small volume of PBS, to give approx. 3×10^8 CFU ml^{-1}. This stock suspension was diluted to ~1×10^7 CFU ml^{-1} and immediately used for the photosensitization experiments.

Photosensitization and bacterial survival assay

Aliquots (10 ml) of bacterial suspension (~1×10^7 CFU ml^{-1} in 0·1 M PBS buffer) with appropriate concentration of ALA (3 and 7·5 mmol l^{-1}) were incubated in a plastic 50 ml bottle in the dark in the shaker (120 rev min^{-1}) at 37°C for (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). The LED based light source used for illumination was developed by the Institute of Applied Sciences of Vilnius University. The surface of samples were illuminated at λ = 400 nm with an intensity of 20 mW cm^{-2}. Light dose was calculated as light intensity multiplied by illumination time. Light power density measurements were performed with a light energy measured by three Sigma meter ('Coherent') equipped with piro-electrical detector J25LP04. No thermal effect was detected for the experimental conditions tested.

The antibacterial effect of photosensitization on *B. cereus* was evaluated by the spread plate method; 100 μl of appropriate dilutions of bacterial test culture after photosensitization were inoculated onto the surface of LB Agar plates. The bacteria were enumerated after incubation at 37°C for 24 h. Duplicate experiments were carried out for each set of exposures.

Mathematical model

The modelling was carried out into two stages. In the first step (primary modelling), the survival curves were fitting with the Weibull model, as proposed by Peleg and Cole (1998):

$$\ln(N/N_0) = -kt^p \qquad (1)$$

where t is the time of exposure to light, N is the bacterial concentration at time t, N_0 is its initial value at time 0, k and p are the scale and shape parameters of the model. Equation (1) describes a concave downward survival curve if $P > 1$, a convex function if $P < 1$ and a linear function if $P = 1$. In the second stage (secondary modelling), polynomial equations were used to describe the effects of ALA concentration and incubation time on

the parameters k and p of the Weibull model. As the parameters k and p are strongly correlated (see e.g. Couvert *et al.* 2005), in this study, only the shape parameter p was modelled directly as a function of the ALA concentration and the incubation time, the parameter k was calculated from the observed strong correlation. The choice of the parameter to be modelled directly was based on sensitivity analysis; namely the shape parameter was more affected by the measurement errors of the raw data (i.e. the sampling errors in the log cell concentrations), therefore it was important to minimize the error on p.

Bacterial growth curves were fitted using a nonlinear regression module (NLINFIT) of MATLAB ver. 7b (The MathWorks, USA). Linear regressions were performed with the Essential Regression and Experimental Design Version 2.2, a Microsoft Excel add-in available at http://www.geocities.com/SiliconValley/Network/1032/. A backward elimination procedure was adopted to eliminate the insignificant terms.

Results

Effectiveness of ALA-based photosensitization

Following irradiation, the survival fraction decreased sharply (Figs 1 and 2). A clear dependence of photoinactivation efficiency on irradiation time (or total energy dose) as well as incubation time with ALA was observed. The number of pathogens killed reached six orders of magnitude, after 20 min irradiation time and 60 min incubation time.

Modelling of the inactivation of *Bacillus cereus*

The survival curves as a function of irradiation time exhibit different shapes: linear, convex, and concave (Figs 1 and 2). At a low concentration of ALA (3 mmol l^{-1}) and for short incubation times (2 and 15 min), the survival curves have convex or linear shapes. However, for incubation times longer 15 min, all the curves showed a concave shape. The same observation was made with 7·5 mmol l^{-1} ALA: as the incubation time increases, the curves have a more pronounced concave shape. Increasing incubation time induces an increase of the mortality in the first minutes of irradiation. In this case, however, surviving cells are also more resistant to further irradiation. For example, a reduction of 5·1 log is observed following 5 min of irradiation after incubation for 60 min in the presence of 7·5 mmol l^{-1} ALA, compared with only 2·2 log reduction after incubation for 30 min. A subsequent irradiation of another 15 min induces a further reduction of only 0·6 log (incubation time 60 min) against 2·8 log (incubation time 30 min).

As can be seen in Figs 1 and 2, eqn (1) successfully described the different shapes of the observed survival curves. Table 1 gives the fitted parameters for k and p. It can be observed that k increases with decreasing p and suggests a strong correlation between the two parameters. This is confirmed by Fig. 3 which clearly shows a linear

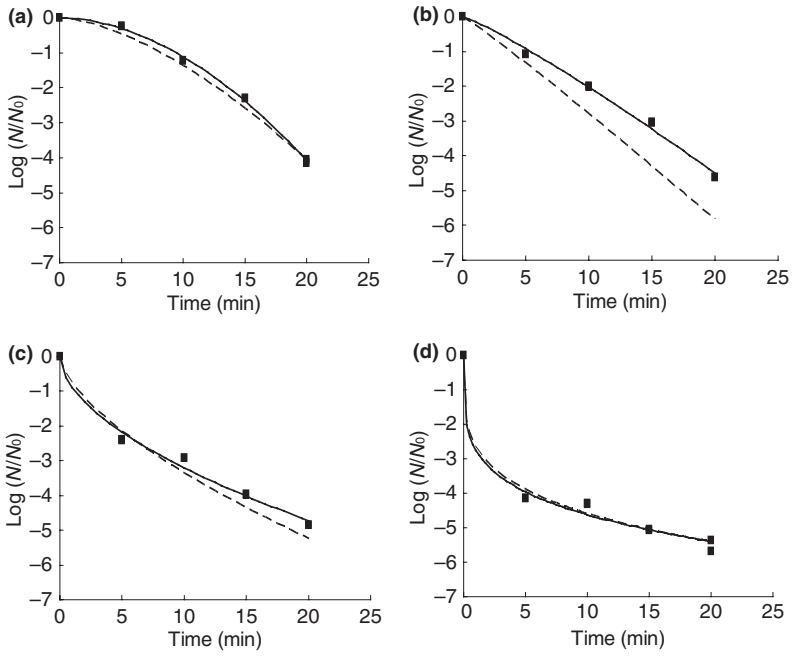

Figure 1 Survival of *B. cereus* after incubation in the presence of 3 mmol l^{-1} ALA for (a) 2 min, (b) 15 min, (c) 30 min and (d) 60 min. Straight line: fit of the Weibull model (eqn 1), dotted line: prediction of eqn (2) and (3), ■ bacterial log counts.

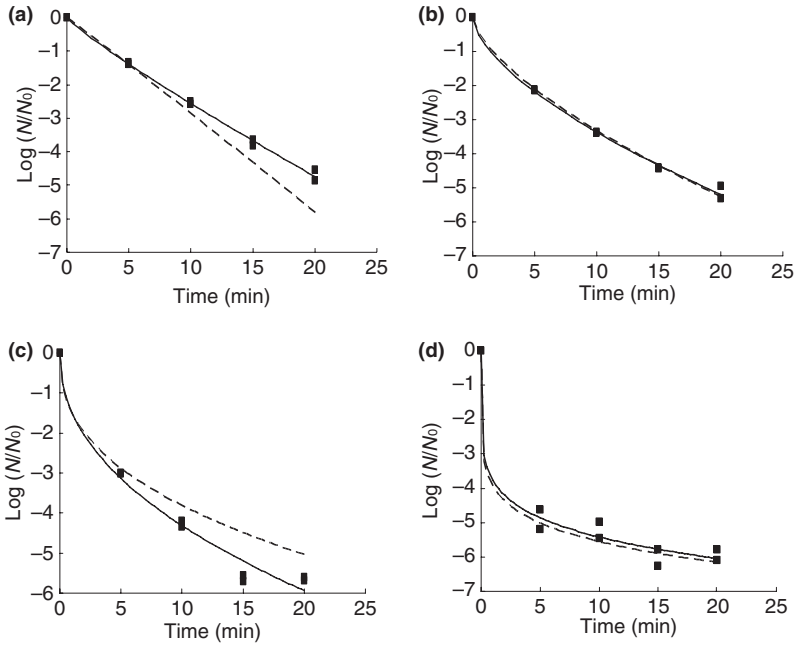

Figure 2 Survival of *B. cereus* after incubation in the presence of 7·5 mmol l^{-1} ALA for (a) 2 min, (b) 15 min, (c) 30 min and (d) 60 min. Straight line: fit of the Weibull model (eqn 1), dotted line: prediction of eqn (2) and (3), ■ bacterial log counts.

Table 1 Fitted parameters of eqn (1)

ALA concentration (mmol l^{-1})	Incubation time (min)	Fitted values	
		k	*P*
3	2	0·034 (0·020–0·049)	1·87 (1·73–2·01)
3	15	0·33 (0·19–0·47)	1·14 (1·00–1·29)
3	30	2·03 (1·40–2·67)	0·56 (0·44–0·67)
3	60	6·37 (5·04–7·70)	0·22 (0·14–0·30)
7·5	2	0·76 (0·59–0·93)	0·89 (0·81–0·97)
7·5	15	1·83 (1·52–2·14)	0·63 (0·56–0·69)
7·5	30	3·43 (2·33–4·54)	0·46 (0·34–0·58)
7·5	60	8·68 (6·64–10·72)	0·16 (0·06–0·25)

correlation between ln (*p*) and sqrt (*k*). These results are in accordance with those of Couvert *et al.* (2005) who found a structural correlation between *p* and *k* in their study on the thermal resistance of *Bacillus pumilus*. The quadratic model was fitted for the effects of ALA concentration and incubation on ln(*p*) (R^2_{adj} = 0·972). Square terms and interaction terms proved to be insignificant (α = 5%) and the final equation is:

$$\ln(p) = 0{\cdot}877 - 0{\cdot}105\ \text{Conc\_ ALA} - 0{\cdot}0333\ \text{Inc\_Time} \quad (2)$$

where Conc_ALA is the concentration of ALA (mmol l^{-1}) and Inc_Time is the incubation time (min). As shown in Fig. 4, the model provides an accurate description of the effect of incubation time at ALA concentrations of 3 and 7 mmol l^{-1}. Sqrt(*k*) was modelled, using the linear

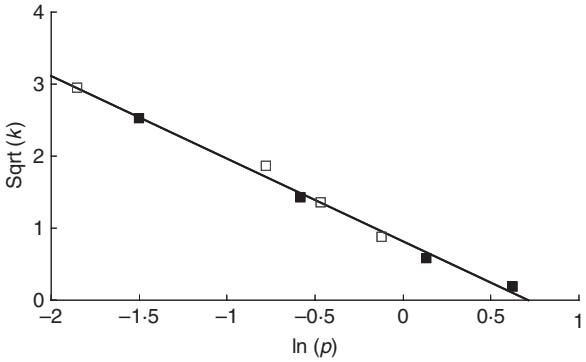

Figure 3 Linear relationship between ln (*p*) and sqrt (*k*), where *p* and *k* are the scale and shape parameters of the Weibull model (eqn 1). ■ and □ are the observations after incubation in the presence of 3 and 7·5 mmol l^{-1} ALA, respectively.

correlation between ln(*p*) and sqrt(*k*). The fitted equation (R^2_{adj} = 0·972) is written as:

$$\text{Sqrt}(k) = 0{\cdot}816 - 1{\cdot}151\ln(p) \quad (3)$$

Equations (2) and (3) were used to predict the survival curves for the experimental conditions studied in this work. Comparison between the predicted and fitted survival curves shows that the model usually provides a good overall description of the data (Figs 1 and 2). Note that when we modelled sqrt(*k*) and then calculated ln(*p*) by using eqn (3), the predictions for the log cell

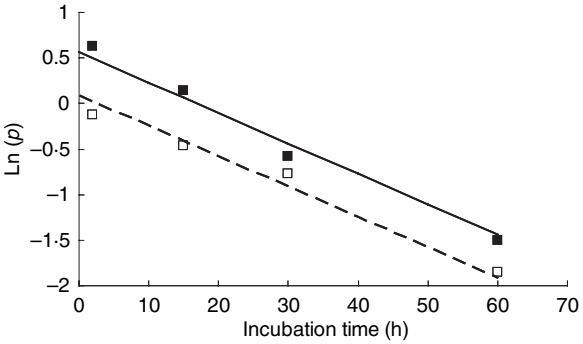

Figure 4 Predicted effect of incubation time on the natural logarithm of *p*, the shape parameter of the Weibull model (eqn 1) at 3 (solid line) and 7·5 mmol l^{-1} (dashed line) ALA. ■ and □ represent the values of ln (*p*) at 3 and 7·5 mmol l^{-1} ALA respectively.

concentrations were much poorer. The reason is that the shape parameter p is more sensitive to errors than k. Therefore, if the modelling follows the $k - p$ order, not only the error in the observed log counts affects p but also the error in the $p = f(k)$ function.

Discussion

The inactivation of pathogens on food or food-related surfaces by photosensitization is a novel and pioneering approach. The selected photosensitizer must have suitable photophysical and photochemical properties and be easy to produce and effective against foodborne pathogens. It must be either a food constituent or at least be nontoxic at the concentrations required for microbial inactivation. The study shows that *B. cereus* can be inactivated by an order of 4–6 log by photosensitization with 3 and 7·5 mmol l^{-1} ALA. ALA is a naturally occurring substance, produced by e.g. mammals and plants. As ALA is planned to be used as a photosensitizer on the surface of ready to eat food products before the product is washed, these concentrations of ALA must not be toxic for humans (Luksiene 2003). However, food additives should be used in as low concentrations as possible. Considering the satisfactory six log reduction observed after exposure to concentrations of 7·5 mmol l^{-1} ALA, the effects of higher concentrations were not investigated in this work. However, further research will investigate the applicability of the protocol used in this work to the decontamination of the surface of fruits and vegetables.

The Weibull model was able to describe the different shapes of the curves encountered in the experiments. A concave shape is generally the result of rapid elimination of the sensitive members of the population, leaving the remaining cells with an increased resistance (Corradini and Peleg 2007). Our results suggest that increasing ALA concentration and incubation time increases the rates at which the sensitive cells are killed in the first minutes of irradiation. However, the survivors also become more resistant to light irradiation. Mathematical modelling can help selecting the combination of ALA concentration, incubation and irradiation times to reach the desired log reduction of pathogens.

Secondary modelling focuses on the effect of the environmental factors on the main parameters of the primary model. One problem which arises in the secondary modelling is the correlation between the parameters of primary models. This correlation is frequently observed in predictive microbiology, for example, between growth rate and lag time (e.g. Cooper 1963; Chandler and McMeekin 1985; Smith 1985; Fu *et al.* 1991; Baranyi and Roberts 1994; Rosso 1995). These authors reported that for the same preincubatory conditions the lag times are inversely proportional to the specific growth rates. This simple relationship has often been utilized to estimate the lag from the growth rate model (e.g. Rosso 1995; Ross and Dalgaard 2004).

A similar problem arises in the secondary modelling for bacterial inactivation with the correlation between the parameters of the Weibull model. Couvert *et al.* (2005) solved this problem by using a single *P*-value estimated from the whole data set (therefore, the secondary model developed by the authors describes only the effects of temperature on the scale parameter k). This was possible since the temperature has little effect on the shape parameter (van Boekel 2002). Despite a slight loss of goodness of fit, this modification improved the robustness of the model. This technique was also suggested by other authors (Mafart *et al.* 2002; Corradini and Peleg 2007). However, in our work the shape parameter p varies greatly with the experimental conditions (with values ranging from 0·15 to 1·15). It was therefore impossible to use a single *P*-value for the whole data set. Instead of modelling independently two parameters mutually dependent on each other, we modelled only one and then estimated the second parameter from their correlation. To our best knowledge, it is the first time that a secondary model has been developed for both parameters of the Weibull model, p and k, using the correlation observed between those two parameters. In this work, the best results were obtained by (i) model ln(p) as a function of ALA concentration and incubation time and (ii) model sqrt(k) as a function of ln(p).

Conclusion

The data presented clearly indicates for the first time that ALA-based photosensitization can be effective against the foodborne pathogen *B. cereus*. Survival curves of *B. cereus* estimated after inactivation by ALA-based

photosensitization can be successfully described by the Weibull model. The secondary model developed can be a useful tool to determine the most effective combination of ALA concentration and incubation time to reach the desired target for the reduction of *B. cereus*. However, further work is needed to assess the applicability of the model and the effectiveness of the ALA-based photosensitization treatment in a real food scenario.

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ORIGINAL ARTICLE

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

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Keywords
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 $\lambda = 400$ 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

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},

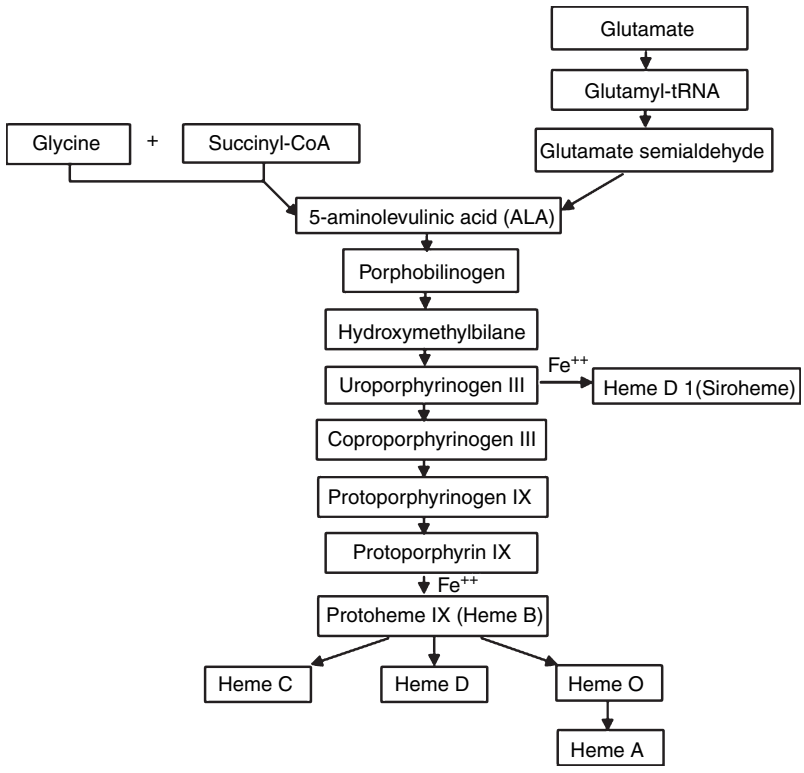

Figure 1 Bacterial biosynthetic pathway of haeme (adapted from Hamblin and Hasan 2004).

OD_{540} = 1·3] in a shaker (120 rev min^{-1}). Bacterial optical density was determined in a 10·01 glass cuvette at λ = 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 $\sim 2{\cdot}5 \times 10^9$ CFU ml^{-1}. This stock suspension was diluted accordingly to $\sim 1 \times 10^7$ CFU ml^{-1} and immediately used for the photosensitization experiments.

Photosensitization

Aliquots (10 ml) of bacterial suspension [$\sim 1 \times 10^7$ 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 λ = 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 [$\sim 1 \times 10^7$ 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, λ = 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 (λ = 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

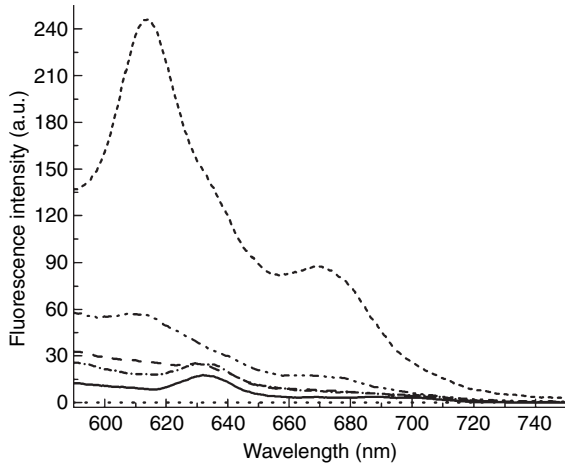

Figure 2 Fluorescence spectra of endogenous porphyrins produced by *Salmonella enterica* Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2) after incubation with 7·5 mmol l^{-1} ALA for different time interval: (·····) control; (——) –2 min; (-·-·-) –15 min; (– – –) –30 min; (-·-·-) –60 min; (– – – –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

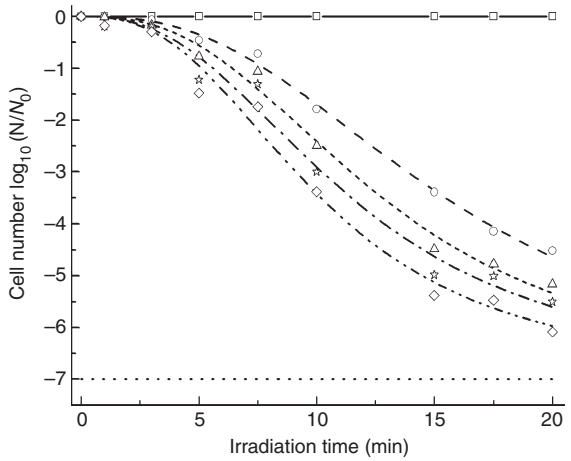

Figure 3 Inactivation of *Salmonella enterica* Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2) by 7·5 mmol l^{-1} ALA - based photosensitization as function of irradiation time, when different incubation with ALA time was used: (–□–) control; (-○-) –2 min; (·△·) –15 min; (-☆·) –30 min; (-◇·) –60 min.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

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

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:

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

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.

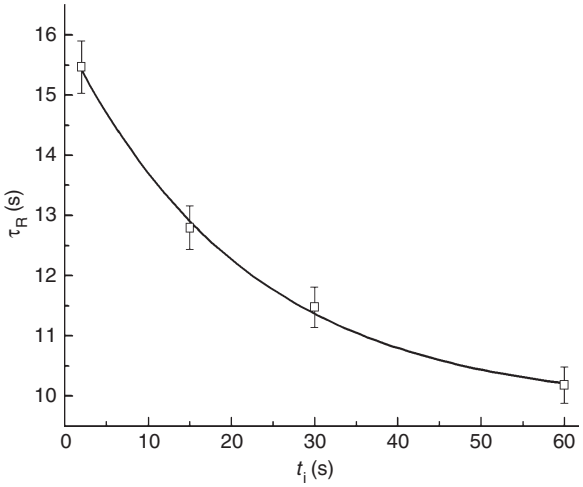

Figure 4 Time parameter τ_R dependency on incubation time approximates by first order exponential decay.

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, foodborne 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)

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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ORIGINAL ARTICLE

Inactivation of food pathogen *Bacillus cereus* by photosensitization *in vitro* and on the surface of packaging material

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Keywords
5-aminolevulinic acid, *Bacillus cereus*, decontamination technology, inactivation, photosensitization.

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Abstract

Aims: The study was focused on the possibility to inactivate food pathogen *Bacillus cereus* by 5-aminolevulinic acid (ALA) – based photosensitization *in vitro* and after adhesion on the surface of packaging material.
Methods and Results: *Bacillus cereus* was incubated with ALA (3–7·5 mmol l^{-1}) for 5–60 min in different environment (PBS, packaging material and wheat grains) and afterwards illuminated with visible light. The light source used for illumination emitted light at λ = 400 nm with energy density at the position of the cells, 20 mW cm^{-2}. The illumination time varied from 0 to 20 min, and subsequently a total energy dose was between 0 and 24 J cm^{-2}. The obtained results indicate that *B. cereus* after the incubation with 3–7·5 mmol l^{-1} ALA produces suitable amounts of endogenous photosensitizers. Following illumination, micro-organism inactivated even by 6·3 log. The inactivation of *B. cereus* after adhesion on the surface of food packaging by photosensitization reached 4 log. It is important to note that spores of *B. cereus* were susceptible to this treatment as well; 3·7-log inactivation *in vitro* and 2·7-log inactivation on the surface of packaging material were achieved at certain experimental conditions.
Conclusions: Vegetative cells and spores of Gram-positive food pathogen *B. cereus* were effectively inactivated by ALA-based photosensitization *in vitro*. Moreover, the significant inactivation of *B. cereus* adhered on the surface of packaging material was observed. It was shown that photosensitization-based inactivation of *B. cereus* depended on the total light dose (illumination time) as well as on the amount of endogenous porphyrins (initial ALA concentration, time of incubation with ALA).
Significance and Impact of the Study: Our previous data, as well as the one obtained in this study, support the idea that photosensitization with its high selectivity, antimicrobial efficiency and nonthermal nature could serve in the future for the development of completely safe, nonthermal surface decontamination and food preservation techniques.

Introduction

Microbiological food safety is an increasing worldwide problem. An estimated 76 million cases of food-borne disease outbreaks occur annually in the United States, costing 6·5–34·9 billion dollars in medical care and lost productivity (Mead *et al.* 1999).

Clostridium botulinum, *Bacillus cereus* and *Listeria monocytogenes* are the main Gram-positive pathogens causing food-borne diseases (Altekruse *et al.* 1997). Even spore-forming *B. cereus* has been identified as the cause of 27 000 cases of food-borne illnesses in the United States (Mead *et al.* 1999). *Bacillus cereus* is naturally found in soil environments, because it can contaminate wide range of foods: cereals, fresh vegetables, berries and fruits. Additionally, this pathogen was found in ready-to-eat foods and sauces (Rosenquist *et al.* 2005).

Heat-stable toxins generated by Gram-positive *Staphylococcus aureus*, *Clostridia perfringens* and *B. cereus* are the common causes of sporadic point-source outbreaks of gastroenteritis (Gill and Hamer 2001).

Several types of antibacterial treatments, traditionally and naturally occurring food antimicrobials are used in food manufacturing. Most of the emerging preservation techniques are based on nonthermal decontamination of food and include ionizing radiation, high hydrostatic pressure, high-intensity pulsed light and natural antimicrobials. Most of them act by slowing down or inhibiting microbial growth, and none can eliminate pathogens totally from the food matrix (Elmnasser *et al.* 2007; Gomez-Lopez *et al.* 2007). Taking this into consideration, novel, effective, nonthermal food decontamination technique without any effects on nutritional and organoleptic characteristics of food matrix is of great demand. May be, one of them could be photosensitization.

Since the beginning of the 20th century, when photosensitization-based destruction of highly proliferating living systems was discovered, many attempts have been made in order to understand the mechanism of action of this phenomenon (Hamblin and Hasan 2004; Jori 2006). Recently, it has been accepted that photosensitization (photodynamic therapy, when treating infectious diseases or cancer) is based on the interaction of three factors: photoactive compound (photosensitizer), light and oxygen. As photosensitizers can easily accumulate inside the cells (cancer cells, inflammatory cells or bacteria) with high metabolic activity, after their illumination with visible light, photophysical and photochemical processes inside the cell can induce cell death (Collaud *et al.* 2004). In detail, a photosensitizer after absorption of light in picoseconds transfer excitation energy from singlet state (S1) to lower excited triplet state T1 with a longer lifetime (milliseconds). Relaxation of photosensitizer from the T1 state results in the induction of two types of photo-oxidative reactions. Type I pathway involves electron or hydrogen atom transfer, producing radical forms of the photosensitizer or the substrate. Type II mechanism is mediated by an energy transfer process to ground state oxygen ($3O_2$). It is worth nothing that the destruction of a cell in this case is strictly localized because of a very short half-life of 102 ns and consequently short diffusion distance of 20 nm (Moan 1990). As a consequence, a plethora of cytotoxic reactions is triggered in the cells. Mostly, cell injuries induced by photosensitization include disruption of cell membrane, inactivation of different enzymes and damage of DNA (Demidova and Hamblin 2005; Luksiene 2005; Jori 2006).

Various studies have demonstrated that some pathogenic micro-organisms can produce endogenous photosensitizers from exogenously applied precursor 5-aminolevulinic acid (ALA; Fig. 1) (Luksiene 2005; Jori 2006; Fotinos *et al.* 2008). It is clear from Fig.1 that ALA is an endogenous component in the heme biosynthesis pathway, and according to literature ALA is ubiquitous in nearly all cells (Fotinos *et al.* 2008). Moreover, ALA is transported to cytosol via different active transport mechanisms. Even in Gram-negative bacteria, ALA is able to enter periplasm through the outer membrane porins and is transported to the cytosol by dipeptide permease (Elliott 1993). The synthesis of the porphyrins in the cytosol is strictly feedback controlled (Dietel *et al.* 2007).

This feature of micro-organisms looks very attractive, especially from the point of view of decontamination of food or food-related surfaces. Only living micro-organisms can metabolize colourless and odourless ALA to endogenous photosensitizers, which appears to be a way of gaining selectivity (Collaud *et al.* 2004). Meanwhile, no data exist on the evaluation of capacity of vegetative cells and spores of *B. cereus* to uptake ALA and to produce endogenous porphyrins. Moreover, there is no data on susceptibility of this bacterium to ALA-based photosensitization *in vitro* or on the different surfaces. Thus, the aim of this study was to focus on the possibility to inactivate vegetative forms and spores of food pathogen *B. cereus* by ALA-based photosensitization *in vitro* as well as on the packaging material.

Materials and methods

Chemicals

Stock solution of ALA (Fluka, Rehobot, Israel) was prepared by dissolving ALA in 100 mmol l^{-1} phosphate-buffered saline (PBS, pH 7·2) up to the concentration of 200 mmol l^{-1}, and NaOH was used to adjust pH level of the solution to 7·2. ALA stock solutions were made instantly before use and sterilized by filtration through 0·20 μm filter (Roth, Karlsruche, Germany) (Luksiene *et al.* 1999).

Bacterial growth

Bacillus cereus ATCC 12826 was kindly provided by the National Centre of Public Health (Vilnius, Lithuania). The bacterial culture was grown at 37°C and maintained onLuria Bertani agar (LBA; Liofilchem, Roseto degli Abruzzi, Italy).

The bacterial culture was grown overnight (*c.* 14 h) at 37°C in 20 ml of Luria-Bertani medium (LB) (Liofilchem), with aeration of 120 rev min^{-1} (Environmental Shaker–Incubator, model ES-20; Biosan, Riga, Latvia). The overnight bacterial culture grown in LB medium was diluted 20 times with the fresh LB medium. The initial

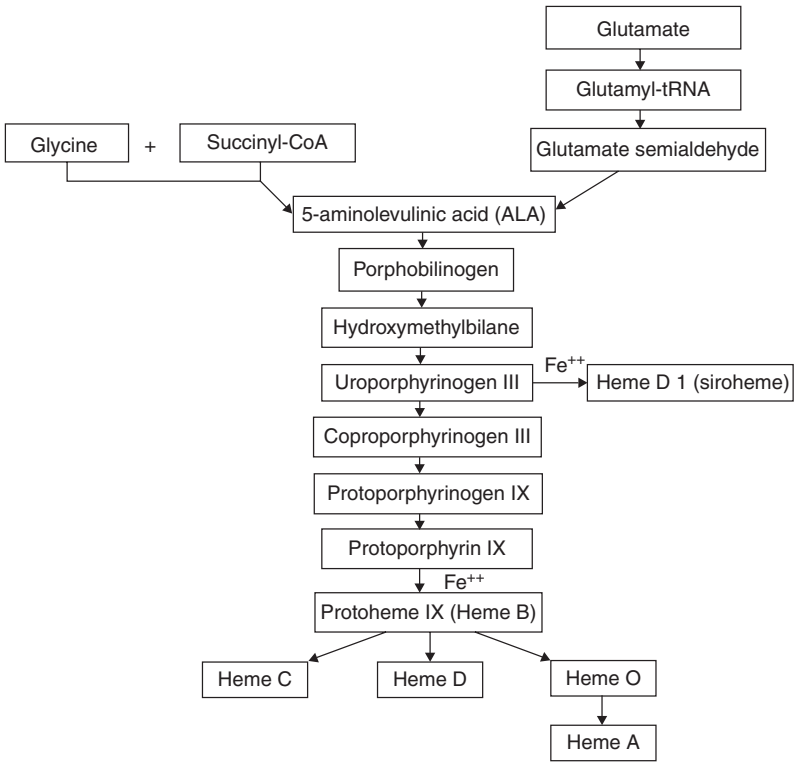

Figure 1 Bacterial biosynthetic pathway of heme (adapted from Hamblin and Hasan 2004). Synthesis of endogenous porphyrins from 5-aminolevulinic acid.

optical density (OD) of bacteria culture was 0·164 at $\lambda = 540$ nm (Heλios Gamma and Delta spectrophotometers; ThermoSpectronic, Cambridge, UK). Bacteria were grown at 37°C to the midlog phase (*c.* 6×10^7 CFU ml^{-1}, $OD_{540} = 1$; bacterial count established by spread plate method) in a shaker (120 rev min^{-1}). Afterwards, the bacteria were harvested by centrifugation (10 min, 5000 ***g***) and resuspended to *c.* $3{\cdot}015 \times 10^8$ CFU ml^{-1} final concentration in 100 mmol l^{-1} PBS (pH 7·2). This stock suspension was accordingly PBS-diluted to *c.* 1×107 CFU ml^{-1} and immediately used for the photosensitization experiments.

Photosensitization *in vitro*

Aliquots (10 ml) of bacterial suspension (*c.* 1×10^7 CFU ml^{-1} in 100 mmol l^{-1} PBS buffer, pH 7·2) with appropriate concentrations of ALA (3 and 7·5 mmol l^{-1}) were incubated in the dark in a 50 ml plastic bottle for cell culture cultivation at 37°C. For the following experiments, the cells were incubated in the shaker (120 rev min^{-1}) for different periods (0–60 min). After incubation, 150 μl aliquots of bacterial suspension were withdrawn, placed in sterile flat bottom wells and exposed to light for different periods (0–20 min) (Nitzan *et al.* 2004). Light emitting diodes (LED)-based light source (constructed in the Institute of Applied Sciences of Vilnius University) for emitted light $\lambda = 400$ nm with the intensity of 20 mW cm^{-2} on the surface of samples. Light dose was calculated as light intensity multiplied on time. Light power density measurements were observed with a light energy measure by 3 sigma meter (Coherent, Santa Clara, CA, USA) equipped with piro-electrical detector J25LP04. No thermal effects were detected at the exploited experimental conditions.

Fluorescence measurements of intracellular porphyrins

The cell suspensions for measurements of endogenous porphyrins from ALA were prepared as follows. Cells (*c.* 1×10^7 CFU ml^{-1} in 100 mmol l^{-1} PBS, pH 7·2) were incubated in the dark at 37°C with 3 and 7·5 mmol l^{-1} ALA concentrations for the indicated period. Then, 2 ml aliquots of bacterial suspension were withdrawn, resuspended in PBS and afterwards used for fluorescence measurements. PerkinElmer model LS-55 fluorescence spectrophotometer (Beaconsfield, UK) was used for the fluorescence detection. Scan range parameters are as follows:

i Excitation wavelength – 390 nm
ii Emission – 590–750 nm
iii Excitation slit – 2·5 nm

iv Emission slit – 15 nm
v Scan speed (nm min^{-1}) – 200.

Evaluation of endogenous porphyrins produced by cells was performed according to the 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).

Photoinactivation of *Bacillus cereus* spores *in vitro*

For preparation of inocula of *B. cereus* ATCC 12826 spores, culture was grown for 3 days at 37°C in brain heart infusion broth (Liofilchem) containing (per litre) 0·05 mg manganese until 80–90% sporulation was obtained. Sporulation intensity was determined by Wirtz–Conklin staining method (Schaeffer and MacDonald 1933). Spore suspension was then washed three times by suspending the spores in 5 ml of sterile distilled water, centrifuging at 6000 ***g*** for 20 min and removing the supernatant. After washing, the spores were resuspended in 1 ml of distilled water, transferred to a sterile, plastic test tube and heated to 80°C for *c.* 15 min to inactivate any remaining vegetative cells. Aliquots (10 ml) of spore suspension (*c.* 1×10^8 CFU ml^{-1} in 100 mmol l^{-1} PBS buffer, pH 7·2) with appropriate concentrations of ALA (3 and 7·5 mmol l^{-1}) were incubated in the dark in a 50 ml plastic bottle for cell culture cultivation at 37°C for 30 min; initial spore count was established by spread plate method. After incubation, 150 μl aliquots of bacterial suspension were withdrawn, placed in sterile flat bottom wells and exposed to light for 15 min. Afterwards, the purity of spores was tested by looking for the absence of vegetative cells using the Wirtz–Conklin staining method.

Photoinactivation of bacteria after adhesion to the surface of packaging material

Packaging yellow trays were provided by LINPAC (West Yorkshire, UK). In order to simplify experiments, the packaging samples for photosensitization experiments were cut into 4 × 8 cm pieces and each soaked in 50 ml *B. cereus* ATCC 12826 suspension (*c.* 1×10^7 CFU ml^{-1}) for cell adhesion. Afterwards, the packaging samples were kept in laminar 30 min for further bacterial adhesion. Then, appropriate packaging samples were incubated in the dark with the 3 mmol l^{-1} concentration of ALA for different periods (5, 10 and 20 min). The control samples were incubated with PBS (7·2 pH).

After incubation with ALA, all packaging samples were dried in a laminar at room temperature for 20 min. Dried samples were placed in the treatment chamber and exposed to light for different time (5, 10, 15 and 20 min). The control sample was not illuminated.

Photoinactivation of *Bacillus cereus* spores after adhesion to the surface of packaging material

Bacillus cereus spore suspension was prepared as described previously. Packaging samples (2·5 × 4 cm) were placed in sterile plastic tubes, and spore solution (*c.* 1×10^8 CFU ml^{-1}) was added until all samples were completely submerged for 30 min at 37°C. Afterwards, coupons were withdrawn and kept in laminar 30 min for further spore adhesion. Then, samples were placed in plastic tubes containing 25 ml of ALA solution with the concentration of 3–7·5 mmol l^{-1} 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 laminar at room temperature for 20 min. Dried samples were placed in the treatment chamber and exposed to light for 15 min. The control samples were not illuminated.

Bacterial cell survival assay

The antibacterial effect of photosensitization on *B. cereus* was evaluated by the spread plate method. Thus, 100 μl of appropriate dilutions of bacterial test culture after photosensitization *in vitro*, using the spread plate method, was surface inoculated on the separate LBA plates. Afterwards, the bacteria were kept 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^{-1} in the untreated culture and N is the number of CFU ml^{-1} in the treated culture.

After treatment, each packaging sample was mixed with 30 ml of 100 mmol l^{-1} PBS buffer and homogenized with a BagMixer (model MiniMix 100 VP; Interscience, St Nom, France). Then, 100 μl of appropriate dilutions (0·9% NaCl) of suspension was placed on LBA plates. The colonies were counted after 24 h incubation at 37°C. The surviving cell populations were enumerated and expressed as $\log_{10}$ (CFU ml^{-1} and CFU cm^{-1}).

Statistical analysis

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. The data were analysed with Origin 7.5 software (OriginLab Corporation).

Results

Detection of endogenous porphyrins synthesized from ALA in *Bacillus cereus* cells

In fact, not every bacterium can synthesize endogenous porphyrins from ALA in the amounts necessary for photosensitization-based inactivation (Fotinos *et al.* 2008). Thus, in the first stages of this study, it was necessary to evaluate the potential of Gram-positive bacterium *B. cereus* to produce endogenous porphyrins from extrinsically applied ALA. According to Szocs *et al.* (1999), the spectral region with maximum at $\lambda = 612–615$ nm is attributed to the presence of endogenously synthesized porphyrins including uroporphyrins and coproporphyrins. For this purpose, fluorescence emission spectra in the region of 590–680 nm were analysed after incubation of cells with 3–7·5 mmol l^{-1} ALA in the dark. Figure 2(a) shows the excitation spectrum of endogenous porphyrins produced by *B. cereus* after incubation with 3 mmol l^{-1} ALA. Thus, the following fluorimetric analysis indicated that the intensity of fluorescence emission of endogenous porphyrins after incubation of cells with 3 mmol l^{-1} ALA for 0–60 min was increasing in a time-dependent manner (Fig. 2b). Fluorescence intensity of endogenous porphyrins was insignificant after 2 min of incubation with ALA but increased and reached more than 500 fluorescence intensity units after 30–60 min incubation. In order to understand whether a suitable concentration of ALA solution was used, we increased ALA concentration to 7·5 mmol l^{-1}. Analysis of the fluorescence emission spectra of endogenous photosensitizers indicated that there was some increase (up to 650 units) in fluorescence intensity of endogenous porphyrins when the same incubation time (60 min) was used (Fig. 2c).

Inactivation of *Bacillus cereus* by ALA-based photosensitization

Analysis of fluorescence emission spectra of endogenous porphyrins indicates that this Gram-positive bacterium can effectively synthesize endogenous photosensitizers from ALA. It means that the main premise for the possibility to inactivate the troublesome food pathogen by ALA-based photosensitization was identified. Thus, the obtained results have prompted us to evaluate the applicability of ALA-based photosensitization for the inactivation of *B. cereus*. The data obtained revealed that toxicity of ALA alone (3–7·5 mmol l^{-1}), without illumination, on viability of *B. cereus* was, as usual, negligible. The following illumination of bacterial cells (0–20 min) decreased the survival fraction fairly sharply (Fig. 3a,b). It is important to note that a very short illumination time of

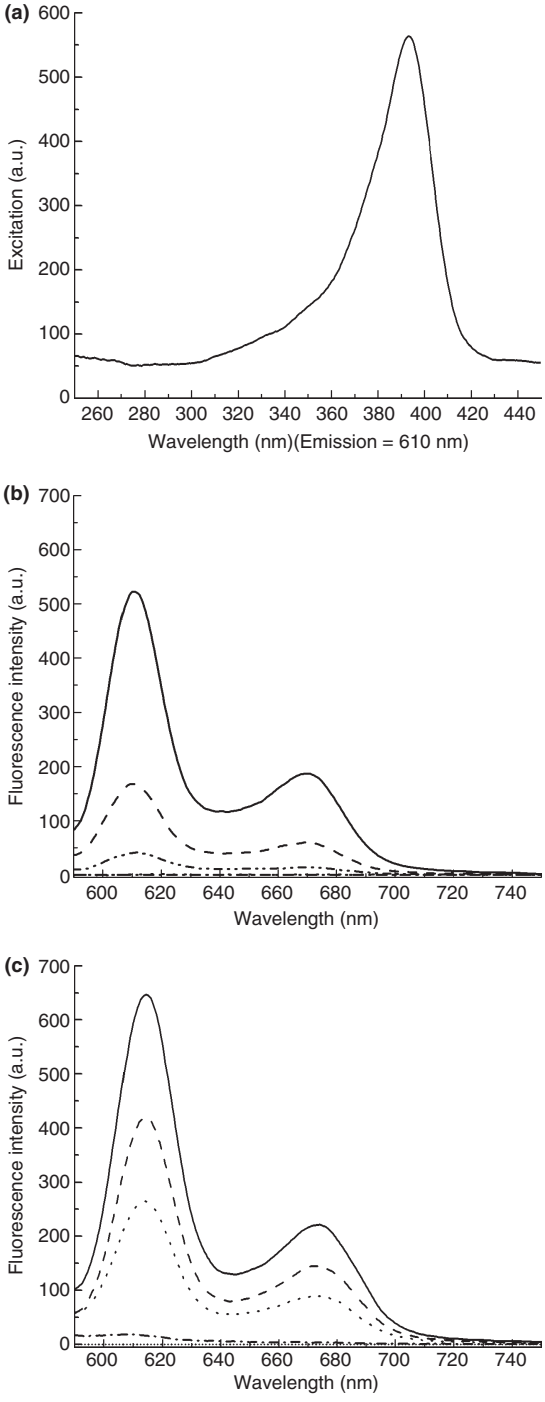

Figure 2 Excitation spectrum of endogenous porphyrins produced by *Bacillus cereus* after incubation with 3 mmol l^{-1} 5-aminolevulinic acid (ALA) (a); fluorescence spectra of endogenous porphyrins produced by *B. cereus* after incubation with 3 mmol l^{-1} ALA for different time intervals (b); fluorescence spectra of endogenous porphyrins produced by *B. cereus* after incubation with 7·5 mmol l^{-1} ALA for different time intervals (c).

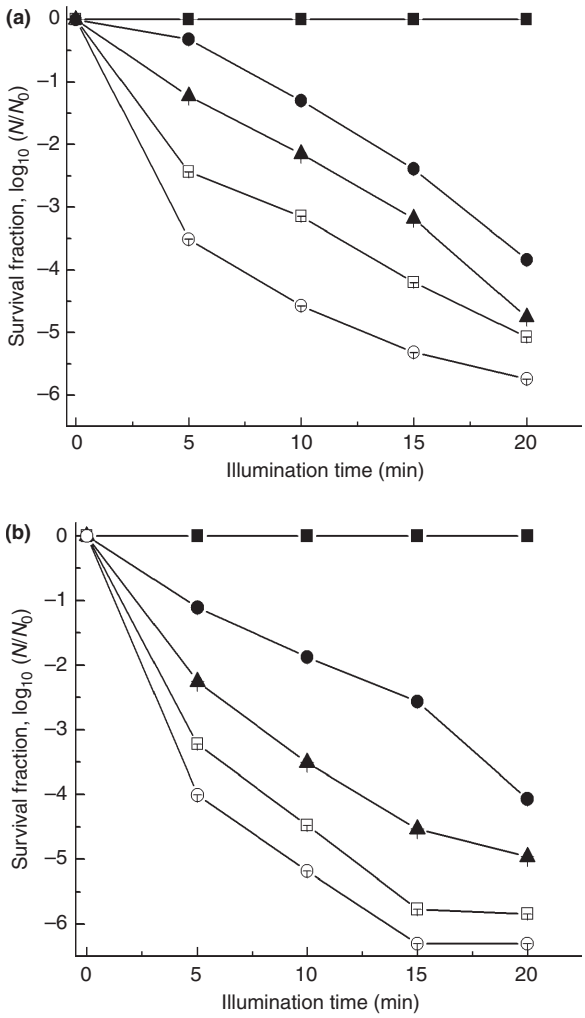

Figure 3 Inactivation of *Bacillus cereus* by 3–7·5 mmol l^{-1} 5-aminolevulinic acid (ALA)-based photosensitization as function of illumination time (a, b); bacteria were incubated with ALA solution for different periods (2–60 min): (■) control; (●) 2 min; (▲) 15 min; (□) 30 min and (○) 60 min. Every point is the average of 3–6 experiments, and error bars sometimes are too small to be more visible.

0–5 min was not effective, as not all endogenous porphyrins were excited by light to S_1 to produce cytotoxic reactions because of the lack of photons delivered during short time.

From the data presented in Fig. 3a, it is clear that the prolongation of period of incubation (from 2 to 60 min) with 3 mmol l^{-1} ALA increased the rate of bacterial inactivation. The use of 60 min incubation time and 20 min illumination time resulted in 5·75-log inactivation of *B. cereus*. As a matter of fact, photosensitization efficiency can be expressed as a function of accumulated photosensitizer multiplied on the total light energy delivered to the bacterial cells. Taking this into account, we investigated the inactivation of *B. cereus* by ALA-based photosensitization as a function of time, using higher ALA concentration (7·5 mmol l^{-1}). Data presented in Fig. 3b reveal that higher ALA concentration (7·5 mmol l^{-1}) can enhance the inactivation of *B. cereus*. This inactivation of bacteria strongly depends on the period of incubation with ALA as well. A short incubation time (2 min) is not enough to produce endogenous porphyrins from ALA, whereas 15–20 min incubation time is much more suitable for the development of perspective antibacterial technology.

Inactivation of *Bacillus cereus* spores by ALA-based photosensitization

In the next step, sensitivity of *B. cereus* spores to ALA-based photosensitization was tested. According to Demidova and Hamblin (2005), shorter incubation time and lower temperature are less effective, suggesting that photosensitizer that penetrates into the spores by passive diffusion process data, presented in Fig. 4, indicated that the incubation of spores with ALA (30 min) did not induce significant changes in their number. Following illumination (15 min) of spores can drastically reduce their population from 8 log in the control to 4·3 log in the treated samples. The inactivation rate of spores, as usually, strongly depended on mentioned ALA concentration. For instance, the use of 3 mmol l^{-1} ALA concentration at given experimental conditions can inactivate spores about 2 log, whereas 7·5 mmol l^{-1} ALA concentration inactivates spores by 3·7 log.

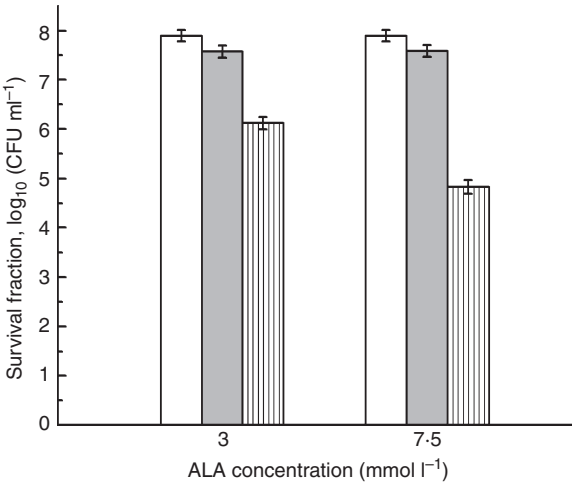

Figure 4 Inactivation of *Bacillus cereus* spores *in vitro* by 5-aminolevulinic acid (ALA)-based photosensitization: (□) control; (■) ALA without light and (▥) ALA + 15 min light.

Decontamination of food-related packaging from *Bacillus cereus* by ALA-based photosensitization

In order to evaluate antibacterial efficiency of ALA-based photosensitization for decontamination of surface, *B. cereus* was attached to food packaging material (yellow trays) provided by LINPAC. Packaging samples (10 × 10 cm) after soaking in ALA solution (3 mmol l^{-1}) for 10 min were illuminated 5–20 min with 405 nm, 20 mW cm^{-2} light. Afterwards, treatment-survived bacteria were counted. Data, depicted in Fig. 5a, describe the ALA-based photoinactivation of *B. cereus* as a function of illumination time. It is clear that 20 min illumination is more than enough to inactivate *B. cereus* by 4 log, when the period of incubation with ALA is 10 min. Results, presented in Fig. 5b indicate, that antibacterial efficiency of ALA-based photosensitization is a function of the period of incubation with ALA as well. When the period of incubation with ALA is short (5 min), the production of endogenous photosensitizers in bacteria is insufficient, and consequently inactivation of *Bacillus* reached just 3·3 log. Further prolongation of the period of incubation with ALA up to 10 or 20 min increases the antibacterial efficiency up to 4 log. Results, depicted in Fig. 5a,b, indicated that selected experimental parameters (period of incubation with ALA, illumination time) were optimal to decontaminate packaging materials from *B. cereus* at certain experimental conditions.

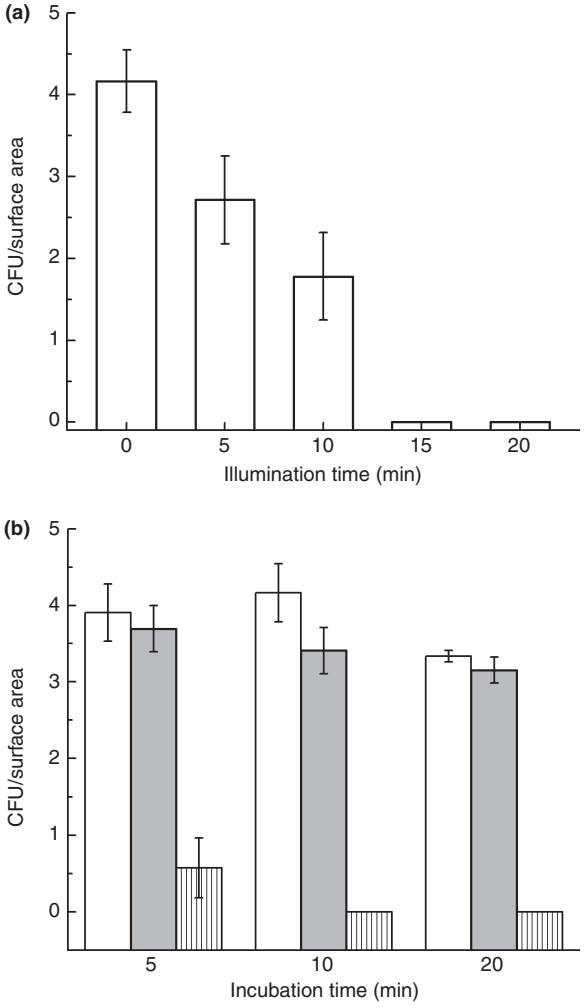

Figure 5 (a, b) Inactivation of *Bacillus cereus* ATCC 12826 attached on packaging samples by 3 mmol l^{-1} 5-aminolevulinic acid (ALA)-based photosensitization as function of illumination time. Incubation time with ALA was 10 min. (a) Inactivation of *Bacillus cereus* by 3 mmol l^{-1} ALA-based photosensitization onto packaging samples as function of incubation time. (b) Illumination time was 20 min: (□) control; (▩) 3 mmol l^{-1} ALA without light and (▥) 3 mmol l^{-1} ALA + 20 min light.

Decontamination of food-related packaging from *Bacillus cereus* spores by ALA-based photosensitization

In order to estimate the decontamination efficiency of ALA-based photosensitization, food packaging material was submerged in *B. cereus* spore solution. Different concentrations of ALA solution (3 and 7·5 mmol l^{-1}) were exploited for experiments. Illumination parameters were as follows: λ = 405 nm, 20 mW cm^{-2} and 15 min. Data, shown in Fig. 6, indicate that *B. cereus* spores are able to attached to plastic food-related packaging material and can be inactivated by ALA-based photosensitization as well as *in vitro* experiments. ALA itself only slightly

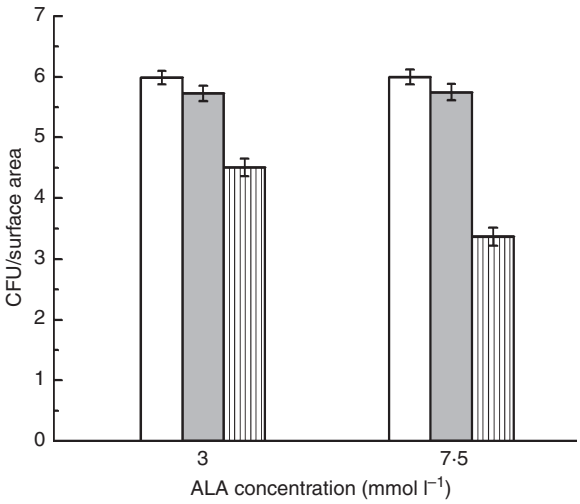

Figure 6 Decontamination of food-related packaging from *Bacillus cereus* spores: (□) control; (▩) 5-aminolevulinic acid (ALA) without light and (▥) ALA + 15 min light.

influences spores inactivation. After 30 min incubation, only 0·3-log decrease is received, whereas 15 min light exposure ensures 1·5- and 2·7-log reductions.

Discussion

Antimicrobial photosensitization-based technique has been developed especially during the last 25 years (Jori 2006). The main prerequisite for the rise was the growing concerns about the emergence of antibiotic-resistant bacterial strains. It is worth emphasizing that the phototoxic action on pathogens is caused by the combined action of two factors: photosensitizer and visible light that separately are devoid of any detectable toxicity at used doses (Jori 2006).

One of the most important advantages of photosensitization in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment (Nitzan and Ashkenazi 2001), because this process is free radical mediated (Polio *et al.* 2002). Moreover, because of the fact that a photoactive compound is localized inside micro-organisms, all cytotoxic reactions are just local. It allows us to achieve an extensive decrease in the population of pathogens with minimal damage effects on the surrounding matrix (Jori 2006). Eventually, it was confirmed that photosensitization, in contrast to ionizing radiation, has no mutagenic or carcinogenic effects on living systems (Luksiene 2005; Luksiene *et al.* 2006). ALA-based photosensitization was found to induce a more pronounced antibacterial effect on Gram-positive bacteria than on Gram-negative ones. Nitzan *et al.* (2004) reported that ALA and 50 J cm^{-2} light intensity resulted in five orders of decrease in *Staphylococcus* strains as opposed to 1–2 orders of decrease in Gram-negative strains.

The data obtained in this study clearly indicate that Gram-positive *B. cereus* can effectively produce endogenous photosensitizers from exogenously applied ALA even at 3 mmol l^{-1} concentration. It was shown in our previous studies that Gram-negative food pathogen *Salmonella enterica* as well as Gram-positive pathogen *L. monocytogenes* can produce endogenous porphyrins in amounts suitable for effective photosensitization just at 7·5 mmol l^{-1} ALA concentration. A comparative analysis of fluorescence intensity of endogenous photosensitizers reveals that *B. cereus*, during 60 min incubation with ALA, is producing endogenous porphyrins ten times more efficient than *L. monocytogenes* or *Salm. enterica* (Buchovec *et al.* in press). As a consequence, *B. cereus* is more susceptible to ALA-based photosensitization than *Salm. enterica* or *L. monocytogenes*. Really fast and significant inactivation (6·5 log) of this micro-organism can be achieved after ALA-based photosensitization, whereas most resistant biofilm forming food pathogen *L. monocytogenes* after 2 h incubation with ALA and 20 min illumination was inactivated by four orders of magnitude (Buchovec *et al.* in press).

Because of very high resistance of bacterial spores to UV (7–50 times more resistant than vegetative cells) (Nicholson *et al.* 2000), germicidal lamps are insufficient to ensure sterilization of surfaces, including packaging materials. More than 90% of packaging contamination is composed of aerobic, spore-forming bacteria (Pirttijarvi *et al.* 1996). In some cases, hydrogen peroxide solution (35% v/v) is sprayed onto the surface of packaging and subsequently removed by the stream of hot sterile air (Holdsworth 1992). Hence, there is a recognized risk of chemical food contamination by residues of hydrogen peroxide, relatively high concentration of this compound is employed.

Decontamination of packaging material from *B. cereus* adhered to the surface by this treatment seems really promising. More than 4-log inactivation was achieved after ALA-based photosensitization. Moreover, obtained data indicated that the *Bacillus* spores are susceptible to this treatment as well. Even 3·1-log reduction in spore population was observed after ALA-based photosensitization *in vitro* and 2·7 log on the surface of packaging material.

Decontaminating food or food-related surfaces by photosensitization is novel and pioneering approach. Undoubtedly, the main premise in this case is the used photosensitizer, which besides its suitable photophysical and photochemical properties must be a pure and water soluble compound with a stable shelf-life, not bleaching and easy to produce. Moreover, it must be food constituent or food additive. When colourless and odourless ALA is used, just living micro-organisms on the matrix surface can metabolize it to endogenous porphyrins that act as photosensitizers. Thus, this appears to be a way of gaining selectivity. After illumination of such surface with bacteria, reactive oxygen species can be selectively generated within the microbes without oxidative damage to food constituents and unwanted colouration. Moreover, other question 'how does ALA interact with food matrix' arises. Our previous experiments on the decontamination of wheat sprouts by ALA-based photosensitization indicate that ALA stimulates the growth of wheat seedlings and roots without impairing the vigor of germination and the viability of seeds. Moreover, ALA increases the rate of photosynthesis and the activities of antioxidant enzymes, what can be associated with enhanced cellular capacity to detoxify reactive oxygen species (Luksiene *et al.* 2007). Moreover, ALA is an essential precursor of such tetrapyrole compounds like vitamin B12 and hemes, which serve as prosthetic groups of respiratory enzymes and

chlorophyll in plants (Granick 1961). Suitable ALA concentrations have promotive effects on the growth rates. Crop yields were enhanced by the application of ALA at the leaf-stage for rice, barley, potato and garlic (Tanaka *et al.* 1992). Foliar application of ALA (100 mg ml^{-1}) on date palm has promoting effects on the fruit weight, volume and sugar content (Hotta *et al.* 2002).

Conclusions

The data obtained in this work clearly indicated that *B. cereus* can effectively produce endogenous photosensitizers from exogenously applied ALA even at very low concentrations (3 mmol l^{-1}). Really significant inactivation (6·3 log) of this micro-organism can be achieved after ALA-based photosensitization *in vitro*. The decontamination of packaging material from adhered *B. cereus* after ALA-based photosensitization reached 4 log. Of importance to note, that spores of *B. cereus* are susceptible to this treatment and can be inactivated by 3·1 log *in vitro* or 2·7 log on the surface of packaging material.

In conclusion, our previous data, as well as the one obtained in this study, support the idea that photosensitization is not 'magic bullet' against all pathogens, but its high selectivity, antimicrobial efficiency and nonthermal nature, may be, will serve in the future for the development of completely safe, nonthermal surface decontamination and food preservation technique.

Acknowledgement

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ORIGINAL ARTICLE

Inactivation of *Bacillus cereus* by Na-chlorophyllin-based photosensitization on the surface of packaging

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Keywords
Bacillus cereus, decontamination technology, Na-chlorophyllin, photosensitization, surface.

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Abstract

Aims: This study was focused on the possibility to inactivate food-borne pathogen *Bacillus cereus* by Na-chlorophyllin (Na-Chl)-based photosensitization *in vitro* and after attachment to the surface of packaging material.

Methods and Results: *Bacillus cereus in vitro* or attached to the packaging was incubated with Na-Chl ($7{\cdot}5 \times 10^{-8}$ to $7{\cdot}5 \times 10^{-5}$ mol l^{-1}) for 2–60 min in phosphate buffer saline. Photosensitization was performed by illuminating cells under a light with a λ of 400 nm and an energy density of 20 mW cm^{-2}. The illumination time varied 0–5 min and subsequently the total energy dose was 0–6 J cm^{-2}. The results show that *B. cereus* vegetative cells *in vitro* or attached to the surface of packaging after incubation with $7{\cdot}5 \times 10^{-7}$ mol l^{-1} Na-Chl and following illumination were inactivated by 7 log. The photoinactivation of *B. cereus* spores *in vitro* by 4 log required higher ($7{\cdot}5 \times 10^{-6}$ mol l^{-1}) Na-Chl concentration. Decontamination of packaging material from attached spores by photosensitization reached 5 log at $7{\cdot}5 \times 10^{-5}$ mol l^{-1} Na-Chl concentration. Comparative analysis of different packaging decontamination treatments indicates that washing with water can diminish pathogen population on the surface by <1 log, 100 ppm Na-hypochlorite reduces the pathogens about 1·7 log and 200 ppm Na-hypochlorite by 2·2 log. Meanwhile, Na-Chl-based photosensitization reduces bacteria on the surface by 4·2 orders of magnitude.

Conclusions: Food-borne pathogen *B. cereus* could be effectively inactivated (7 log) by Na-Chl-based photosensitization *in vitro* and on the surface of packaging material. Spores are more resistant than vegetative cells to photosensitization-based inactivation. Comparison of different surface decontamination treatments indicates that Na-Chl-based photosensitization is much more effective antibacterial tool than washing with water or 200 ppm Na-hypochlorite.

Significance and Impact of the Study: Our data support the idea that Na-Chl-based photosensitization has great potential for future application as an environment-friendly, nonthermal surface decontamination technique.

Introduction

Food-borne diseases are a common, distressing and life-threatening problem around the world (Mead *et al.* 1999). The US Center for Disease Control and Prevention (CDC) estimates that 76 million people suffer from food-borne illnesses every year. Gram-positive spore-forming *B. cereus* species have been identified as the cause of 27 000 cases of food-borne illnesses in the United States (Mead *et al.* 1999). There are about 48 known species in the genus *Bacillus*, but only *B. cereus* and *Bacillus anthracis* are associated with human diseases. *Bacillus cereus* causes food-borne illnesses with characteristic nausea, vomiting and diarrhoea (Altekruse *et al.* 1997). It can

contaminate a wide range of foods including cereals, fresh vegetables, berries and fruits. Additionally, this pathogen has been found in ready-to-eat foods and sauces (Rosenquist *et al.* 2005). The food poisoning is a result of ingesting heat-stable toxin (cereulide) or consumption of food contaminated by spores of *B. cereus*, which cause illness by growing and producing toxin in the gut (Gill and Hamer 2001).

Food-borne pathogens are generally eliminated by conventional treatments during food processing. However, during postprocessing, contamination can occur via contaminated working and packaging surfaces, equipment and environment. It has been determined that contamination with *B. cereus* and its highly resistant spores can occur via the food packaging (Pirttijarvi *et al.* 1996; Nicholson *et al.* 2000).

In general, the current methods of surface decontamination include chemical washing, heat treatment and irradiation. Each of these methods has proven to be effective for certain situations, but each has specific disadvantages. Chemical washing is environmentally unsound and has associated occupational and operational hazards. Heat treatment in autoclaves is not practical for large objects and harmed by heat equipment. Ionizing irradiation equipment is rather expensive, requires more space and highly qualified personal. Ultraviolet light (UV) has been used for decontamination of the food-related surfaces. However, high microbial resistance to this treatment, the long exposure time and enormous light intensities required and the degradation of exposed materials including food itself limit the wide application of this technique (Guerrero-Beltran and Barbosa-Canovas 2004). Thus, for reasons of cost, portability, environmental impact or safety the existing methods have limited practicality and attractiveness (Luksiene and Zukauskas 2009).

Considerable efforts are underway to find effective decontamination treatments. A new promising approach to decontaminate surfaces from micro-organisms is photosensitization (Luksiene and Zukauskas 2009). This treatment is based on the administration of a photosensitizer, which is preferentially accumulated in the microbial cells. Subsequent illumination with visible light, in the presence of oxygen (not obligatory), lethally injures the cell (Demidova and Hamblin 2005). It is accepted that the main cytotoxic agent produced after absorption of light by photosensitizer is singlet oxygen ($^1O_2$). As a result, plenty of cytotoxic reactions which are triggered in the cell disrupt cell membrane, inactivate enzymes and damage DNA (Luksiene 2005; Jori 2006). One of the most important advantages of photosensitization in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment (Nitzan and Ashkenazi 2001), as the process is free radical-mediated (Polio *et al.* 2002). Moreover, cytotoxic action is local, as photosensitizer accumulates inside micro-organisms and its diffusion distance is <20 nm (Moan 1990). It allows us to achieve an extensive decrease in the population of pathogens with minimal damage to the surrounding matrix or thermal effects (Jori 2006; Fotinos *et al.* 2008).

Chlorophylls, bacteriochlorophylls and their derivatives are widely known photosensitizers in photodynamic therapy. Unlike natural chlorophyll, chlorophyllin is water-soluble food additive (E141) and is used as food colourant, in dietary supplements and in cosmetics (Lopez-Carballo *et al.* 2008). Although different classes of porphyrins have been tested against Gram-positive and Gram-negative bacteria, chlorophyllin-based photosensitization and following inactivation of food pathogens have been never investigated.

This paper is focused on the possibility to inactivate *B. cereus* by chlorophyllin-based photosensitization *in vitro* and on the surface of packaging material polyolefine.

Materials and methods

Chemicals

Stock solution of Na-chlorophyllin (Na-Chl) (Roth, Karlsruhe, Germany) was prepared by dissolving in 0·9% NaCl up to the concentration $7{\cdot}5 \times 10^{-5}$ to $7{\cdot}5 \times 10^{-8}$ mol l^{-1}. The absorption spectrum of this compound was measured by spectrophotometer (Heλios Gamma & Delta spectrophotometers; ThermoSpectronic, Cambridge, UK), whereas excitation and fluorescence spectra were obtained by a PerkinElmer model LS-55 fluorescence spectrophotometer (Beconsfield, UK). Scan range parameters are as follows: excitation wavelength – 395 nm; emission – 400–750 nm; ex Slit – 2·5 nm; em Slit – 2·5 nm; scan Speed (nm/min) – 200 (Luksiene *et al.* 2001).

Bacterial strain and culture conditions

Bacillus cereus ATCC 12826 was kindly provided by the National Centre of Public Health (Vilnius, Lithuania). Bacterial culture was grown at 37°C and all maintained on Luria–Bertani Agar (LBA; Liofilchem, Roseto degli Abruzzi, Italy).

The bacterial culture was grown overnight (*c.* 14 h) in 20 ml of Luria–Bertani medium (LB; Liofilchem), with agitation of 120 rev min^{-1} (Environmental Shaker – Incubator ES – 20; Biosan, Riga, Latvia). This culture was then diluted 20 times in the fresh LB medium. The initial optical density (OD) of bacterial culture was 0·164 ± 0·005 at $\lambda = 540$ nm (Heλios Gamma & Delta spectrophotometers; ThermoSpectronic). Bacteria were grown to the late mid-log phase [*c.* 6×10^7 colony

forming units (CFU) per ml, $OD_{540} = 1$ in a shaker (120 rev min^{-1})]. Afterwards the bacteria were harvested by centrifugation (10 min, 5000 ***g***; PC-6, Moscow, Russia) and resuspended to *c.* $3{\cdot}015 \times 10^8$ CFU ml^{-1} final concentration in 0·1 mol l^{-1} phosphate buffer saline (PBS; pH 7·2). These stock suspensions were then diluted in PBS to *c.* 1×10^7 CFU ml^{-1} and immediately used for the photosensitization experiments.

Preparation of *Bacillus cereus* spores

For preparation of inocula of *B. cereus* ATCC 12826 spores, culture was grown for 3 days at 37°C in brain heart infusion broth (Liofilchem) containing (per litre) 0·05 mg manganese until 80–90% sporulation was obtained (sporulation strength was determinate by Wirtz–Conklin staining method (Murray *et al.* 1999)). Spore suspension was then washed three times by suspending the spores in 5 ml of sterile distilled water, centrifuging at 6000 ***g*** for 20 min, and removing the supernatant. After washing, the spores were resuspended in 1 ml of distilled water, transferred to a sterile, plastic test tube, and heated to 80°C for *c.* 15 min to inactivate remaining vegetative cells. We expect the same levels of inactivation with non-heat-treated spores.

Photosensitization *in vitro*

A volume of 10 ml solution of bacterial vegetative cells or spores suspension with appropriate concentration of Na-Chl ($7{\cdot}5 \times 10^{-5}$ to $7{\cdot}5 \times 10^{-8}$ mol l^{-1}) was incubated in the dark in 50-ml plastic tubes for 2 min at 37°C for the rapid accumulation of photosensitizer. After incubation, 150-μl aliquots of bacterial suspension was withdrawn, placed into sterile flat-bottom flasks 96-well microtitre plates and exposed to light for different time duration (0–5 min) (Nitzan *et al.* 2004). A light-emitting diode-based light source constructed in the Institute of Applied Sciences of Vilnius university emitted light $\lambda = 405$ nm with intensity 20 mW cm^{-2} at the surface of samples. Light dose was calculated as light intensity multiplied by time. Light power density measurements were performed with a light energy measure by 3 Sigma meter (Coherent, Santa Clara, CA, USA) equipped with piro-electrical detector J25LP04. Precision Celsius temperature sensors (Deltha Ohm, Padua, 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 ±1/4°C at room temperature. No thermal effects were detected under experimental conditions investigated.

Photoinactivation of bacteria and spores after adhesion to the surface of packaging material

Yellow packing trays (polyolefine-mixture of polyethylene and polypropylene, PP) were provided by LINPAC (West Yorks, UK). To simplify the experiments, the packaging samples for photosensitization experiments were cut into 4 cm × 8 cm pieces, cleaned well with 96% ethyl alcohol and soaked in 50 ml *B. cereus* ATCC 12826 vegetative cell suspensions for further adhesion (30 min). Afterwards, the packaging samples were kept in sterile box for 30 min for further adhesion. Appropriate packing samples were then incubated in the dark with the $7{\cdot}5 \times 10^{-7}$ mol l^{-1} concentration of photosensitizer for 2 min. The control samples were incubated with 0·1 mol l^{-1} PBS (7·2 pH) buffer. After incubation with Na-Chl solution, all packaging samples were dried in a sterile box at room temperature for 20 min. Dry samples were placed in the treatment chamber and exposed to light for 2 min. The control sample was not illuminated.

For inactivation of spores, the packaging samples were placed in sterile plastic tubes, and spores (*c.* 1×10^8 CFU ml^{-1}) were added until all samples were completely immersed for 30 min at 37°C. Afterwards, samples were withdrawn and kept in sterile box for 30 min (experimentally estimated as optimal) to allow further spore adhesion to take place. Then samples were then placed in plastic tubes containing 25 ml of $7{\cdot}5 \times 10^{-5}$ to $7{\cdot}5 \times 10^{-6}$ mol l^{-1} Na-Chl solutions and incubated in the dark for 5 min. The control samples were incubated with sterile 0·1 mol l^{-1} PBS. After incubation, all packaging samples were dried in a sterile box at room temperature for 20 min. Dry samples were placed in the treatment chamber and exposed to light for 5 min. The control samples were not illuminated.

Bacterial cell survival assay

The antibacterial effect of photosensitization onto packaging material was evaluated by the spread plate method. Each packing sample was mixed with 30 ml of 0·1 mol l^{-1} PBS buffer and homogenized in a BagMixer (model MiniMix 100 VP; Interscience, St. Nom, France). Hundred microlitres of appropriate dilutions (in 0·9% NaCl) of suspension was then spread plated on LBA plates. Colonies were counted after 24-h incubation at 37°C. The surviving cell populations were enumerated and expressed as $\log_{10}$ (CFU cm^{-2}).

Statistical analysis

The experiments were carried out in triplicate for each set of exposure. A standard error was estimated for every

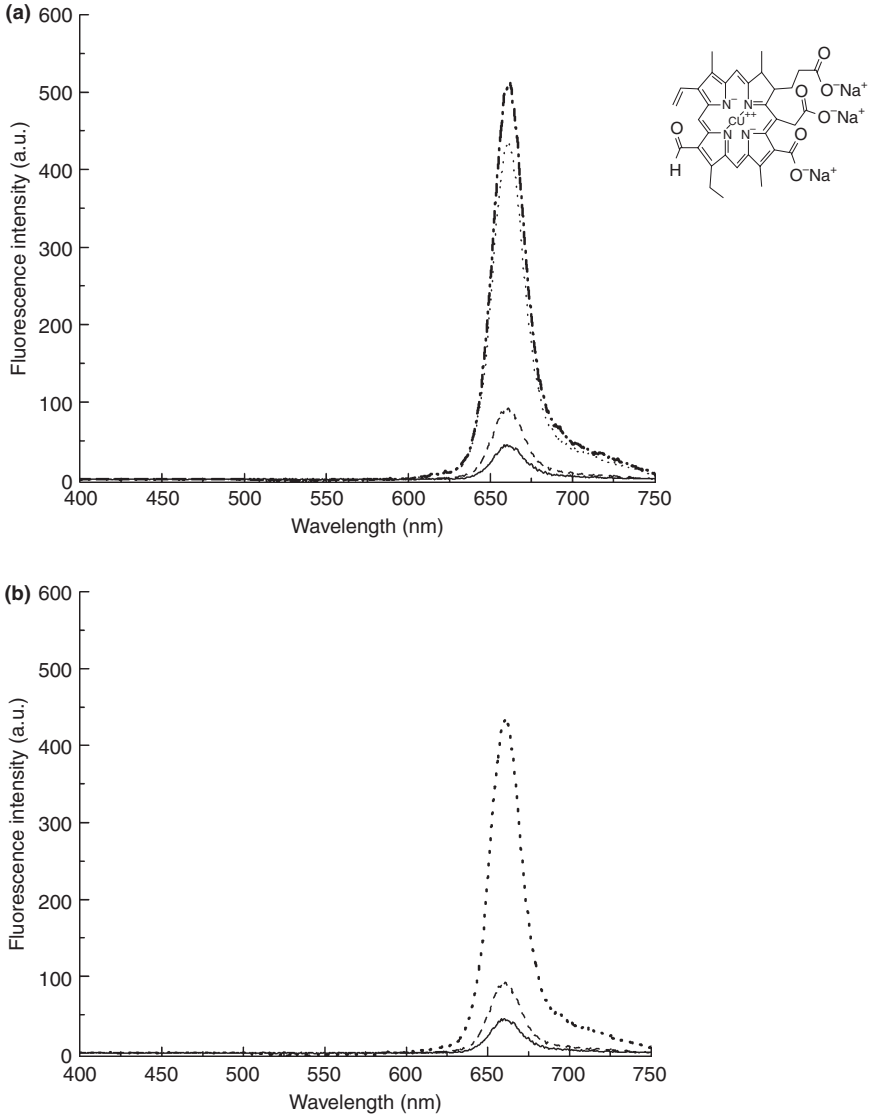

Figure 1 (a, b) Na-chlorophyllin chemical structure, absorption (a) and fluorescence (b) spectra: excitation – 395 nm [(——)$7{\cdot}5 \times 10^{-8}$ mol l^{-1}; (- - -) $7{\cdot}5 \times 10^{-7}$ mol l^{-1} and (••••) $7{\cdot}5 \times 10^{-6}$ mol l^{-1}].

experimental point and marked in a figure as an error bar. Sometimes the bars were too small to be visible. The data were analysed with ORIGIN 7.5 software (OriginLab Corporation, Northampton, MA 01060, USA).

Comparative analysis of different antibacterial treatments

To compare the efficacy of different decontamination techniques, the packaging samples with inoculated *B. cereus* ATCC 12826 were treated with sterile water and 100–200 ppm Na-hypochlorite. Packaging samples (4 × 8 cm) were completely submerged in containers with appropriate sanitizers and left for 1 min (standard treatment). Afterwards, decontamination efficiency of water and hypochlorite were compared with that of Na-Chl-based photosensitization.

Results

Absorption and fluorescence spectra of Na-Chl

Data presented in Fig. 1a indicate that Na-Chl at the concentrations $7{\cdot}5 \times 10^{-6}$ to $7{\cdot}5 \times 10^{-8}$ mol l^{-1} has absorption maximum at $\lambda = 405$ nm. In addition, Na-Chl is

highly fluorescing compound, and its fluorescence maximum is about 660 nm (Fig. 1b). The absorption and fluorescence spectra of Na-Chl indicate that in the concentration range $7{\cdot}5 \times 10^{-6}$ to 10^{-8} mol l^{-1}, this compound is mostly monomeric, not aggregated and can be used for the photoinactivation of bacteria. After incubation of *B. cereus* cell suspension with different concentrations of Na-Chl solution ($7{\cdot}5 \times 10^{-5}$ to 10^{-7} mol l^{-1}), no changes in fluorescence parameters (Fig. 2) of cell-bound Na-Chl were detected: fluorescence maximum and fluorescence intensities exhibit no difference in comparison with fluorescence spectra of the same concentrations of Na-Chl in PBS (Fig. 1b).

Inactivation of the *Bacillus cereus* cells by Na-Chl-based photosensitization *in vitro*

As no data exist on the photoinactivation of *B. cereus* by Na-Chl, we examined the cytotoxic effect of wide range of Na-Chl concentrations: from highest $1{\cdot}5 \times 10^{-5}$ mol l^{-1} to lowest $7{\cdot}5 \times 10^{-8}$ mol l^{-1}. For this purpose, two incubation times were chosen: 2 min and 60 min. Experimental data reveal clearly that light alone had negligible effects on viability of bacteria. The incubation of bacterial cells with different concentrations of Na-Chl ($1{\cdot}5 \times 10^{-5}$ to $7{\cdot}5 \times 10^{-8}$ mol l^{-1}) for 2–60 min without following illumination had no impact on their viability. Photosensitization drastically reduced it (data not shown). Obviously, that incubation of bacteria with the lowest $7{\cdot}5 \times 10^{-8}$ mol l^{-1} Na-Chl concentration for shortest 2-min incubation time is sufficiently effective as it results in 7 log inactivation of *B. cereus* cells. Clear dependence of inactivation efficiency on the illumination time (light dose) or used Na-Chl concentration was observed (Fig. 3).

Inactivation of *Bacillus cereus* spores by Na-Chl-based photosensitization *in vitro*

Data presented in Table 1 indicate that a 1-log reduction of *B. cereus* spores was achieved when spores were incubated with $7{\cdot}5 \times 10^{-7}$ mol l^{-1} Na-Chl and illuminated 5 min with a light of $\lambda = 405$ nm. Taking it into account, a higher Na-Chl concentration (of $7{\cdot}5 \times 10^{-6}$ mol l^{-1}) was evaluated. The data obtained indicate that the spore population decreased by 4 log at this concentration.

Decontamination of food-related packaging from *Bacillus cereus* by Na-Chl-based photosensitization: comparison of the efficiencies of different packaging decontamination techniques

Data presented in Fig. 4 allow us to compare efficiencies of different surface decontamination techniques. Photosensitization was compared with conventional surface treatments such as washing with sterile water or Na-hypochlorite. For this purpose, *B. cereus* was attached to the surface of a packaging material. Samples of the packaging material with *B. cereus* attached to it were treated by photosensitization, sterile water or Na-hypochlorite. It was determined that washing with water could only

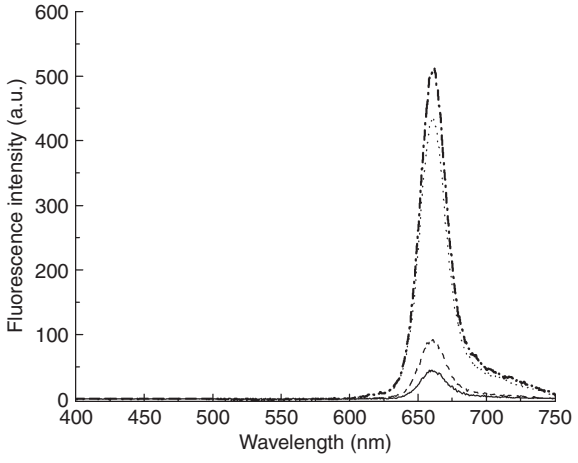

Figure 2 Na-chlorophyllin fluorescence spectrum (PerkinElmer LS55 fluorescence spectrophotometer). Bacterial cells – *Bacillus cereus* ATCC 12826: incubation time with photosensitizer 2 min., Ex – 395 nm [(——) $7{\cdot}5 \times 10^{-8}$ mol l^{-1}; (- - -) $7{\cdot}5 \times 10^{-7}$ mol l^{-1}; (•••••) $7{\cdot}5 \times 10^{-6}$ mol l^{-1} and (-•-•-) $7{\cdot}5 \times 10^{-5}$ mol l^{-1}].

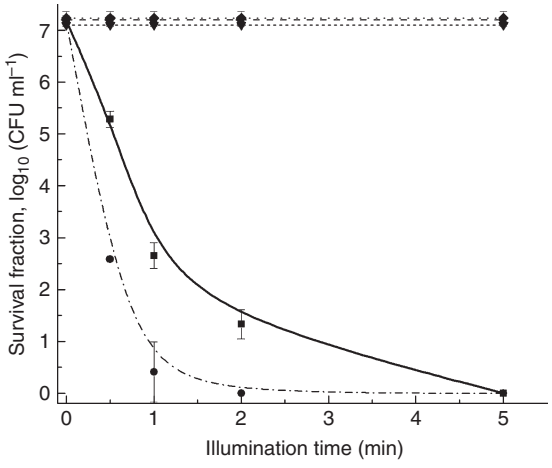

Figure 3 Photoinactivation of *Bacillus cereus* ATCC 12826 when Na-chlorophyllin was used as photosensitizer ($7{\cdot}5 \times 10^{-7}$ and $7{\cdot}5 \times 10^{-8}$ mol l^{-1}) *in vitro*. (-◆-) Control; (—▼—) $7{\cdot}5 \times 10^{-7}$ mol l^{-1} Na-Chl without light; (-▲-) $7{\cdot}5 \times 10^{-8}$ mol l^{-1} Na-Chl without light; (-•-•-) $7{\cdot}5 \times 10^{-7}$ mol l^{-1} Na-Chl with light; (—■—) $7{\cdot}5 \times 10^{-8}$ mol l^{-1} Na-Chl with light, 2-min incubation).

Table 1 Inactivation of *Bacillus cereus* ATCC 12826 spores by different Na-chlorophyllin (Na-Chl) concentration

Na-Chl concentration (mol l^{-1})	*In vitro** (Log_{10}, CFU ml^{-1})	Onto packaging material* (Log_{10}, CFU cm^{-2})
0 (control sample)	8 ± 0·03	6 ± 0·07
$7·5 \times 10^{-7}$ (without light)	8 ± 0·02	NT
$7·5 \times 10^{-7}$ (with light, 5 min)	7 ± 0·04	NT
$7·5 \times 10^{-6}$ (without light)	8 ± 0·03	6 ± 0·03
$7·5 \times 10^{-6}$ (with light, 5 min)	4 ± 0·05	4 ± 0·07
$7·5 \times 10^{-5}$ (without light)	NT	6 ± 0·03
$7·5 \times 10^{-5}$ (with light, 5 min)	NT	1 ± 0·04

NT, not tested.
*Value ± standard error.

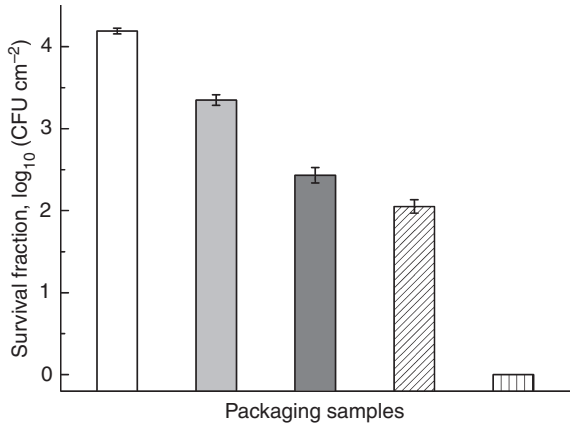

Figure 4 Comparative analysis of different antimicrobial tools: efficiencies of inactivation of *Bacillus cereus* ATCC 12826 attached on the surface of packaging samples: (▭) control; (▬) washing with water; (▬) 100 ppm Na-hypochloride, 1-min treatment; (▨) 200 ppm Na-hypochloride, 1-min treatment; (▥) $7·5 \times 10^{-7}$ mol l^{-1} Na-chlorophyllin with light, 2 min.

diminish the surface *B. cereus* population on the packaging samples by <1 log, whereas washing with 100 and 200 ppm Na-hypochlorite reduces the surface population by 1·7 and 2·2 log, respectively. Meanwhile, Na-Chl-based photosensitization was most effective as it significantly ($P < 0·05$) reduced the *Bacillus* population by 4·2 log.

Decontamination of food-related packaging from *Bacillus cereus* spores by Na-Chl-based photosensitization

As described in Table 1, the $7·5 \times 10^{-6}$ mol l^{-1} Na-Chl-based photosensitization can reduce the population of *B. cereus* spores attached to the surface of polyolefine by 2 log. An increase in Na-Chl concentration to $7·5 \times 10^{-5}$ mol l^{-1} reduced the spore population by 5 log. This large degree of inactivation of spores on the surface of packaging material indicates that photosensitization has great potential for use as surface decontamination technique.

Discussion

One of the most attractive feature of photosensitization as antimicrobial tool is the absence of any bacterial resistance to this treatment (Nitzan and Ashkenazi 2001; Buchovec *et al.* 2010 in press). Moreover, photosensitization has high selectivity because target is bacterial cell, not surrounding matrix. To date, neither mutagenic nor carcinogenic effects of photosensitization on living systems were observed (Luksiene 2005).

Much work has been carried out on the photoinactivation of food pathogens using endogenous photosensitizers produced by micro-organisms metabolizing colourless and odourless aminolevulinic acid (ALA) to endogenous porphyrins (Buchovec *et al.* 2009, 2010; Le Marc *et al.* 2009; Luksiene *et al.* 2009). Effective decontamination of packaging by ALA-based photosensitization from *B. cereus*, *Salmonella* Typhimurium, *Listeria monocytogenes*, accordingly spores and biofilms indicate that this treatment is promising (Luksiene and Zukauskas 2009). Our previous experiments on the decontamination of wheat sprouts from microfungi by ALA-based photosensitization revealed that besides significant antimicrobial properties of this treatment, ALA could stimulate the growth of wheat seedlings and roots, increase photosynthesis rate and activity of antioxidant enzymes without impairing the vigour of germination and the viability of seeds (Luksiene *et al.* 2007).

Meanwhile, ALA is comparatively expensive compound for use in food industry. Moreover, synthesis of endogenous porphyrins from ALA in the bacteria requires additional time (20–60 min) that increases the duration and the costs of technology (Luksiene and Zukauskas 2009).

An optimal photosensitizer for decontamination of food or food-related surfaces should be endowed with specific features (in addition to the expected photophysical characteristics, i.e. generation of singlet oxygen) such as a large affinity for microbial cells, a broad spectrum of activity and ability to damage bacteria without the development of mutagenic processes or resistant strains.

Data obtained in this study reveal that Na-Chl being water soluble, highly fluorescing food additive exhibits excellent photosensitizing properties. Theoretically, in Gram-positive bacteria the outer wall (15–80 nm thick) contains up to 100 peptidoglycan layers, which are

associated with lipoteichoic and negatively charged teichuronic acids. This wall is permeable to most photosensitizers whose molecular weight does not exceed 1500–1800 Da (Nikaido 1994). Thus, after accumulation of Na-Chl by cell and following photosensitization, *B. cereus* was diminished by 7 log using very low photosensitizer concentration ($7{\cdot}5 \times 10^{-8}$ mol l^{-1}) *in vitro*. A faster inactivation rate was observed when higher $7{\cdot}5 \times 10^{-7}$ mol l^{-1} Na-Chl concentration was used. In a nutshell, the efficiency of photoinactivation depends on the used photosensitizer concentration and illumination time. In this case, we found that variation of incubation time with Na-Chl for 2–60 min does not influence the photoinactivation efficiency when all other parameters were kept constant [Na-Chl concentration, illumination time (dose)]. These data are in line with previously published results (Jori and Brown 2004) indicating that the extension of the incubation time from 2 min to 1–2 h has no effect on the amount of photosensitizer bound to the microbial cell. Our preliminary data indicate that about 50% of Na-Chl is bound to the cells in solution. By no means, more detail quantitative evaluation of cell-bound photosensitizer would give additional information about kinetics of this process. The comparison of ALA- and Na-Chl-based photoinactivation of *B. cereus* indicates some advantages of later treatment. First, when we use Na-Chl as photosensitizer, we can shorten the incubation time from 20 to 2 min. Second, we can reduce the illumination time from 20 to 5 min. Third, Na-Chl is effective photosensitizing compound and 'works' at extremely low concentrations ($7{\cdot}5 \times 10^{-8}$ mol l^{-1}), whereas ALA 'working' concentration was much higher ($7{\cdot}5 \times 10^{-3}$ mol l^{-1}) (Luksiene *et al.* 2009). Spores require a higher concentration of Na-Chl than vegetative cells for the same level of inactivation to be attained. However, treatment with even a low concentration of $7{\cdot}5 \times 10^{-6}$ mol l^{-1} Na-Chl was able to give a 4-log reduction in spore counts. It is important to note that spores were susceptible to ALA-based photosensitization as well (Luksiene *et al.* 2009). As a role, the higher Na-Chl concentrations induce higher inactivation levels. This decontamination efficiency is comparable with that obtained by high-intensity pulsed light (Dunn *et al.* 1997). Finding is particularly important because spores are usually resistant to commonly employed antibacterial agents, hence photosensitization could represent an innovative approach in sterilization of packaging contaminated by bacterial spores.

Lopez-Carballo *et al.* (2008) studied the phototoxic effect of Na-Chl-incorporated gelatin-based edible films against *Staphylococcus aureus* and *L. monocytogenes*. Results revealed that photoactivated Na-Chl immobilized in gelatin film had a strong bactericidal effect on the viability of *Staph. aureus*: those counts decreased by 4 log after Na-Chl-based photosensitization. Similar results were obtained when stationary phase cultures of *L. monocytogenes*, *Escherichia coli* and *Salmonella* were used. Alone, neither Na-Chl nor visible light are toxic to micro-organisms. In contrary, Na-Chl exhibits antimutagenic and anticarcinogenic properties (Surh 1998). An appropriate combination of a suitable concentration of Na-Chl and light dose can induce bactericidal effects (Lopez-Carballo *et al.* 2008).

Ulatowska-Jarza *et al.* (2006) analysed the efficiency of $Chlorine_{e6}$ (Photolon)-based photosensitization on *E. coli* strains isolated from poultry and cows. They found that Photolon can reduce *E. coli* population by 1 log.

Kreitner *et al.* (2001) examinated the photosensitivity of Gram-positive bacteria and yeasts to hematoporphyrin and Na-Chl-based photosensitization. *Staph. aureus*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Rhodotorula mucilaginosa* were susceptible to treatment (3–5 log inactivation) after incubation with 10^{-5} mol l^{-1} photosensitizer and illumination for 1 h with light. In our work, we found the 'lowest working' Na-Chl concentration ($7{\cdot}5 \times 10^{-8}$ mol l^{-1}) and lowest incubation time (2 min) necessary to inactivate *B. cereus* by 7 log *in vitro*.

Considering the application of photosensitization to decontaminate food. Our previous work indicates that a plethora of mesophylic and pathogenic bacteria and micromycetes naturally distributed on food surface are susceptible to porphyrin-based photosensitization (Luksiene *et al.* 2005; Luksiene 2010). It appears to be an effective antimicrobial tool. Meanwhile, not every photosensitizer which can be used against infections is possible to use for food decontamination. First of all, every photosensitizer interacts with the food matrix and has its own colour, odour, etc. ALA solutions, however, have no colour and odour. Luksiene *et al.* (2007) determined that spraying onto food (e.g. wheat sprouts) did not change the foods organoleptic properties.

Conclusions

Food pathogen *B. cereus* can be effectively inactivated (7 log) by Na-Chl-based photosensitization *in vitro* and attached on the surface of packaging material polyolefine. *Bacillus* spores *in vitro* or attached to the surface were susceptible to this treatment as well but required higher Na-Chl concentration. Comparison of different surface decontamination treatments reveal that Na-Chl-based photosensitization is much more effective against *B. cereus* attached on the surface than washing with water or up to 200 ppm Na-hypochlorite.

Our results show that Na-Chl-based photosensitization has great potential for use as an environment-friendly, nonthermal surface decontamination technique.

Acknowledgements

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Inactivation of several strains of *Listeria monocytogenes* attached to the surface of packaging material by Na-Chlorophyllin-based photosensitization

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ABSTRACT

This study was focused on the possibility to inactivate thermosensitive *Listeria monocytogenes* $ATC_{L3}C$ 7644 and thermoresistant 56 Ly strain by Na-Chlorophyllin (Na-Chl)-based photosensitization *in vitro* and on the surface of packaging. Comparative analysis of antimicrobial efficiency of photosensitization with conventional surface cleaning was performed.

Data indicate that both *Listeria* strains, after incubation with Na-Chl and following illumination (λ = 400 nm, 20 mW cm^{-2}), were inactivated by 7 log *in vitro*. This treatment cleaned both *Listeria* strains from packaging surfaces. Comparative analysis indicates that washing with water diminishes pathogens by less than 1 log, 200 ppm Na-hypochlorite by 1.7 log, Na-Chl-based photosensitization by 4.5 log. *Listeria* biofilms were totally removed from the surface by photosensitization at higher photosensitizer concentrations and longer incubation times.

In conclusion, both strains of *L. monocytogenes* can be effectively inactivated by photosensitization *in vitro* and on the surface of packaging. *Listeria* biofilms are susceptible to this treatment as well. Comparison of different surface decontamination treatments reveals that photosensitization is much more effective against both *Listeria* strains than washing with water or 200 ppm Na-hypochlorite.

Our data support the idea that Na-Chl-based photosensitization is an effective antimicrobial tool which may serve in the future for the development of human and environmentally friendly surface decontamination techniques.

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1. Introduction

The US Center for Disease Control and Prevention (CDC) estimates that 76 million people suffer from food-borne illnesses every year [1]. Recent reports from the World Health Organization (WHO) have also concluded that the incidence of food-borne diseases is a growing public health problem in both developed and developing countries [2]. Human listeriosis infections are mostly (98%) food-borne and account for about 2500 cases of illness and approximately $200 million in monetary loss in the US annually [3]. Listeriosis is considered to be among the most frequent causes of food-borne death, with a mortality rate of 20–30% [1]. Despite world wide efforts by research organizations and the food industry to reduce the incidence of listeriosis, it remains a critical threat to human health and the safety of the food supply.

Listeria monocytogenes is the causative agent of listeriosis, a disease that mainly affects pregnant women, newborn children, the elderly and immunocompromised people. In healthy people, *L. monocytogenes* infection usually presents as diarrhoea or flu-like symptoms. Immunocompromised patients with a malignancy, an autoimmune disease or diabetes mellitus are at higher risk of invasive listeriosis, likely to develop into septicaemia, meningo-encephalitis and death [4]. In fact, the robust ability of *L. monocytogenes* to survive and multiply outside a host, tolerance to refrigerated temperature, relatively acidic pH, high salt concentration, formation of biofilms on different biotic or abiotic surfaces are crucial to its transmission, which typically occurs via the consumption of contaminated foods. *L. monocytogenes* poses a threat to the food processing industry since it can colonize and persist on the surface of food processing equipment and has been found on gaskets, conveyor belts, slicing, dicing, packaging machines, containers, knives, tables drains, floors and walls [5]. Thus, the effective control of *Listeria* biofilm formation on surfaces within food processing facilities is foremost in reducing contamination of food.

Traditional decontamination methods can be divided into thermal and non-thermal sterilization. Thermal sterilization can inactivate pathogens efficiently but induces side-effects (reduction of nutritive and organoleptic properties of foods) and cannot be applied to some foods or materials [6]. To overcome these disadvantages, non-thermal sterilization methods were developed including chemical treatments, ultraviolet light, ionizing irradiation, high pressure, etc. However, these processes also have certain disadvantages which include high costs of application, require-

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ments for specialized equipment, generation of undesirable residues, extended processing time, degradation of exposed materials, microbial resistance and lower efficiency. Irradiation is known as one of the best non-thermal sterilization methods to destroy pathogenic and spoilage micro-organisms. However, it needs special facilities, highly trained personnel and is not acceptable to consumers [4,7]. Atmospheric pressure plasma (APP) treatment is effective for inactivation of *L. monocytogenes* and applicable for disposable food containers. However, the type of material is crucial and appropriate treatment conditions should be considered for achieving satisfactory inactivation levels [8].

Thus, considerable efforts are underway to find effective decontamination treatments. A new and promising approach to decontaminate surfaces from micro-organisms can be photosensitization [7]. This treatment is based in the administration of a photosensitizer, which is preferentially accumulated in the microbial cells. The subsequent illumination with visible light, in the presence of oxygen, specifically produces cell damage that inactivates the micro-organisms [9]. It is known that the main cytotoxic agent produced after absorption of light by photosensitizer is singlet oxygen ($^1O_2$). As a result, many cytotoxic reactions are triggered in the cell which disrupt cell membranes, inactivate enzymes and damage DNA [10,11]. One of the most important advantages of photosensitization in comparison with other antibacterial tools is the absence of any bacterial resistance to this treatment [12], as the process is free radical-mediated [13]. Moreover, the cytotoxic action is local, as the photosensitizer is accumulated only in micro-organisms and its diffusion distance is less than 20 nm [14]. It allows us to achieve an extensive decrease in the population of pathogens with minimal damage or thermal effects on the surrounding matrix [11,15].

A lot of work has been done on the photoinactivation of food pathogens using endogenous photosensitizers produced by micro-organisms from colorless and odorless aminolevulinic acid (ALA) [16–19]. Effective decontamination of packaging by ALA-based photosensitization from *Bacillus cereus*, *Salmonella thyphimurium*, *L. monocytogenes*, their spores and biofilms indicate that this treatment is promising [7]. However, ALA is a rather expensive compound and it requires a longer time (20 min) to produce endogenous porphyrins thus raising the cost of every ALA-based decontamination technology [7].

Actually, the optimal photosensitizer for decontamination of food or food-related surfaces should be endowed with specific features (in addition to the expected photophysical characteristics, for instance high yield of generation of singlet oxygen) such as high affinity for microbial cells, a broad spectrum of inactivation and the damage of bacteria without the development of mutagenic processes or resistant strains.

Chlorophyll and its derivatives are widely known photosensitizers in photodynamic therapy. Although different classes of porphyrins have been tested against Gram-positive and Gram-negative bacteria, chlorophyllin-based photosensitization and the concomitant inactivation of food pathogens has been never investigated.

This paper is focused on the possibility to inactivate several strains of *L. monocytogenes* by Na-Chl-based photosensitization *in vitro* and on the surface of the packaging material polyolefine. In addition, comparative analysis of different conventional surface decontamination techniques with Na-Chl-based photosensitization was performed.

2. Materials and methods

2.1. Chemicals

Stock solution of Na-Chlorophyllin (Roth, Karlsruhe, Germany) was prepared by dissolving in 0.9% NaCl up to the concentration 7.5×0^{-5}–7.5×10^{-8} M. Absorption spectrum of this compound was performed by spectrophotometer (Helios Gamma & Delta spectrophotometers ThermoSpectronic, Great Britain) whereas excitation and fluorescence spectra were obtained by PerkinElmer model LS-55 fluorescence spectrophotometer (Germany). Scan range parameters were as follows: excitation wavelength – 395 nm; emission – 400–750 nm; ex slit – 2.5 nm; em slit – 2.5 nm; scan speed (nm min^{-1}) – 200.

2.2. Bacterial strain and culture conditions: L. monocytogenes

$ATC_{L3}C$ 7644 was kindly provided by the National Veterinary Laboratory (Vilnius, Lithuania). Thermoresistant strain *L. monocytogenes* 56 Ly was kindly provided from UNIBO (prof. E. Guerzoni, Italy). The bacterial cultures were grown at 37 °C and maintained on Tryptone Soya Agar supplemented with 0.6% Yeast Extract (TSYEA) (Liofilchem, Italy).

The bacterial cultures were grown overnight (∼14 h) at 37 °C in 20 mL of Tryptone Soya medium supplemented with 0.6% Yeast Extract (TSYE) (Liofilchem, Italy), with agitation at 120 rpm (Environmental Shaker-Incubator ES-20; Biosan, Latvia). This culture was then diluted 20 times with the fresh medium (OD = 0.164) and grown at 37 °C to approximately 1.16×10^9 CFU mL^{-1}, OD = 0.9 in a shaker (120 rpm; Biosan). Bacterial optical density was determined in a 10.01 mm glass cuvette at λ = 540 nm (Helios Gamma & Delta spectrophotometers; ThermoSpectronic, Cambridge, Great Britain). Afterwards the bacteria were harvested by centrifugation (20 min, 5000*g*) and resuspended to $\sim 5.8 \times 10^9$ CFU mL^{-1} final concentration in 0.1 M phosphate buffered saline (PBS) (pH = 7.2). This stock suspension was accordingly PBS-diluted to $\sim 1 \times 10^7$ CFU mL^{-1} and immediately used for the photosensitization experiments.

2.3. Photosensitization in vitro

Ten milliliter solutions of bacterial cell suspensions with appropriate concentrations of Na-Chl (7.5×10^{-5}–7.5×10^{-8} M) were 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 times (0–30 min). A light emitting diode (LED)-based light source (constructed in the Institute of Applied Sciences of Vilnius University) emitted light λ = 405 nm with intensity 20 mW cm^{-2} at 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 3 Sigma meter (Coherent, Santa Clara, USA) equipped with a piro-electrical detector J25LP04. No thermal effects were detected in these experimental conditions.

2.4. Photoinactivation of bacteria after adhesion to the surface of packaging material

Yellow packing trays (polyolefine) were provided by LINPAC (West Yorks, United Kingdom). In order to simplify experiments, the packaging samples for photosensitization experiments were cut into 4 cm × 8 cm pieces, cleaned well with 96% ethyl alcohol and soaked in 50 mL of a *L. monocytogenes* vegetative cell suspension for cell adhesion. Afterwards, the packaging samples were kept in sterile box for 30 min for further adhesion. Then appropriate packing samples were incubated in the dark with the 7.5×10^{-7} M concentration of photosensitizer for 2 min. The control samples were incubated with 0.1 M l^{-1} PBS (7.2 pH) buffer. After incubation with Na-Chl solution, all packing samples were dried in a sterile box at room temperature for 20 min. Dry samples

were placed in the treatment chamber and exposed to light for 2 min. The control sample was not illuminated.

2.5. Photoinactivation of bacterial biofilms on the surface of packaging material

L. monocytogenes biofilms were prepared according to the method of Pan et al. [19]. In short, the packaging material samples (2.5 cm × 4 cm) were placed in sterile plastic tubes to keep them separated from each other. Then, these tubes were filled *L. monocytogenes* cell suspension ($\sim 1 \times 10^7$ CFU mL^{-1}) until all samples were completely submerged and kept for 3 h at 37 °C. Afterwards, the cell suspension was removed by aspiration and samples were separately washed three times with sterile PBS to exclude weakly adhered cells. Then the plastic samples were transferred into plastic tubes containing 25 mL of TSYE medium, with each tube containing one sample.

These tubes were incubated at 22.5 °C (according to Pan et al., [20]) for 48 h for biofilm development. Then samples were washed with sterile PBS three times, positioned in plastic tubes containing 25 mL of 1.5×10^{-5}–1.5×10^{-4} M Na-Chl solutions, and incubated in the dark for 2 min. The control samples were incubated with sterile PBS. After incubation with Na-Chl, all packaging samples were dried in a sterile environment (sterile box) 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.

2.6. Bacterial cell survival assay

The antibacterial effect of photosensitization on packaging material was evaluated by the spread plate method. Each packing sample was mixed with 30 mL 0.1 mol L^{-1} PBS buffer and churned with a BagMixer (model MiniMix 100 VP, Interscience, St. Nom, France). Then, 100 μL of appropriate dilutions (in 0.9% NaCl) of suspension were placed on LBA plates. The colonies were counted after 24 h incubation at 37 °C. The surviving cell populations were enumerated and expressed as $\log_{10}$ (CFU cm^{-2}).

2.7. Statistical analysis

The experiments were carried out in triplicate for each set of exposure. 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 7.5 software (OriginLab Corporation, Northampton, MA 01060, USA).

2.8. Comparative analysis of different antibacterial treatments

In order to compare the efficacy of different decontamination techniques, the packaging samples with inoculated *L. monocytogenes* were treated with sterile water and 100 or −200 ppm Na-hypochlorite. Packaging samples (4 × 8 cm) were completely submerged in containers with appropriate sanitizers and left for 1 min. Afterwards, decontamination efficiency of water and hypochlorite were compared with that of Na-Chl-based photosensitization.

3. Results

3.1. Inactivation of L. monocytogenes cells by Na-Chlorophyllin-based photosensitization in vitro

Chlorophyllin (Na-Chl) is a semi-synthetic mixture of sodium copper salts derived from chlorophyll. During the synthesis of chlorophyllin, the magnesium atom at the center of the ring is replaced with copper and the phytol tail is lost (Fig. 1a). Data presented in Fig. 1b and c indicate that Na-Chl at the concentrations 7.5×10^{-6}–7.5×10^{-8} M has an absorption maximum at λ = 405 nm fluorescence maximum is about 660 nm. We examinated the photocytotoxic effect of several Na-Chl concentrations (from 7.5×10^{-6} M L^{-1}–7.5×10^{-7} M L^{-1}) and found that to inactivate listeria 7.5×10^{-7} M L^{-1} Na-Chl concentration is optimal (data not shown). Experimental data reveal clearly that light alone had negligible effects on the viability of bacteria. The incubation of bacterial cells with Na-Chl (7.5×10^{-7} M) for 2 min had no impact on their viability, but following illumination of bacteria with visible light (405 nm, 0–30 min) drastically reduced viability (Fig. 2). It is evident that incubation of thermosensitive *L. monocytogenes* $ATC_{L3}C$ 7644 bacteria with 7.5×10^{-7} M Na-Chl concentration for 2 min and illumination for 5 min is effective enough to produce inactivation of bacteria by 7 log. The inactivation of the thermoresistant 56 Ly *Listeria* strain by photosensitization exhibited another "shape" as its inactivation rate was slower. Despite this, the thermoresistant strain was inactivated by 7 log as well – however adequate inactivation needed a longer illumination time (30 min). Clear dependence of inactivation efficiency on the illumination time (light dose) for both *Listeria* strains was observed (Fig. 2).

Decontamination of food-related packaging from *L. monocytogenes* by Na-Chl-based photosensitization: comparison of the efficiencies of different packaging decontamination techniques.

Data presented in Fig. 3a and b allow us to compare efficiencies of different surface decontamination techniques. Photosensitization was compared with conventional surface treatments such as washing with sterile water or Na-hypochlorite. It is obvious that washing with water can diminish *L. monocytogenes* $ATC_{L3}C$ 7644 population on the surface of packaging by less than 1 order of magnitude, washing with 100 ppm Na-hypochlorite is more effective and reduces the pathogen population about 1.2 orders of magnitude. When a higher Na-hypochlorite concentration was used (200 ppm), the reduction of bacterial population on the surface of packaging reached 1.8 log. Meanwhile Na-Chl-based photosensitization seems most effective as it reduced *Listeria* population by 4.5 log. In the case of inactivation of thermoresistant *L. monocytogenes* 56 Ly strain on the surface of packaging material we again found total elimination of bacteria from the surface, however it required a higher Na-Chl concentration (1.5×10^{-4} M) (Fig. 3b).

3.2. Inactivation of L. monocytogenes biofilms by Na-Chlorophyllin-based photosensitization

Data presented in Fig. 4 indicate that *L. monocytogenes* biofilms incubated with 1.5×10^{-5}–1.5×10^{-4} M Na-Chl and illuminated 15 min with 405 nm light showed a reduced population of about 1.5 log. It means that biofilms are less susceptible to photosensitization in comparison with vegetative cells, thus the higher Na-Chl concentration (1.5×10^{-4} M) had to be used to inactivate *L. monocytogenes* biofilms by 4.5 log.

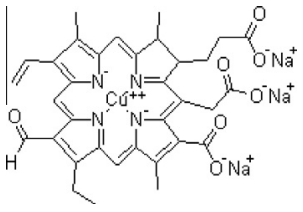

Fig. 1. Na-Chlorophyllin chemical structure.

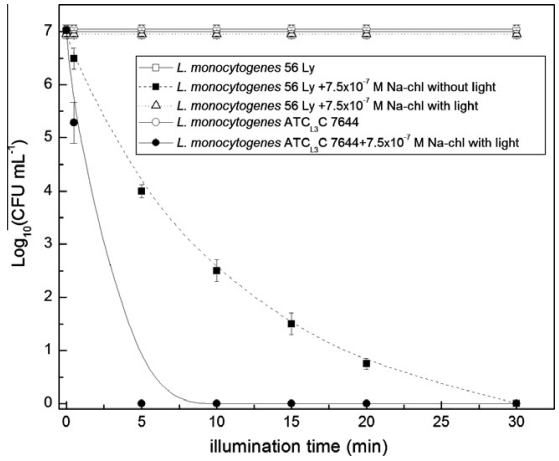

Fig. 2. Inactivation of *L. monocytogenes* $ATC_{L3}C$ 7644 and *L. monocytogenes* 56 Ly by 7.5×10^{-7} M Na-Chlorophyllin-based photosensitization as a function of illumination time (incubation time 2 min).

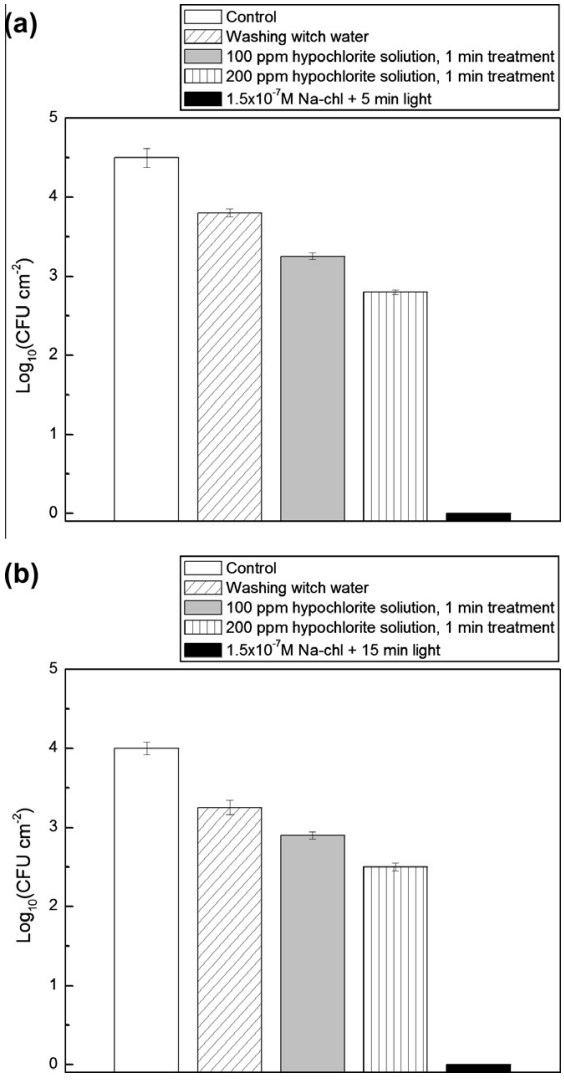

Fig. 3. (a and b) Comparative analysis of different antimicrobial tools: efficiencies of inactivation of *L. monocytogenes* $ATC_{L3}C$ 7644 (a) and *L. monocytogenes* 56 Ly (b) on the surface of packaging samples.

4. Discussion

Recently there is plenty of chemical and physical antimicrobials meanwhile micro-organisms mostly are able to develop resistance to them. One of the most attractive features of photosensitization as an antimicrobial tool is the absence of any bacterial resistance to this treatment [11,12].

A lot of work has been done on photosensitization-based inactivation of micro-organisms which induce infectious diseases [9,11,12,15]. In our studies for the first time photosensitization was applied for inactivation of food pathogens [10,16–19,23], microfungi and yeasts [24–26].

Data obtained in this study reveal that Na-Chl, being a water soluble, highly fluorescing food additive (E141), distinguished for its anti-mutagenic and anti-carcinogenic action [21] exhibits excellent photosensitizing properties. Theoretically, in Gram (+) bacteria the outer wall (15–80 nm) contains up to 100 peptidoglycan layers which are associated with lipoteichoic and negatively charged teichuronic acids. This wall is not a permeability barrier for most photosensitizers with molecular weight 1500–1800 Da including Na-Chl [22]. Thus, after accumulation of Na-Chl by the cell, and following photosensitization, *L. monocytogenes* was inactivated by 7 log using a very low photosensitizer concentration (7.5×10^{-7} M L^{-1}) *in vitro*. A slower inactivation rate was characteristic for the thermoresistant *Listeria* strain: to achieve 7 log inactivation we had to illuminate samples for 30 min whereas the thermosensitive strain was inactivated after 5 min illumination. Further deep investigation of bacterial resistance is necessary to understand why one strain is more susceptible to photosensitization than other. Incubation time with Na-Chl (2 min) was selected from our previous work on photoinactivation of *Bacillus cereus* [23]. The comparison of ALA- and Na-Chl-based photoinactivation of *L. monocytogenes* indicates some advantages for the latter treatment in terms of antimicrobial efficiency, lower photosensitizer concentration and shorter incubation and illumination time [18]. Our data are in line with Kreitner et al. [27] who examinated the photosensitivity of Gram-positive bacteria and yeasts to Na-Chl-based photosensitization and found that *S. aureus, B. subtilis, S. cerevisiae, Rhodotorula mucilaginosa* were susceptible to this treatment (3–5 log inactivation) after incubation with 10^{-5} M L^{-1} photosensitizer and 1 h illumination. Ulatowska-Jarza et al. [28] analyzed the efficiency of other chlorophyll-type photosensitizer Chlorine e6 (Photolon)-based photosensitization on *Escherichia coli* strains isolated from poultry and cows. They found that Pholoton can reduce *E. coli* population by 90%.

The photodynamic bactericidal effect of the photoactive dyes acriflavine neutral, rose bengal, phloxine B, and malachite green (oxalate salt) against two Gram-negative strains (*Escherichia coli* LJH 128 and *Salmonella typhimurium* C1058), two Gram-positive strains (*Bacillus* sp. C578 and *Listeria monocytogenes* LJH 375), and yeast (*Saccharomyces cerevisiae* C1172) was investigated in [29]. Data indicate that Acriflavine neutral, rose bengal were active against all bacteria whereas malachite green and phloxine B resulted in a significant decline in cell numbers for Gram-positive bacteria.

In order to decrease microbial contamination, the fresh-cut industry commonly uses NaOCl and acids as disinfection agents, but by-products such as trihalometanes and chloramines are potentially harmful for humans [7]. Inactivation of *L. monocytogenes* on

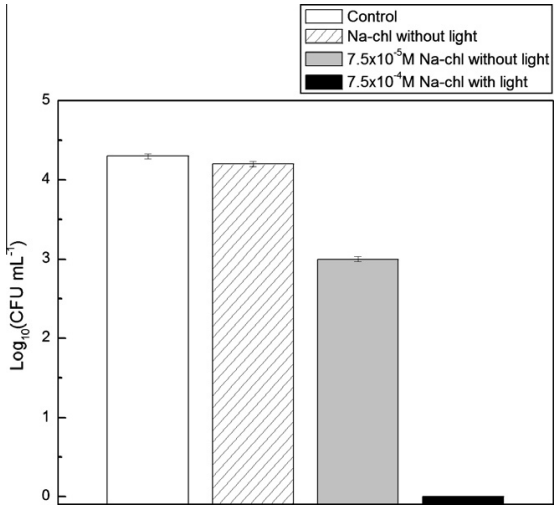

Fig. 4. Inactivation of *L. monocytogenes* $ATC_{L3}C$ 7644 biofilms by Na-Chlorophyllin-based photosensitization on packaging samples.

the surface of packaging material by Na-Chl-based photosensitization indicates that this treatment can totally clean *Listeria* from surfaces. Decontamination of packaging material from thermoresistant *L. monocytogenes* 56 Ly cells adhered to the surface reveals that a higher Na-Chl concentration (1.5×10^{-4} M) is necessary to reduce the bacterial population from 4 log in control to 0 log in treated sample. Comparative analysis of efficiencies of different antimicrobial tools indicates that washing with sterile water or Na-hypochlorite is less effective than photosensitization in the case of thermosensitive and thermoresistant *Listeria* strains. Amongst different approaches such as the use of chlorine dioxide, ozone, ultraviolet (UV)-radiation, and advanced filtration processes, photocatalytic oxidation is considered to be the most convenient and least expensive [30], but *Listeria monocytogenes* is rather resistant to this treatment [31].

Listeria biofilms can be defined as a structured community of cells enclosed in a self-produced polymeric matrix and adherent to the surface. In general, it is difficult to remove biofilms from the surface by conventional cleaning methods as their resistance to antimicrobials is very high [32]. Data presented in Fig. 4 reveal that *Listeria* biofilms are also susceptible to photosensitization and surfaces can be totally cleaned from them when higher 1.5×10^{-4} M Na-Chl concentration is used. This finding is particularly important since biofilms are usually resistant to commonly employed antibacterial agents. Hence, photosensitization could represent an innovative approach in sterilization of packaging contaminated by bacterial biofilms. Thus, the Na-Chl-based photosensitization is an effective antibacterial tool. Its efficiency is comparable with antimicrobial efficiency of high intensity pulsed light which is approved by Food and Drug Administration (FDA) as surface decontamination technique [33].

5. Conclusions

Na-Chl is a water soluble food additive (E141) which exerts high photosensitizing activity and inactivates thermoresistant and thermosensitive *L. monocytogenes* strains after illumination by 7 log at extremely low concentrations (7.5×10^{-8} M). Comparison of different surface decontamination treatments reveal that Na-Chl-based photosensitization is much more effective against *L. monocytogenes* attached on the surface of packaging material polyolefine than washing with water or 200 ppm Na-hypochlorite.

Our data support the idea, that Na-Chl-based photosensitization with its high antimicrobial efficiency and non-thermal nature, may well serve in the future for the development of human and environmentally friendly, non-thermal surface decontamination techniques.

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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

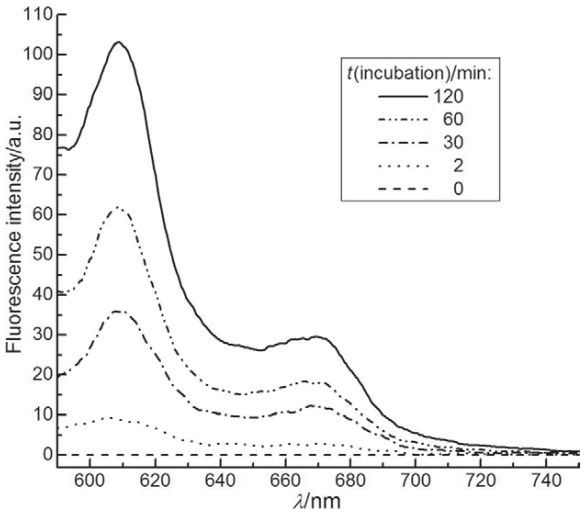

Fig. 1. Fluorescence spectra of endogenous porphyrins produced by *Listeria monocytogenes* ATCL3C 7644 after incubation with 7.5 mM ALA for different time intervals

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).

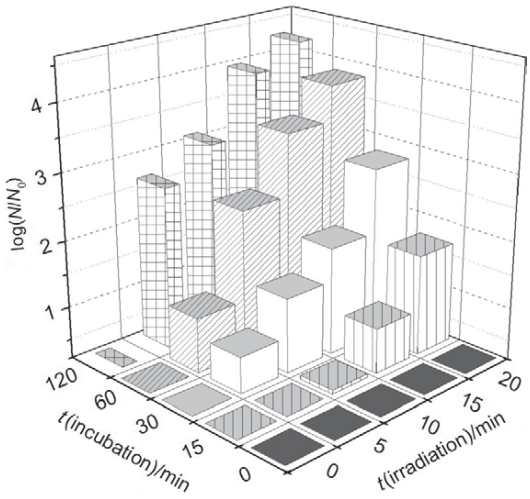

Fig. 2. Inactivation of *Listeria monocytogenes* ATCL3C 7644 by 7.5 mM ALA-based photosensitization when different illumination time and time of incubation with ALA were used

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.

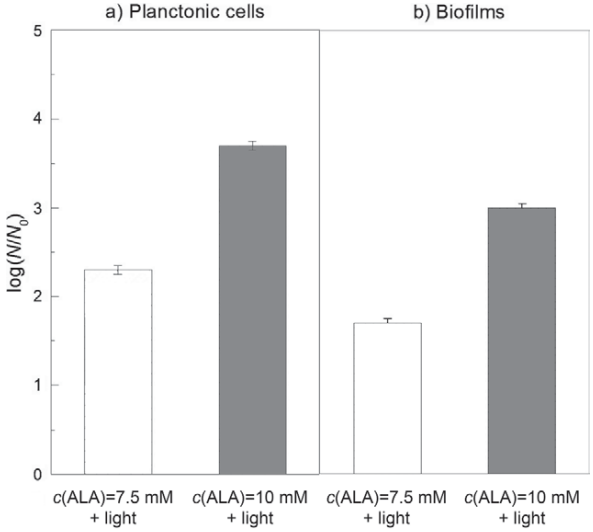

Fig. 3. Susceptibility of *Listeria monocytogenes* ATCL3C 7644 to ALA-based photosensitization: (a) cells and (b) biofilms adhering to the surface of packaging material. Illumination time 15 min, total light dose 18 J/cm^2. Control, untreated sample (log (N/N_0)=0)

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.

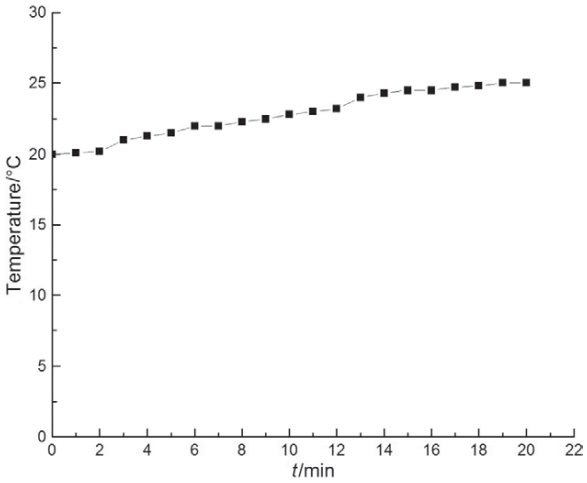

Fig. 4. The increase of temperature in the chamber of LED-based light source during 20 min of illumination

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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Photosensitization-based inactivation of food pathogen *Listeria monocytogenes in vitro* and on the surface of packaging material

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ABSTRACT

The study was focused on the susceptibility of *Listeria monocytogenes* ATCL3C 7644 cells and biofilms to non-thermal antimicrobial treatment – photosensitization *in vitro* and after adhesion to the surface of packaging material.

L. monocytogenes was incubated with 5-aminolevulinic acid (ALA) (7.5 mM) for 0–2 h and illuminated with visible light. The LED-based light source used for the illumination emitted light $\lambda = 400$ nm with energy density 20 mW/cm^2. The illumination time varied 0–20 min, and a total light dose reached 0–24 J/cm^2.

The obtained data indicate that *L. monocytogenes* produces endogenous porphyrins after incubation with 7.5 mM ALA. Subsequent illumination of cells remarkably inactivates (4 log) them *in vitro*. Photosensitization diminished population of Listeria cells adhered onto the packaging material by 3.7 log and inactivated bacterial biofilms by 3.1 log. It was shown that antimicrobial efficiency of photosensitization depended on the illumination time, incubation with ALA time as well as on the used ALA concentration. In conclusion, cells and biofilms of *L. monocytogenes* ATCL3C 7644 can be effectively inactivated by ALA-based photosensitization in the solution as well as adhered onto the surface of packaging material. Obtained data support the idea, that photosensitization as non-thermal and effective antimicrobial treatment has potential to develop into environmentally safe, surface decontamination technique.

1. Introduction

Listeria monocytogenes is highly pathogenic bacteria [1]. The food-borne 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, newborns, and elder people with weakened immune systems [3]. The Center for Disease Control and Prevention estimates that 2,500 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 food-borne illness-related hospitalization and 27.6% of food-borne disease-related deaths [5].

One of the threats posed by *L. monocytogenes* is its adaptation to stress such as high salt concentration (10% NaCl), broad pH range ($4.5 < pH < 9$). The bacterium is capable to grow and multiply even at 1.7 °C [6]. Another threat is their extremely strong adherence to the different surfaces in food-processing industry (stainless steel, polypropylene, aluminium, and glass).

As a natural biofilm community is functioning through collective behaviour and coordinated activity, which assists survival of individual cells in stressful conditions, biofilms are more than 100 times resistant to antibacterial treatment than planktonic cells and 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 equipments [8].

Most of the traditional food safety technologies invoke thermal or chemical effects, which usually induce undesirable physical and chemical changes in the food. Thus, the development of modern, non-thermal, ecologically friendly and cost-effective antimicrobial technology is of importance.

In our opinion, one of them could be photosensitization [9]. Photosensitization is based on the interaction of three non-toxic agents: photoactive compound (photosensitizer), light and oxygen [10]. Numerous investigators have confirmed that different microorganisms including bacteria, viruses and microfungi can be killed after their treatment with an appropriate photosensitizer and light [11–15]. The antibacterial efficiency of this treatment depends on many factors, including physiological state of bacteria, cellular structure and organization, physico-chemical properties of the photosensitizers and their capacity to bound 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].

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

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 exogenously applied (ALA).

This study is focused on the possibility to inactivate *L. monocytogenes* by ALA-based photosensitization. In addition it seems important to evaluate the efficiency of this treatment against surface-attached bacterial cells and biofilms.

2. Material and methods

2.1. Chemicals

Stock solution of 5-aminolevulinic acid hydrochloride (ALA) (Fluka, Israel) was prepared by dissolving ALA in 0.1 M PBS buffer (pH = 7.2). NaOH (0.2 M) 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) [17].

2.2. Bacterial growth

L. monocytogenes ATCL3C 7644 was kindly provided by the National Veterinary Laboratory (Vilnius, Lithuania). The bacterial culture was grown at 37 °C and maintained onto Tryptone Soya Agar supplemented with 0.6% Yeast Extract (TSYEA) (Liofilchem, Italy).

The bacterial culture was grown overnight (~14 h) at 37 °C in 20 mL of Tryptone Soya medium supplemented with 0.6% Yeast Extract (TSYE) (Liofilchem, Italy), with agitation at 120 rpm (Environmental Shaker-Incubator ES–20; Biosan, Latvia). This culture afterwards was diluted 20 times by the fresh medium (optical density, A = 0.164) and grown at 37 °C to approximately 1.16 × 109 CFU mL^{-1}, A = 0.9 in a shaker (120 rpm; Biosan). Bacterial optical density was determined in a 10.01 mm glass cuvette at λ = 540 nm (Helios Gamma & Delta spectrophotometers; ThermoSpectronic, Cambridge, Great Britain). Afterwards the bacteria were harvested by centrifugation (20 min, 5000 g) and resuspended to ~5.8 × 109 CFU mL^{-1} final concentration in 0.1 M phosphate buffer saline (PBS) (pH = 7.2). This stock suspension was accordingly PBS-diluted to ~1 × 107 CFU mL^{-1} and immediately used for the photosensitization experiments.

2.3. Photosensitization

10 ml aliquots of bacterial suspension (~1 × 107 CFU mL^{-1} 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 (120 rpm; Biosan) with 7.5 mM concentration of ALA for different periods (0–2 h) [18]. 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 intensity P = 20 mW cm^{-2} and wavelength λ = 400 nm.

2.4. 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 (1 × 107 CFU mL^{-1} in 0.1 M PBS (pH = 7.2)) were incubated in dark at 37 °C with 7.5 mM ALA concentration for the indicated time. Then 2 mL aliquots of bacterial suspensions were withdrawn by centrifugation (10 min, 5000*g*) and afterwards used for cell-bounded porphyrins fluorescence measurements [18]. PerkinElmer LS 55 fluorescence spectrophotometer (Beaconsfield, United Kingdom) was used for the fluorescence detection. Scan range parameters were as follows:

1. Excitation wavelength: 390 nm.
2. Emission: 590–750 nm.
3. Excitation Slit (nm): 2.5 nm.
4. Emission Slit (nm): 15 nm.
5. Scan Speed (nm/min): 200.

2.5. Photoinactivation of bacteria adhered to the surface of packaging material

Packing yellow trays (polyolefine, a mixture of polyethylene/polypropylene) were provided by LINPAC (West Yorks, United Kingdom).

In order to simplify experiments and minimize illumination square, the packaging coupons for photosensitization experiments were cut into 2.5 cm × 4 cm pieces. To adhere the bacteria on the surface, each packaging sample was soaked in 25 mL *L. monocytogenes* suspension (~1 × 107 CFU mL^{-1}). The soaked samples were kept in a box-laminar for 30 min for better attachment of the bacterial cells. Then appropriate packing coupons with the adhered bacteria were incubated in the dark with the 7.5–10 mM ALA concentration for 15 min. The control samples were incubated with PBS (7.2 pH) buffer. After incubation with ALA, all packaging samples were dried in a box-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.

2.6. Photoinactivation of bacterial biofilms on the surface of packaging material

L. monocytogenes biofilms were prepared according to the method of Pan et al. [19]. In short the packaging material samples (2.5 cm × 4 cm) were placed in sterile plastic tubes to keep separated from each other. After these tubes were filled *L. monocytogenes* cell suspension (~1 × 107 CFU mL^{-1}) until all samples were completely submerged for 3 h at 37 °C. Afterwards the cell suspension was removed by aspiration and samples were separately washed three times with sterile PBS to exclude weakly adhered cells. Then the plastic coupons were 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 samples were washed with sterile PBS three-times positioned in plastic tubes containing 25 mL of 7.5–10 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 environment (box-laminar) 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.

2.7. 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 0.1 M sterile

PBS buffer and "washed" 60 s 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, was 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^{-1} in the untreated culture and N is the number of CFU mL^{-1} in the treated one. CFU cm^{-2} was used for planctonic cells and biofilms adhered to plastic coupons.

2.8. Statistical analysis

Bacterial populations were transformed from CFU mL^{-1} and CFU cm^{-2} into log 10 mL^{-1} and log 10 cm^{-2}. All experiments were repeated three times, every experiment was carried out in triplicate. A standard deviation was estimated for every experimental point and marked in a figure as an error bar. Sometimes the bars were too small to be visible (SE = 0–0.32). The fluorescence data were analysed with Origin 7.5 software (OriginLab Corporation, Northhampton, USA).

3. Results

3.1. Fluorescence measurements of endogenous porphyrins synthesized from ALA in L. monocytogenes

In our previous paper [18] the production of endogenous porphyrins from ALA in *Salmonella enterica* was investigated. Using the same methodology we found, that Gram-positive bacterium *L. monocytogenes* did produce endogenous porphyrins from extrinsically applied ALA as well. Thus, the cells were incubated with 7.5 mM ALA in the dark for 0–2 h. Afterwards, in order to detect the production of the 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 [20]. The data, presented in Fig. 1 indicate that relative fluorescence intensity of endogenous porphyrins increases with increasing 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 expansion of the incubation time to 2 h, fluorescence intensity increased more than 10 orders of magnitude. In order to develop surface decontamination technology, our aim was to detect the synthesis of endogenous porphyrins in selected pathogens within shortest time without any deeper analysis of specific endogenous porphyrins produced by cells.

3.2. Susceptibility of L. monocytogenes to ALA-based photosensitization in vitro

Experimental data, presented in Fig. 2a and b reveal clearly that the incubation of cells with ALA (dark toxicity of ALA) does not change the viability of *L. monocytogenes*. Light alone, without any incubation of cells with ALA has no toxicity on bacteria. Even incubation of cells with ALA and subsequent illumination decreases the surviving fraction fairly sharply, especially when longer incubation times are used. Clear dependence of inactivation efficiency on the illumination time (or total energy dose) as well as incubation with ALA time (or concentration of produced endogenous porphyrins) is observed. The number of killed *L. monocytogenes* reaches even four

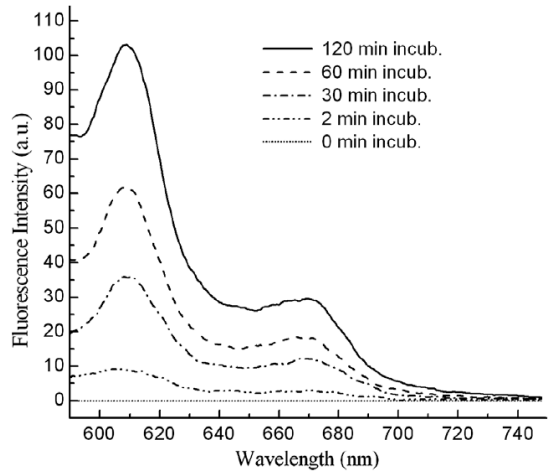

Fig. 1. Fluorescence spectra of endogenous porphyrins produced by *Listeria monocytogenes* $ATC_{L3}C$ 7644 after incubation with 7.5 mM ALA for different time interval.

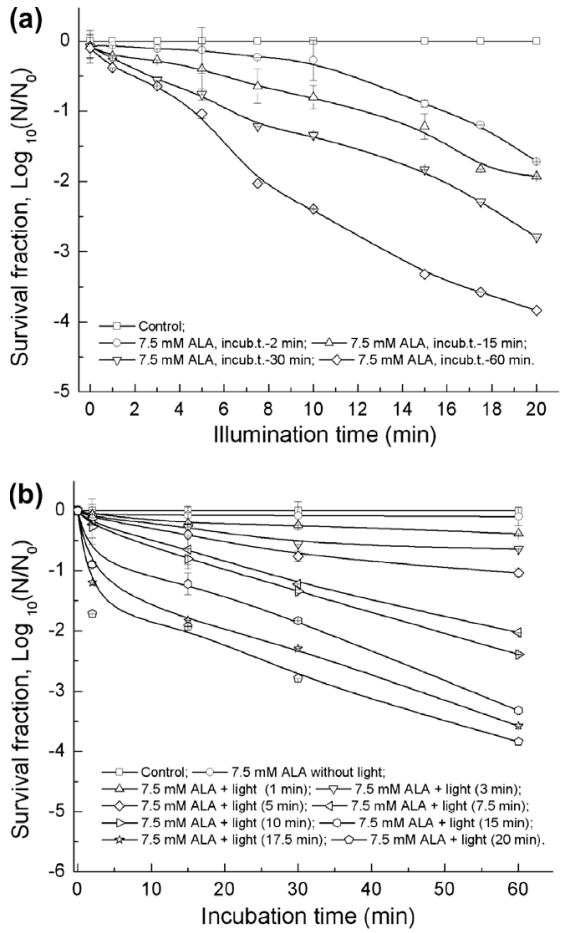

Fig. 2. Inactivation of *Listeria monocytogenes* $ATC_{L3}C$ 7644 by 7.5 mM ALA-based photosensitization when different illumination (a) and incubation with ALA (b) time was used.

orders of magnitude, when 20 min illumination time and 2 h incubation time are used. It is evident that at a given illumination dose the inactivation effect can be modified by ALA incubation time. The data, depicted in Fig. 3, show the interrelation between two factors important for inactivation efficiency: illumination time and incubation time. Contours curves are based on approximation of experimental results and show what amount of bacteria ($\log(N/N_0)$) is killed at different illumination (t_R) and incubation (t_i) times. It is easy to understand that the points joined in one black curve exhibit the shortest distance from coordinate axes and suggest us what is the optimal ratio of incubation time and irradiation time for the most effective bacterial inactivation. According to these data, antibacterial efficiency of treatment is optimal, when the increase of incubation time (amount of cell bound endogenous porphyrins) is proportional to the increase of illumination time (amount of photons for activation of porphyrins).

3.3. Different susceptibilities 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. monocotogenes* cells adhered onto one coupon of packaging material reached over 4.6 log. Afterwards coupons of packaging material with the adhered bacteria were soaked in 7.5–10 mM ALA solution and illuminated with light $\lambda = 400$ nm. Illumination time reached 15 min. and a total light dose did not exceed 18 J/cm^{-2}. The data, depicted in Fig. 4a, clearly indicate that the inactivation of cells after photosensitization treatment decreases from 2.3 log up to 3.7 log, depending on the ALA concentration used.

Afterwards, bacterial biofilms were adhered on 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 by 7.5–10 mM ALA in the dark did not exhibit any citotoxic effect against bacterial cells. Light alone had no toxic effect as well. Just incubation with ALA and subsequent illumination of these bacteria by light ($\lambda = 400$ nm) reduced significantly the formation of biofilms. Depending on the used ALA concentration, (7.5–10 mM) inactivation of biofilm-associated cells decreased from 1.7 log to 3 log respectively.

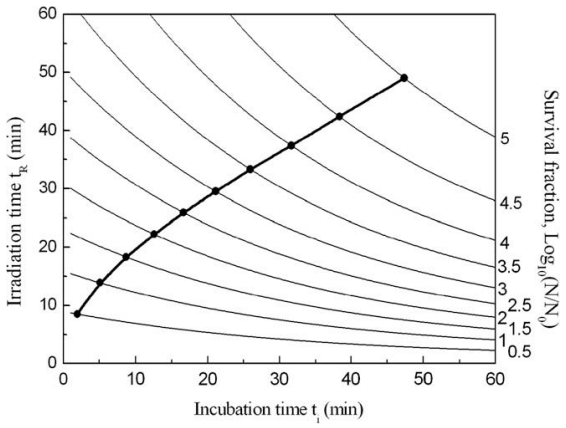

Fig. 3. Mathematical modeling of interrelation between incubation time (t_i) and irradiation time (t_R) for the constant inactivation levels ($\log(N/N_0)$) (cotours curves). Black curve connects points of optimal incubation and irradiation times for each level (0.5–5 log) of inactivation.

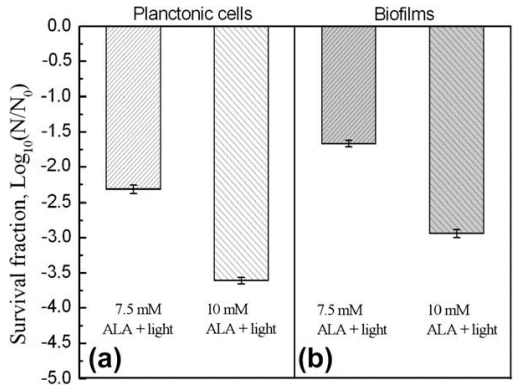

Fig. 4. Susceptibility of *Listeria monocytogenes* $ATC_{L3}C$ 7644 to ALA-based photosensitization: cells (a) and biofilms (b) adhered to the surface of packaging material. ALA concentration 7.5–10 mM, illumination time – 15 min, total light dose 18 J/cm^2. Control, not treated sample = 0 $\log(N/N_0)$.

4. Discussion

The foods implicated in outbreaks of listeriosis include raw milk, cheeses, ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats (all types), raw and smoked fish [6,21]. Meanwhile, the risk is highest in ready-to-eat (RTE) food, as bacteria may contaminate cooked RTE products before packaging, during transportation, or during post-cooking handling [22].

The inactivation of *L. monocytogenes* was performed using several emerging antibacterial technologies. Ultrahigh hydrostatic pressure inactivated *Listeria* by 2.76 log in the liquid [23]. Pulsed UV light technology can inactivate *Listeria* by 6 log, but with some thermal effects [24,25]. Pulsed electric field [26] has a very limited effect on the reduction of *L. monocytogenes*. Hot-water post-packaging pasteurization reduced by 2 log *L. monocytogenes* on food surface [27]. Ionizing radiation reduced the amount of biofilm-associated *L. monocytogenes* cells by 6.4–8.6 log CFU mL^{-1}, but the irradiation values ranged from 0.380 to 0.682 kGy, which were somewhat higher than usable in food industry [28].

Chemical sanitizers like hypochlorite, iodine, ozone and chloramines are largely used in food industry to avoid formation of biofilms [29]. Jeyasekaran et al. [30] defined the efficiency of sodium hypochlorite on the inactivation of *L. monocytogenes* biofilm adhered on plastic material: 100 pm and 200 pm hypochlorite gave 2.0 and 3.0 log reduction of bacterial viability respectively.

Efficiency of other sanitizer ozone confirmed the same killing efficiency as hypochlorite [31]. Peroxide reduced the biofilm formation on PVC surface by 2.0 log. Some authors claim that ultrasonication enhanced the effectiveness of chlorine or quaternary ammonium sanitizers [32].

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* was inactivated by 4 log (Fig. 2). The efficiency of inactivation strongly depends on the used ALA concentration, incubation with ALA time and light dose delivered to the bacteria (Fig. 3). 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. 4a reveal that inactivation of cells after photosensitization can reach in

2.3–3.7 log. No measurable changes were observed in cell inactivation after their incubation with 7.5–10 mM ALA solution or only illumination alone. The biofilms of *Listeria* at the same experimental conditions exhibit lower susceptibility to ALA-based photosensitization. Meanwhile, inactivation of biofilms by 1.7–3.1 log indicate that this treatment has potential to combat biofilms, usually more resistant to environmental effects than their planktonic counterparts [33].

According to obtained results, ALA-based photosensitization can inactivate listeria biofilms to less extent than the vegetative cells adhered to the same surface of packaging material. This effect can be explained by biofilm 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 decrease the effectiveness of the photosensitizing process [34,35].

Considering the application of ALA-based photosensitization to decontaminate food matrix, the question arises how ALA interacts with food matrix. Our previous experiments on the decontamination of wheat sprouts by ALA indicated that ALA could stimulate the growth of wheat seedlings and roots without impairing the vigor of germination and the viability of seeds [36]. 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 [36]. 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 [37]. 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 [38]. In addition, foliar application of ALA (100 mg/ml) on date palm has promoting effects on the fruit weight, volume and sugar content [39].

The effective photoinactivation of different physiological forms of *L. monocytogenes* obtained *in vitro* and onto packaging materials looks promising and may be can serve as a background for the further development of a novel non-thermal or hurdle technology for decontamination of foods 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 potential to work and is patented as antimicrobial tool to decontaminate surfaces [40].

5. Conclusions

The present data for the first time clearly indicate that food pathogen *L. monocytogenes* in the presence of ALA can produce endogenous porphyrins, which after illumination with light can induce lethal photochemical reactions in the bacteria and as consequence inactivation by 4 log *in vitro*. After adhesion of cells to the surface of packaging material, photosensitization inactivated them by 3.7 log. It is important to note that normally more resistant to environmental factors *Listeria* biofilms are susceptible to this treatment and could be inactivated by 3.1 log. In this context, photosensitization may constitute a suitable process to combat both antimicrobial and biofilm-related resistance. Our previous data as well as obtained in this study allow us to forecast that main food pathogens and microfungi on some food and food-related surfaces can be eliminated by photosensitization-based technique, which has potential to develop in the future into an effective, non-thermal, completely safe and environmentally friendly antimicrobial technology.

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Antimicrobial efficiency of photoactivated chlorophyllin-chitosan complex

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The aim of this study was to assess the antimicrobial efficiency of a photoactivated chlorophyllin–chitozan complex against the food pathogen *Salmonella enterica*.

Salmonella enterica was used for experiments. Aliquots of bacterial suspensions (~1×10^7 CFU/ml in 0.9 % NaCl) with 0.001 % chlorophyllin–0.1 % chitosan complex (Chl–CHS) were incubated in the dark at 37 °C for 0–120 min. For the photoactivation, the samples were exposed to light (λ = 405 nm) for 30 min (light dose 17.3 J/cm^2). Microbial viability was evaluated by the spread plate method.

The Chl-based photosensitization treatment reduced the bacterial population by 1.39 log. An extremely high antibacterial efficiency was demonstrated after photoactivation of the Chl–CHS complex (7.3 log reduction of microbial population).

Experimental data support the idea that a photoactivated CHS–Chl complex in a slightly acidic environment can be a useful tool against the gram-negative bacteria *S. enterica*.

Key words: photoactivation, chitosan–chlorophyllin complex, *Salmonella*, food safety

Introduction

Microbiological food safety is an increasing problem worldwide. *Salmonella enterica* is one of the most important foodborne pathogens in many countries. Each year in the United States 1.0 million people are infected with non-typhoidal *Salmonella*, resulting in 19 336 hospitalizations and 378 deaths [1]. In 2010, a total of 1 962 confirmed cases of salmonellosis were reported in Lithuania [2]. The high resistance of *Salmonella* to disinfecting agents has led to the development of alternative antimicrobial technologies. In this context, the photosensitization treatment seems promising. Several studies have demonstrated that bacteria, as well as micromycetes and viruses, could be inactivated by photosensitization *in vitro* [3–5].

Na-chlorophyllin (Chl) is a water-soluble food additive (E140) and food component used as a food colourant, in dietary supplements and in cosmetics [6]. According to our previous results, photosensitized Chl exhibited a very high antimicrobial activity against gram-positive food pathogens *in vitro* and *in vivo* [5, 7, 8]. Meanwhile, photoactivated Chl might be less effective against gram-negative bacteria, which are characterized by a more complex cell wall [9].

Chitosan (CHS) is a natural cationic linear polysaccharide [10]. It is characterized as a nontoxic antimicrobial tool which can be applied in food technologies, agriculture, medicine, and environment protection [11, 12].

The aim of this study was to assess the antimicrobial efficiency of photoactivated chlorophyllin–chitozan complex against the *S. enterica* food pathogen.

Materials and methods

Chemicals

Not coperized chlorophyll sodium salt (Chl) was obtained from ROTH, Karlsruhe, Germany. Low molecular weight chitosan (CHS, Brookfield, viscosity of 1 % solution in 1 % acetic acid at 20 °C 140 cP) was obtained from *Aldrich.* Aqueous stock solution of CHS (pH 2.4 at 20 ºC) containing 1 % of CHS and 0.18 % of HCl was prepared dissolving in water appropriate amounts of HCl and then CHS. The aqueous stock solution of 0.01 % Chl was prepared by Chl dissolution in water. The aqueous stock solution of chlorophyllin–chitosan complex (Chl–CHS) (pH 2.4 at 20 °C), containing 1 % of CHS, 0.01 % of Chl and 0.18 % of HCl, was prepared by a dropwise addition of aqueous 0.05 % Chl solution into a rapidly spinning aqueous solution containing 1.25 % of CHS and 0.23 % of HCl.

Cultivation of the microorganism

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

S. enterica was grown in the Luria–Bertani medium (LB) (*Liofilchem*, Italy) incubated overnight at 37 °C. The

overnight culture was 20 times diluted with fresh LB medium (OD_{540} = 0.164) and grown at 37 °C to the mid-log phase (5×10^8 CFU/ml, OD_{540} = 1.3) in a shaker (Environmental Shaker–Incubator ES–20; Biosan, Latvia) (120 rev/min). Cells were then harvested by centrifugation (10 min, 3420 g) (Hettich Zentrifugen, Mikro-200, Germany) and resuspended in 0.9 % NaCl to give ~2.5×10^9 CFU/ml. These stock suspensions were diluted to ~1×10^7 CFU/ml and immediately used for the experiments.

Effect of chitosan on bacterial growth

Aliquots of 20 ml of bacterial suspensions (~1×10^7 CFU/ml in 0.9 % NaCl) with 0.1 % chitosan (CHS) were incubated in 50 ml flasks for cell culture cultivation in a shaker (120 rev/min) at 37 °C. The samples were removed at 15 min, 60 min and 120 min intervals.

Photosensitization treatment

Aliquots of bacterial suspensions with 10 times diluted CHS–Chl or Chl stock solutions were incubated for cell culture cultivation in a shaker (120 rev/min) in the dark at 37 °C for different periods (0–120 min). Primarily, Chl and Chl–CHS solutions were tested against *S. enterica* in the dark. For the photosensitization treatment, 150 µl of the samples were placed into sterile flat-bottom wells and then exposed to light (λ = 405 nm) for 30 min (light dose 17.3 J/cm^2).

A LED-based light source for the photosensitization was constructed at the Institute of Applied Sciences of Vilnius University. The emission maximum of the light source was 405 nm, and light intensity at the surface of samples reached 9.6 mW/cm^2.

Evaluation of antibacterial activity

The antibacterial activity of photosensitized Chl and Chl–CHS complex against *S. enterica* was evaluated by the spread plate method. Particularly, 100 µl of a diluted bacterial test culture after treatment was surface-inoculated on a separate LB agar (LBA) plate. Afterwards, the LBA plates were kept in a thermostat for 24 h at 37 °C. Bacterial populations were recalculated from CFU/ml into $\log_{10}$/ml.

Statistical analysis

The experiments were triplicated for each set of exposure. A standard error was estimated for each experimental point and marked in a figure as an error bar. The data were analyzed using Origin 7·5 software (OriginLab Corporation, Northampton, MA 01060, USA).

Results and discussion

The results obtained in our previous work have shown that Chl-based photoactivation can inactivate the gram-positive pathogens *Listeria monocytogenes* $ATC_{L3}C$ 7644 and *Bacillus cereus* ATCC 12826 by 7 log *in vitro* and can clean the surface of food-packaging materials made of polyolefines [5, 7]. The antibacterial activity of Chl against *S. enterica* is illustrated in Fig. 1*a*.

It was determined that the dark toxicity of Chl was negligible since the cell number after 120 min of incubation in the dark was reduced only by 0.05 log. The photoactivation treatment (15 min of incubation with Chl and the following illumination) led to a 1.05 log reduction of *S. enterica*. An extension of the incubation time to 120 min favoured the inactivation of *Salmonella* to 1.39 log (Fig. 1*a*). This means that *S. enterica* is rather resistant to Chl-based photosensitization. These data are in line with those reported by Lopez-Carballo et al. [6] who showed that a photoactivated gelatin film with immobilized Chl exhibited a pronounced bactericidal effect against gram-positive *Staphylococcus aureus* (4 log reduction), but the inactivation of gram-negative *Escherichia coli* and *Salmonella* was just marginal.

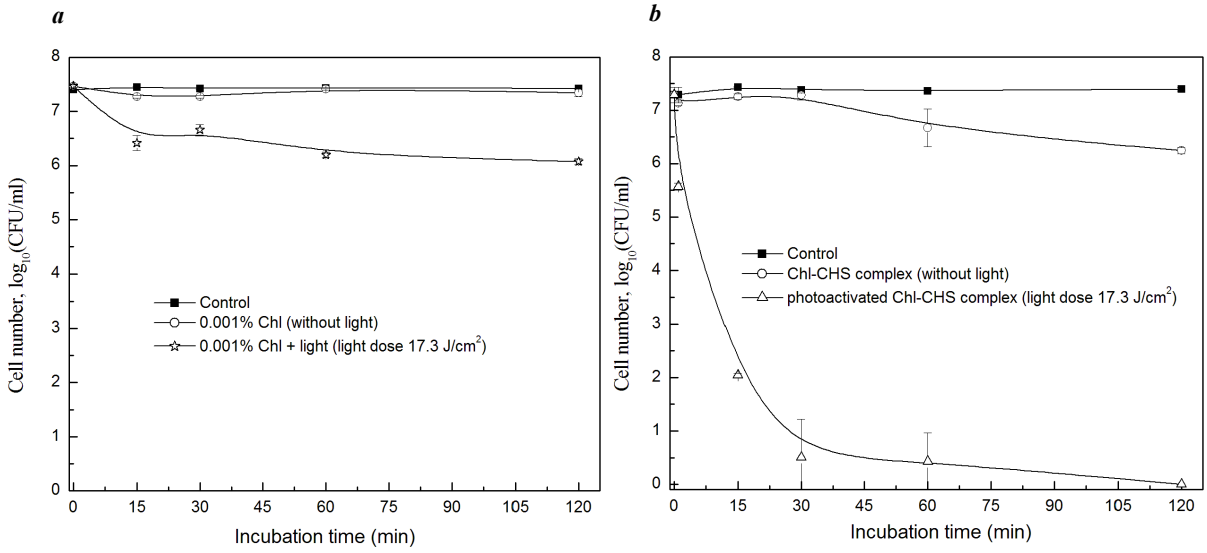

Fig.1. Inactivation of *Salmonella enterica* Serovar Typhimurium strain DS88 (SL 5676 Smr pLM2) by 0.001 % Na-chlorophyllin-based photosensitization treatment (*a*) and by photoactivation of 0.001 % chlorophyllin–0.1 % chitosan complex (*b*)

The antimicrobial efficiency of CHS (0.1%) was assessed in a 0.9 % NaCl suspension. Generally, inactivation of the gram-negative bacteria *S. enterica* by CHS alone after 120 min of incubation reached 3.19 log (data not shown).

We suppose that the synergetic antibacterial effect of CHS and Chl relies on the complexation of these two compounds (Fig. 2). Due to the presence of amino groups in the molecule, CHS is able to form ionic (salt-like) complexes with polyvalent anionic species such as chlorophyllin [13]. The formation of soluble complexes is governed by thermodynamic equilibrium and results in a uniform distribution of the short-chain component among the chains of the oppositely charged long-chain component. Such a case occurs in suspensions containing 0.1 % of CHS and 0.001 % of Chl where the long-chain component chitosan is in high excess.

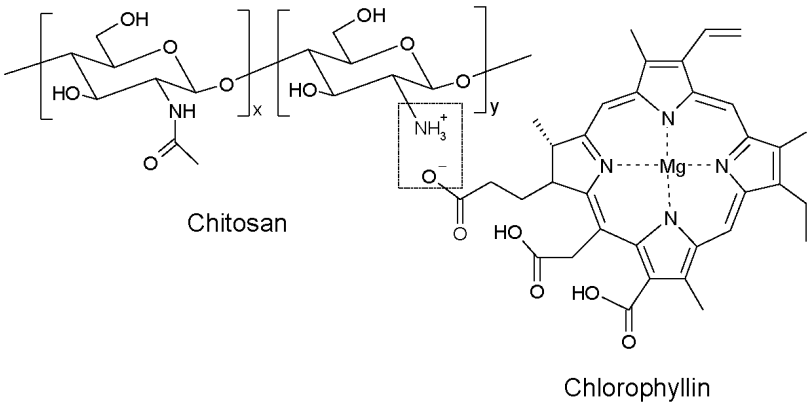

Fig. 2. Hypothetical formula of Chl–CHS complex in 0.9 % NaCl solution at pH 3.5

It is well known that the antibacterial activity of chitosan has been assessed for a wide range of gram-negative and gram-positive bacteria [14]. Liu et al. [15] have shown that protonized chitosan may disrupt the outer but not inner membrane of *E. coli*. This seems to be the first and most important step in chitosan's antimicrobial action. CHS ability to bind to the outer membrane of gram-negative bacteria and to increase membrane permeability should generally sensitize bacteria to other antimicrobial agents.

According to the obtained results, the dark toxicity of the Chl–CHS complex was 1.05 log. On the contrary, the incubation (1 min) of *Salmonella* with a Chl–CHS complex and the following illumination (light dose 17.3 J/cm^2) resulted in a 1.7 log reduction (Fig.1*b*). The use of 60 min incubation and 30 min illumination time resulted in a 6.86 log inactivation of *S. enterica*. An increase of the incubation time to 120 min diminished the bacterial population by 7.3 log.

Conclusions

The gram-negative food pathogen *S. enterica*, being resistant to many antimicrobials, can be effectively inactivated (7.3 log) by a photoactivated 0.001 % Chl–0.1 % CHS complex. Such combination of antimicrobial properties of chitosan and chlorophyllin-based photosensitization seems to be a promising tool to combat gram-negative bacteria which are highly resistant to photosensitization treatment.

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ANTIMIKROBINIS FOTOAKTYVAUS CHLOROFILINO-CHITOZANO KOMPLEKSO POVEIKIS

S a n t r a u k a

Šio darbo tikslas buvo įvertinti fotoaktyvaus chlorofilino-chitozano (Chl-CHS) komplekso antimikrobinį poveikį *Salmonella enterica* maisto patogenui. Bakterijų suspensijos (~1×107 kfv/ml 0,9 % NaCl) buvo 0–120 min inkubuojamos 0,001 % chlorofilino–0,1 % chitozano kompleksu tamsoje. Paskui mėginiai buvo 30 min (šviesos dozė 17,3 J/cm2) švitinami didelės galios reguliuojamo optinio srauto UV šviestukais (λ = 405 nm). Antimikrobinis poveikis buvo įvertintas taikant paskleidimo lėkštelėje metodą.

Įvertinus tyrimų duomenis, buvo nustatyta, kad chlorofilinu indukuota fotosensibilizacija inaktyvuoja bakterijų populiaciją 1.39 log. Antibakterinis poveikis gerokai padidėja fotoaktyvavus Chl-CHS kompleksą (bakterijų populiacija sumažėja 7,3 log). Šio darbo rezultatai parodė, kad fotoaktyvuotas Chl-CHS kompleksas silpnai rūgštinėje aplinkoje efektyviai inaktyvuoja gramneigiamas *S. enterica* bakterijas.

Industrial, medical and environmental applications of microorganisms

Current status and trends

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Novel approach to the microbial decontamination of wheat sprouts: photoactivated chlorophillin-chitosan complex

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Abstract

High resistance of bacteria to disinfecting agents has led to the development of alternative antimicrobial technologies. It is well-known that chitosan itself is an antimicrobial agent, and chlorophyllin is water-soluble food additive (E140) which in the presence of light exerts antimicrobial properties. Complex of these two antimicrobials can serve as background for the development of edible active biodegradable packaging. The aim of this study was to evaluate antimicrobial efficiency of photoactivated chlorophyllin-chitosan complex against food pathogens and microfungi *in vitro* and on the surface of germinated wheat.

Keywords: antimicrobial; photoactivated chlorophyllin-chitosan complex

1. Introduction

The sprouts are outstanding sources of protein, vitamins and minerals. Therefore, germinated seeds are offered as a natural nutritive product which is beneficial to health [1]. However, seed contamination with harmful and pathogenic microorganisms is important as it often reduces product quality and presents a potentially serious health risk to humans. Hence, methods recently applied for inactivation of harmful and pathogenic microorganisms are not always efficient, human and ecologically friendly [1-2]. In this context, photosensitization treatment seems promising. Na-chlorophyllin (Chl) is water-soluble food additive (E140) and food component used as food colourant, in dietary supplements and in cosmetics [3]. According to our previous results, photoactivated Chl exhibited high antimicrobial activity against Gram (+) food pathogens *in vitro* and *in vivo* [4-6]. One of important disadvantages of Chl-based photosensitization is lower killing efficiency of Gram (-) bacteria which have more complicated cell wall structure [7].

Chitosan (CHS), a natural cationic linear polysaccharide, is produced commercially by deacetylation of chitin and is insoluble in water at pH above 6 [8]. It is characterized as nontoxic antimicrobial tool, which do not change pH, color, improves quality of fruits and vegetables and can be applied in food technologies, agriculture, medicine and environment protection [9-10]. In addition, CHS is able to control decay of different fruits and vegetables extending their storage time [11]. Moreover, CHS can form antimicrobial films and serve as carrier of wide range of food additives, including various antimicrobials [12-13]. In this context, chitosan film with inserted Chl may act as more efficient antimicrobial tool to improve food safety and prolong shelf-life of products.

The aim of this study was to evaluate antimicrobial efficiency of photoactivated chlorophyllin-chitosan complex (Chl-KCHS) against food pathogens and microfungi, contamination on wheat.

2. Materials and methods

2.1 Chemicals

Chlorophyll sodium salt (Chl) was purchased from *ROTH*, Karlsruhe, Germany. Low molecular weight chitosan (CHS, degree of deacetylation 72%, Brookfield viscosity of 1% solution in 1% acetic acid at 20 °C 140 cP) was purchased from Aldrich. 2,3-epoxypropyl trimethyl ammonium chloride (EPTMAC) was purchased from Aldrich. Deionized water used in all experiments had specific conductivity less than 1×10^{-6} S/cm.

2.2 Solutions and synthesis of Chl-KCHS complex

N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (KCHS) with 35 mol% degree of cationization was synthesized from equimolar mixture of CHS and EPTMAC according to the procedure described in [14].

Aqueous stock solution of KCHS containing 1% of KCHS and 0.1% of HCl was prepared dissolving in water appropriate amounts of HCl and then KCHS. Aqueous stock solution of 0.01% Chl was prepared by dissolution of Chl in water. Aqueous stock solution of Chl-KCHS complex (pH 2.90 at 20 °C) containing 1% of KCHS, 0.01% of Chl and 0.1% of HCl was prepared by dropwise addition of aqueous 0.05% Chl solution into rapidly spinning aqueous solution containing 1.25% of KCHS and 0.125% of HCl.

2.3 Spectrophotometric measurements

Absorption spectrum of Chl-KCHS solution was recorded by spectrophotometer Heλios Gamma & Delta spectrophotometers, ThermoSpectronic (Great Britain), fluorescence spectrum was recorded by Perkin Elmer fluorescence spectrophotometer LS-55 (Germany). Scan range parameters are as follows: excitation wavelength – 400 nm; emission – 550-750 nm; ex Slit – 10 nm; em Slit – 4 nm; scan speed (nm/min) – 200.

2.4 Cultivation of microorganism

Salmonella typhimurium and *Listeria monocytogenes* were used for experiments *in vitro*. *Salmonella enterica* serovar Typhimurium strain DS88 [SL5676 SmR (pLM32)], resistant to tetracycline, was kindly provided by Prof. D. H. Bamford (University of Helsinki, Finland). *L. monocytogenes* $ATC_{L3}C$ 7644 were kindly provided by the National Veterinary Laboratory (3rd passage of ATCC7644-test organism) (Vilnius, Lithuania). The bacterial culture were grown at 37 °C and maintained Luria Bertani Agar (LBA; Liofilchem, Roseto degli Abruzzi, Italy).

S. enterica and *L. monocytogenes* cultures were grown overnight (~16 h) at 37 °C in 20 ml of Luria-Bertani medium (LB; Liofilchem, Roseto degli Abruzzi, Italy) with agitation of 120 rev/min (Environmental Shaker-Incubator ES–20; Biosan, Latvia). The overnight bacterial cultures were diluted 20 times by the fresh medium containing 0.001% Chl–0.1% KCHS stock solution and used for the experiments (optical density at 540 nm (OD_{540}) was 0.164, *S. enterica* ~2.14×10^8 cfu/ml, *L. monocytogenes* ~1.25×10^8 cfu/ml)

2.5 Evaluation of Chl-KCHS antibacterial activity in laboratory medium

20 ml solution of bacterial cells suspensions with 0.001% Chl–0.1% KCHS were incubated in the dark at 37 °C, with aeration of 120 rev/min. Primarily, Chl-KCHS solution was tested against bacteria in the dark. For the photosensitization treatment, 150 µl of the samples were removed at intervals and placed into sterile flat bottom wells and then exposed to the light (λ=405 nm) for 5 min (*L. monocytogenes*; light dose 2.9 J/cm^2) and 30 min (*S. enterica*; light dose 17.3 J/cm^2).

LED based light source for the photosensitization was constructed at the Institute of Applied Sciences of Vilnius University. The emission maximum of the light source was 405 nm and the light intensity at surface of samples (at 6 cm from the light source) reached 9,6 mW/cm^2. Light dose was calculated as light intensity multiplied on irradiation time. Light intensity was measured by *3 Sigma* power and energy meter 'Coherent' equipped with a piro-electrical detector J25LP04.

The antibacterial effect of Chl-KCH3 onto bacteria was evaluated by the spread plate method. Portions (100 µl) were spread on LBA plates, which were incubated at 37 °C for 48 h, and the numbers of colonies were counted. The surviving cell populations were enumerated and expressed as $\log_{10}$ (cfu/ml).

2.6 Evaluation of Chl-KCHS antibacterial activity *in vivo*

The wheat seeds samples (each sample 100 seeds, ~4.8 g) were soaked in 0.001% Chl-0.1% KCHS solution. The control samples were soaked in 0,9% NaCl. The inoculums were incubated in the shaker (130 rev/min) in the dark at 37 °C for 60 min. Then the seeds samples were decanted in the treatment chamber in a sterile Petri dish without cover where they were exposed to light intensity 21,12 mW/cm^2 at λ=400 nm for 30 min. The control sample was not irradiated.

After treatment each sample (also control samples) was mixed with 0.9% NaCl in a sterile 100 BagPage and homogenized 60 s with a BagMixer. Then, 100 µl of appropriate dilutions (0.9% NaCl) of homogenized seeds suspension placed onto dichloran glycerol (DG18) agar (Liofilchem, Italy). All

plates were placed in the thermostat for 144 h at 30 °C. The surviving cell populations were enumerated and expressed $\log_{10}$ (cfu/g).

2.7 Statistic

The experiments were triplicated 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. The data were analyzed using Origin 7,5 software (*OriginLab Corporation*, Northampton, MA 01060, USA).

3. Results

3.1 Absorption and fluorescence spectra of chlorophyllin-chitosan complex

The representative absorption spectrum of Chl-KCHS suspension in 0.01 M PBS (1×10^{-5}) is presented in Fig. 1. It is obvious, that absorption maximum at 405 nm is characteristic for Chl-KCHS complex at these experimental conditions. Fluorescence spectrum of Chl-KCHS supports the idea that complex molecules tend to form aggregates. Fluorescence of Chl-KCHS complex at 660 nm is negligible but remarkably increases after addition of Triton-X100 which disassembles CHS-Chl complex.

3.2 Antibacterial effect of photoactivated Chl-KCHS complex on *S. enterica* and *L. monocytogenes*

The antibacterial activity of Chl-KCHS complex against *S. enterica* and *L. monocytogenes* is illustrated in Fig. 2. Generally, Gram (-) bacterium *S. enterica* was not susceptible to Chl-KCHS complex treatment in LB medium even at 0.1% concentration and incubation time (0-4 h) (Fig. 2). On the contrary, inactivation of Gram (+) *L. monocytogenes* was more significant and reached 5.4 log. Thus, data obtained in this study confirmed that photoactivated Chl-KCHS complex remarkably inactivated *L. monocytogenes* but had an insignificant effect on the *S. enterica*. Just 1 h incubation and 2.9 J/cm^2 illumination dose lead to 8 log reduction of *Listeria*. In contrast, *Salmonella* was very resistant to photoactivated Chl-KCHS complex treatment: 4 h incubation and following 17.3 J/cm^2 illumination had no effect on *Salmonella* growth (data do not differ from the control).

3.3 Antibacterial effect of photoactivated Chl-KCHS complex on contamination of wheat by total aerobic mesophils, yeast and fungi

The high antimicrobial effect of photoactivated Chl-KCHS complex offers the possibility for new effective wheat decontamination tool. Subsequently, it was determined whether naturally surface-distributed mesophiles, yeast and microfungi were susceptible to photoactivated Chl-KCHS complex treatment.

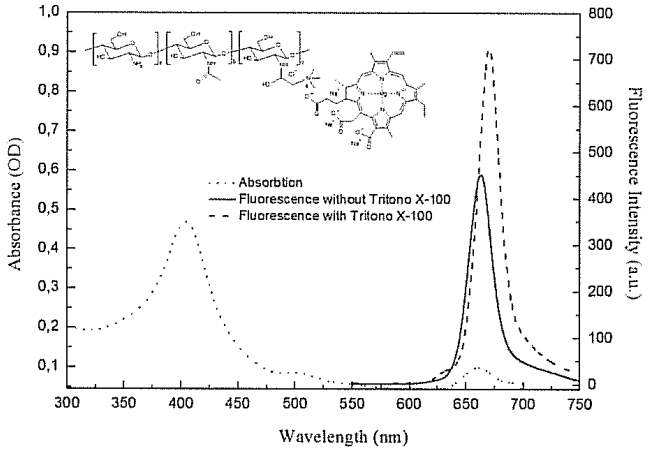


Fig. 1. Chemical formula, absorption and fluorescence spectra of 0.001% Chl–0.1% KCHS complex in 0.01 M PBS.

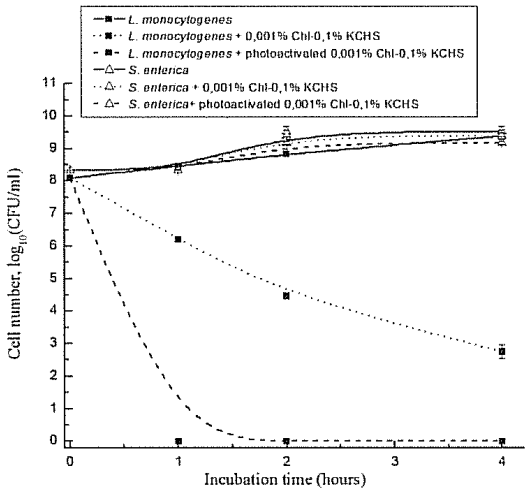


Fig. 2. Inactivation of *Listeria monocytogenes* and *Salmonella enterica* Serovar Typhimurium by photoactivated 0.001% Chl–0.1% KCHS complex in laboratory medium.

Data presented in Fig. 3 indicated that the growth of total aerobic mesophiles, yeast and microfungi on the wheat seeds in control group was 4.81 log. Thus, in treated samples after photoactivated complex treatment (incubation time– 60 min, light dose 38 J/cm^2) the amount of yeasts/ microfungi reduced by 0.68 log.

Fig. 4 illustrates the visual growth of total aerobic mesophils, yeast and fungi in control and after photoactivated Chl-KCHS complex treatment.

In conclusion, chlorophyllin-chitozan complex seems promising in reduction of microbial loud on the surface of germinated wheats.

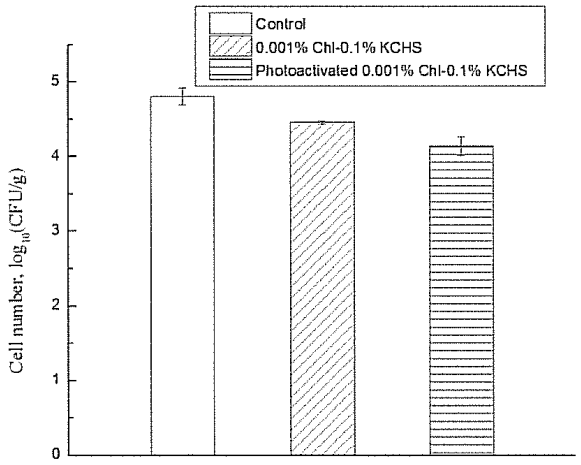
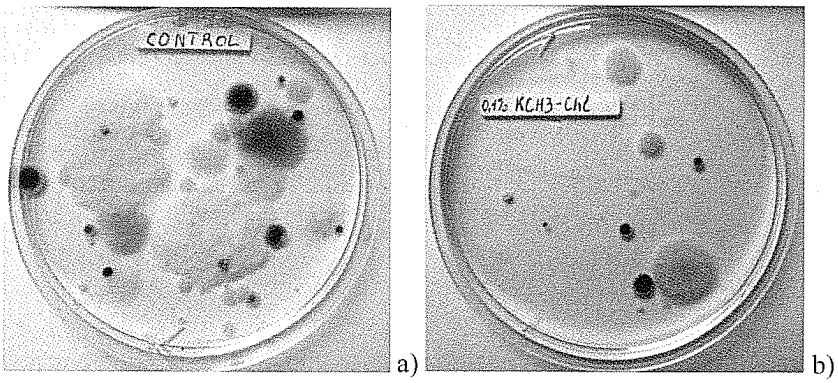


Fig. 3. Inactivation of yeasts/microfungi of the surface of wheat seeds by photoactivated of 0.001% Chl–0.1% KCHS complex.



a) b)

Fig. 4. Decontamination of wheat seeds by photoactivated 0.001% Chl–0.1% KCHS complex: untreated a) and treated b). Incubation time in 0.01 M PBS – 60 min, light dose 38 J/cm^2.

Acknowledgements

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Novel Approach to Control Microbial Contamination of Germinated Wheat Sprouts: Photoactivatedchlorophillin-Chitosan Complex

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Abstract: High resistance of bacteria and fungi to antimicrobial treatments has led to the development of innovative and alternative antimicrobial technologies. It is well-known that chitosan itself is an antimicrobial agent, and chlorophyllin is water-soluble food additive (E140) and food component which in the presence of light exerts antimicrobial properties. Complex of these two antimicrobials can be background for the development of edible active biodegradable coating.

The aim of this study was to evaluate antimicrobial efficiency of photoactivated chlorophyllin-chitosan complex against food pathogens and fungi on the surface of germinated wheat seeds. Obtained data indicate that chlorophyllin-chitosan complex in the presence of visible light (400nm) exhibits strong antimicrobial activity which can be used for decontamination of sprouts.

Keywords: Antimicrobial, Photoactivated chlorophyllin-chitosan complex.

1. INTRODUCTION

The sprouts are outstanding sources of protein, vitamins and minerals. Therefore, germinated seeds are offered as a natural nutritive product which is beneficial to health [1]. Seed contamination with harmful and pathogenic microorganisms is important as it often reduces product quality and shelf-life. Moreover it can be source of food-borne diseases and presents a potentially serious health risk to humans. However, methods recently applied for inactivation of harmful and pathogenic microorganisms are not always efficient and ecologically friendly [1, 2]. In this context, photosensitization treatment seems promising. Antimicrobial photosensitization approach is based on the interaction of three agents: photosensitizer (photoactive compound), light and oxygen. There are two main routes for photosensitizer-cell interaction. In the first case, it could form a tight complex with the surface of the cell wall. In another way, the photosensitizer is transported inside the cell, where it associates with the key structures and irreversibly damages them after photosensitization [3]. Two oxidative mechanisms of photoinactivation are considered to be implicated the inactivation of microbe. The type I pathway involves electron/hydrogen atom-transfer reactions from the photosensitizer triplet state and produce radical ions of surrounding biomolecules. Type II pathway involves energy transfer from triplet state to molecular oxygen and produce singlet oxygen. As a result, a series of cytotoxic reactions occurs in the cell [3].

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Na-chlorophyllin (Chl) is effective photosensitizer, water-soluble food additive (E140) and food component used as food colorant, in dietary supplements and in cosmetics [4]. According to our previous results, photoactivated Chl exhibited high antimicrobial activity against Gram (+) food pathogens *Listeria monocytogenes Bacillus cereus, in vitro* and *in vivo* [5-7]. Inactivation of Gram (-) bacteria with more complex cell wall structure needs longer treatment time [8].

Chitosan (CHS), a natural cationic linear polysaccharide, is produced commercially by deacetylation of chitin and is insoluble in water at pH above 6 [9]. It is characterized as nontoxic antimicrobial tool, which do not change pH, color, improves quality of fruits and vegetables and can be applied in food technologies, agriculture, medicine and environment protection [10, 11]. In addition, CHS is able to control decay of different fruits and vegetables extending their storage time [12]. Moreover, CHS can form antimicrobial films and serve as carrier of wide range of food additives, including various antimicrobials [13, 14]. In this context, chitosan film with inserted Chl may act as more efficient antimicrobial tool to improve food safety and prolong shelf-life of products. The aim of this study was to evaluate antimicrobial efficiency of photoactivetedchlorophyllin-chitosan complex (Chl-KCHS) against food pathogen *Listeria monocytogenes* and microfungus, *Botrytis cinerea*, contaminating wheat sprouts.

2. MATERIALS AND METHODS

2.1. Chemicals

Chlorophyll sodium salt (Chl) was purchased from *ROTH*, Karlsruhe, Germany. Low molecular weight chitosan (CHS, degree of deacetylation 72%,

Brookfield viscosity of 1% solution in 1% acetic acid at 20°C (140 cP) was purchased from *Aldrich*. 2, 3-epoxypropyl trimethyl ammonium chloride (EPTMAC) was purchased from *Aldrich*. Deionized water used in all experiments had specific conductivity less than 1×10^{-6}S/cm.

2.2. Synthesis of Chl-KCHS Complex

N-[(2-hydroxy-3-trimethylammonium)propyl] chitosan chloride (KCHS) with 35 mol% degree of cationization was prepared from equimolar mixture of CHS and EPTMAC according to [15].

Aqueous stock solution of KCHS containing 1% of KCHS and 0.1% of HCl was prepared dissolving in water appropriate amounts of HCl and then KCHS. Aqueous stock solution of 0.01% Chl was prepared by dissolution of Chl in water. Aqueous stock solution of Chl-KCHS complex (pH 2.90 at 20°C) containing 1% of KCHS, 0.01% of Chl and 0.1% of HCl was prepared by dropwise addition of aqueous 0.05% Chl solution into rapidly spinning aqueous solution containing 1.25% of KCHS and 0.125% of HCl.

2.3. Spectrometric Analysis

Absorption spectrum of Chl-KCHS solution was recorded by spectrophotometer Heλios Gamma & Delta spectrophotometers, *Thermo Spectronic* (Great Britain), fluorescence spectrum was recorded by *Perkin Elmer* fluorescence spectrophotometer LS-55 (Germany). Scan range parameters are as follows: excitation wavelength – 400 nm; emission – 550–750 nm; ex Slit – 10 nm; em Slit – 4 nm; scan speed (nm/min) – 200.

2.4. Cultivation of Microorganism

Listeria monocytogenes $ATC_{L3}C$ 7644 were kindly provided by the National Veterinary Laboratory (3rd passage of ATCC7644-test organism, Vilnius, Lithuania). The bacterial culture was grown at 37°C and maintained on aLuria Bertani Agar (LBA; Liofilchem, Rosetodegli Abruzzi, Italy).

L. monocytogenes cultures were grown overnight (~16 h) at 37°C in 20 mLof Luria-Bertani medium (LB; Liofilchem, Rosetodegli Abruzzi, Italy) with agitation of 120 rev/min (Environmental Shaker-Incubator ES–20; Biosan, Latvia). The overnight bacterial cultures were diluted 20 times by the fresh medium containing 0.001% Chl–0.1% KCHS stock solution and used for the experiments (optical density at 540 nm (OD_{540}) was 0.164 for*L. monocytogenes* ~1.25×10^8 CFU/mL)

2.5. Evaluation of Chl-KCHS Antibacterial Activity *in vitro*

20 mL of bacterial cells suspension with 0.001% Chl–0.1% KCHS were incubated in the dark at 37°C, with aeration of 120 rev/min (cultivation). Primarily, Chl-KCHS solution was tested against bacteria in the dark. For the photosensitization treatment, 150 μL of the samples were removed at intervals and placed into sterile flat bottom wells and then exposed to the light (*λ* = 405 nm) for 5 min (*L. monocytogenes*; light dose 2. 9 J/cm^2).

LED based light source for the photosensitization was constructed at the Institute of Applied Sciences of Vilnius University. The emission maximum of the light source was 405 nm and the light intensity reached 9.6 mW/cm^2 (6 cm from the light source) and 11.52 mW/cm^2 (3.5 cm from the light source) at the surface of samples from top and bottom LED, respectively. Light dose was calculated as light intensity multiplied on irradiation time. Light intensity was measured by *3 Sigma* power and energy meter “Coherent” equipped with a piro-electrical detector J25LP04.

The antibacterial effect of Chl-KCH3 on bacteria was evaluated by the spread plate method. Portions (100 μL) were spread on LBA plates, which were incubated at 37°C for 48 h, and the numbers of colonies were counted. The surviving cell populations were enumerated and expressed as $\log_{10}$ CFU/μL.

2.6. Evaluation of Chl-KCHS Antibacterial Activity on Wheat Sprouts

The wheat seeds samples (each sample 100 seeds, ~4.8 g) were soaked in 0.001% Chl-0.1% KCHS solution. The control samples were soaked in 0.9% NaCl. The inoculums were incubated in the shaker (130 rev/min) in the dark at 37°C for 60 min. Then the seeds samples were decanted in the treatment chamber in a sterile Petri dish without cover where they were exposed to 405 nm light for 30 min (light dose 38 J/cm^2). The control sample was not irradiated.

After treatment each sample (also control samples) was mixed with 0. 9% NaCl in a sterile 100 BagPage and homogenized 60 s with a BagMixer. Then, 100 μL of appropriate dilutions (0. 9 % NaCl) of homogenized seeds suspension placed onto dichloran glycerol (DG18) agar (Liofilchem, Italy). All plates were placed

in the thermostat for 144 h at 30°C. The surviving cell populations were enumerated and expressed $\log_{10}$ CFU/g.

2.7. Statistics

All experiments were repeated 3-4 times. 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. The data were analyzed using Origin 7.5 software (*Origin Lab Corporation*, Northampton, MA 01060, USA).

3. RESULTS

3.1. Absorption and Fluorescence Spectra of Chlorophyllin-Chitosan Complex

The representative absorption spectrum of Chl-KCHS suspension in 0.01 M PBS (1×10^{-5}) is presented in the table at Figure **1**. It is obvious, that absorption maximum at 405 nm is characteristic for Chl-KCHS complex at these experimental conditions. Fluorescence spectrum of Chl-KCHS supports the idea that complex molecules tend to form aggregates. Fluorescence of Chl-KCHS complex at 660 nm is negligible but remarkably increases after addition of Triton-X100 which disassembles CHS-Chl complex.

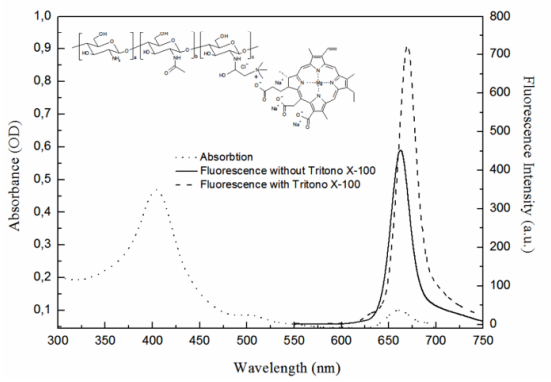


Figure 1: Chemical Formula, Absorption and Fluorescence Spectra of 0.001% Chl–0.1% KCHS Complex in 0.01 M PBS.

3.2. Antibacterial Effect of Photoactivated Chl-KCHS Complex on *L. Monocytogenes*

The antibacterial activity of Chl-KCHS complex against *L. monocytogenes* is illustratedat the Figure **2**. Inactivation of Gram (+) *L. monocytogenes* by this complex in the dark (without light activation)was rather significant and after 4 hours incubation reached 5. 4 log CFU/g. Photoactivated Chl-KCHS complex has remarkably, at much shorter incubation time, inactivated the bacteria. Just 1 h incubation and 2.9 J/cm^2 illumination dose lead to 8 log reduction of viable cell population.

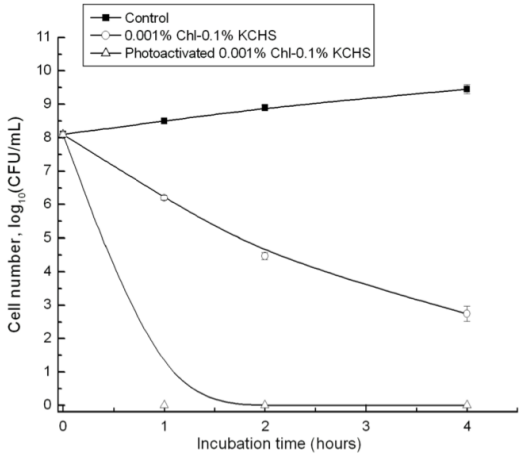


Figure 2: Inactivation of *L. monocytogenes* by photoactivated 0.001% Chl–0.1% KCHS complex *in vitro*.

3.3. Antimicrobial Effect of Photoactivated chl-KCHS Complex

Decontamination of Wheat Sprouts from Total Aerobic Mesophils, Yeasts and Fungi.

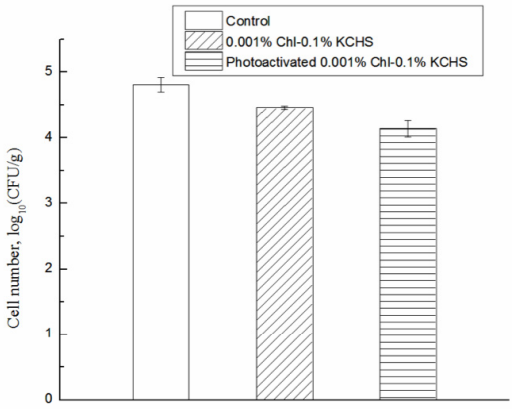
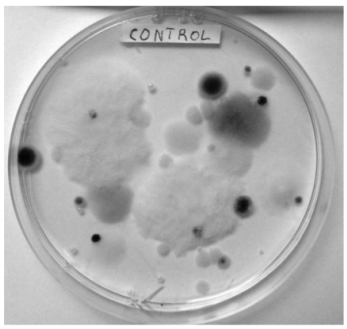
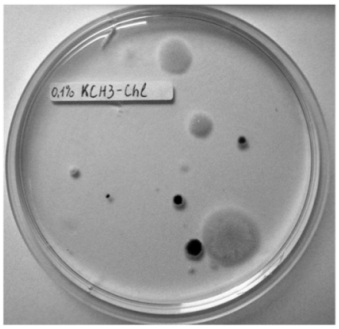


Figure 3: Decontamination of Wheat Seeds from Yeasts/Fungi by Photoactivated of 0.001% Chl–0.1% KCHS Complex.

It was determined whether naturally surface-distributed mesophiles, yeasts and fungi were susceptible to photoactivated Chl-KCHS complex treatment. Data presented in Figure **3** indicated that the growth of total aerobic mesophiles, yeasts and



(a)



(b)

Figure 4: Decontamination of Wheat Seeds by Photoactivated 0.001% Chl–0.1% KCHS Complex: **a**)- Untreated Seeds and **b**)- Treated Seeds. Incubation Time 60 min, Light Dose 38 J/cm^2.

microfungi in control sprout group increases to 4.81 log. In treated sprout samples (incubation with complex– 60 min, light dose 38 J/cm^2), the amount of yeasts/fungi reduced by 0.68 $\log_{10}$ GFU/g sample. Figure **4** illustrates the visual growth of total aerobic mesophils, yeasts and fungi after photoactivated Chl-KCHS complex treatment.

4. DISCUSSION

Despite breath taking progress in biomedical science and technology, the infectious diseases continue to be one of the important problems in the world. Obviously, existing antimicrobial technologies are not enough potent, and development of environmentally friendly and cost-effective antimicrobial technologies is in progress. In this context, photosensitization might serve as an effective and promising antimicrobial tool [16-17].

Na-chlorophyllin (Chl) is water-soluble food additive (E140) and food component used as food colorant, in dietary supplements and in cosmetics [4]. The results obtained in our previous work revealed that Chl-based photosensitization can inactivate Gram (+) pathogens *Listeria monocytogenes*$ATC_{L3}C$ 7644 and *Bacillus cereus* ATCC 12826 by 7 log *in vitro* and can clean the surface of food-packaging materials made from polyolefines [5-6]. The data obtained in the present study (Figure **2**) revealed that the population of *L. monocytogenes* decreased after treatment with chlorophyllin-chitosan complex at shorter incubation time in comparison with inactivation found after photosensitization treatment.Moreover, data presented in Figure **3** indicated that it is possible to decontaminate sprouts from total aerobic mesophiles, yeasts and microfungi, since in treated sprout samples (incubation with complex– 60 min, light dose 38 J/cm^2), the amount of yeasts/fungi reduced by 0.68 $\log_{10}$ GFU/g sample.

It is well-known that chitosan itself is an antimicrobial agent [18-19]. Antibacterial activity of CHS was assessed for a wide range of Gram (-) and Gram (+) bacteria [20]. The actual mechanism of its antimicrobial activity is not yet fully understood. There are various data concerning the ability of chitosan to disrupt outer membrane of Gram (-) bacteria. Je and Kim [21] found that chitosan and its derivatives disrupt inner and outer bacterial cell membrane.

CONCLUSIONS

Combination of antimicrobial properties of chitosan and chlorophyllin-based photosensitization can be valuable tool to combat food pathogens, yeasts and fungi. This phenomenon can be explained by the enhanced interaction of the positively charged polymer-dye complex with negatively charged bacterial cell wall which probably brings photosensitizer closer to the target cell facilitating photodestruction. Such Chlorophyllin-chitosan complexes have potential to be used for the decontamination and disinfection of different surfaces in industrial environment. Moreover such conjugates can be used for the development of biodestructive antimicrobial “smart” packaging in the future.

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Effective photosensitization-based inactivation of Gram (–) food pathogens and molds using the chlorophyllin–chitosan complex: towards photoactive edible coatings to preserve strawberries

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This study is focused on the novel approaches to enhance the inactivation of the Gram (–) food pathogen *Salmonella enterica* and harmful molds *in vitro* and on the surface of strawberries using the chlorophyllin–chitosan complex. *Salmonella enterica* (~1 × 10^7 CFU mL^{-1}) was incubated with chlorophyllin 1.5 × 10^{-5} M (Chl, food additive), chitosan 0.1% (CHS, food supplement) or the chlorophyllin–chitosan complex (1.5 × 10^{-5} M Chl–0.1% CHS) and illuminated with visible light (λ = 405 nm, light dose 38 J cm^{-2}) *in vitro*. Chlorophyllin (Chl)-based photosensitization inactivated *Salmonella* just by 1.8 log. Chitosan (CHS) alone incubated for 2 h with *Salmonella* reduced viability 2.15 log, whereas photoactivated Chl–CHS diminished bacterial viability by 7 log. SEM images indicate that the Chl–CHS complex under these experimental conditions covered the entire bacterial surface. Significant cell membrane disintegration was the main lethal injury induced in Gram (–) bacteria by this treatment. Analysis of strawberry decontamination from surface-inoculated *Salmonella* indicated that photoactivated Chl–CHS (1.5 × 10^{-5} M Chl–0.1% CHS, 30 min incubation, light dose 38 J cm^{-2}) coatings diminished the pathogen population on the surface of strawberries by 2.2 log. Decontamination of strawberries from naturally distributed yeasts/molds revealed that chitosan alone reduced the population of yeasts/molds just by 0.4 log, Chl-based photosensitization just by 0.9 log, whereas photoactivated Chl–CHS coatings reduced yeasts/molds on the surface of strawberries by 1.4 log. Electron paramagnetic resonance spectroscopy confirmed that no additional photosensitization-induced free radicals have been found in the strawberry matrix. Visual quality (color, texture) of the treated strawberries was not affected either. In conclusion, photoactive Chl–CHS exhibited strong antimicrobial action against more resistant to photosensitization Gram (–) *Salmonella enterica* in comparison with Gram (+) bacteria *in vitro*. It reduced significantly the viability of strawberry surface-attached yeasts/molds and inoculated *Salmonella* without any negative impact on the visual quality of berries. Experimental data support the idea that photoactivated Chl–CHS can be a useful tool for the future development of edible photoactive antimicrobial coatings which can preserve strawberries and prolong their shelf-life according to requirements of "clean green technology".

1. Introduction

Recently concerns about microbial food safety have dramatically increased. The center of Disease Control and Prevention (CDC) in the United States (US) reported that 48 million Americans get sick every year due to foodborne illness caused by pathogenic microorganisms.[1] Fresh produce has been increasingly implicated as the vehicle of pathogen transmission and has become the second leading cause of foodborne illnesses, which costs for instance the US economy $6.9 billion of loss in productivity and medical expenses.[2] Strawberry is a major crop with 4–5 million tons of production worldwide.[3] According to the U.S. Food and Drug Administration (FDA) survey, 1 out of 143 imported strawberry samples tested positive for *Salmonella*.[4]

The other challenge is the extremely short postharvest life of strawberries. Due to high susceptibility to mechanical injury and spoilage induced by plant pathogenic fungi[5] losses of the harvest reach 30–40% if no chemical control is applied.[6]

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Therefore, finding innovative and more effective techniques to decontaminate strawberries from foodborne pathogens and molds seems important. Data obtained in our previous study clearly indicate that photosensitization might be a useful non-thermal and non-chemical tool for the decontamination of strawberries from the Gram (+) food pathogen *Listeria*, yeasts, molds and mesophils distributed on the surface of strawberries.[7] Most important is the fact that this treatment can expand the shelf-life of strawberries by 2 days,[7] and it is comparable with the antimicrobial effects of high power pulsed light.[8] Meanwhile, the lower susceptibility of Gram (−) pathogens to photosensitization is well documented, and remains the main disadvantage of this treatment.[9]

Chitosan is a biodegradable, nontoxic polymer produced by chitin deacetylation. It demonstrates antimicrobial activity against a wide variety of bacteria, filamentous fungi and yeasts. The antimicrobial activity of chitosan is a complex superposition of many chemical, physical and environmental factors and by no means depends on the specificity of the microorganism.[10] Most interesting is the fact that chitosan is a nutritional supplement which possesses excellent film-forming properties. Chitosan-based edible coatings reduced microbial contamination of strawberries and slightly extended their shelf-life, maintaining nutritional quality.[11]

The aim of this study is to increase the susceptibility of the Gram (−) food pathogen *S. enterica* to chlorophyllin-based photosensitization combining it with the antimicrobial properties of positively-charged chitosan by immobilization of chlorophyllin into a chitosan polymer. The impact of the photoactivated Chl–CHS coating on microbial contamination and visual quality of strawberries will be evaluated as well.

2. Materials and methods

2.1. Chemicals

Non-copperized chlorophyll sodium salt (Chl) was obtained from Roth (Karlsruhe, Germany). Low molecular weight chitosan (CHS, Brookfield viscosity of 1% (all concentrations in percent refers to w/v) solution in 1% acetic acid at 20 °C 140 cP) was obtained from Aldrich (Saint Louis, USA). Triton X-100 was purchased from MERCK (Darmstadt, Germany). Deionized water used in all experiments had specific conductivity less than 1×10^{-6} S cm^{-1}.

An aqueous stock solution of CHS (pH = 2.4 at 20 °C) containing 1% of CHS and 0.18% of HCl was prepared by dissolving in water appropriate amounts of HCl and then CHS. An aqueous stock solution of 1.5×10^{-5} M Chl was prepared by dissolution of Chl in water. An aqueous stock solution of the chlorophyllin–chitosan complex (Chl–CHS) (pH = 2.4 at 20 °C) containing 1% of CHS, 1.5×10^{-5} M Chl and 0.18% of HCl was prepared by dropwise addition of aqueous 0.05% Chl solution into a rapidly spinning aqueous solution containing 1.25% of CHS and 0.23% of HCl. After addition of the Chl–CHS complex to the bacterial suspension in NaCl, the pH of the final bacterial suspension changed to 3.95.

2.2. Absorption and fluorescence measurements of the Chl–CHS complex

The absorption spectrum of Chl–CHS solution was recorded by using Helios Gamma & Delta spectrophotometers, Thermo-Spectronic (Leicestershire, Great Britain), and the fluorescence spectrum was recorded by using a Perkin Elmer fluorescence spectrophotometer LS-55 (Rodgau, Germany). The scan range parameters were as follows: excitation wavelength – 405 nm; emission – 550–750 nm; ex. slit – 10 nm; em. slit – 4 nm; scan speed (nm min^{-1}) – 200. A 3 mL quartz cuvette (Hellma-analytics QS, Mullheim, Germany) was used for measurements. The 1.5×10^{-5} M Chl–0.1% CHS complex diluted by using 0.9% NaCl was used for absorption and fluorescence measurements. To observe the monomeric Chl forms of this complex 20 μL of Triton X-100 was added to 20 mL of the suspension.

2.3. Cultivation of the microorganism

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).

S. enterica was grown in Luria–Bertani medium (LB) (Liofilchem, Roseto Degli Abruzzi, Italy; pH = 7.1) and incubated overnight at 37 °C. The overnight culture was 20-times diluted with fresh LB medium (optical density at 540 nm (OD_{540}) was 0.164) and grown at 37 °C to the mid-log phase (5×10^8 CFU mL^{-1}, OD_{540} = 1.3). The cells were then harvested by centrifugation (10 min, 6 °C, 3574*g*) (MPW-260R; MPW Med. Instruments, Warsaw, Poland) and resuspended in a 1×10^{-1} M PBS buffer (pH = 7.4) and normal saline 0.9% NaCl (pH 7.3), depending on treatment requirements, to give ~2.5×10^9 CFU mL^{-1}. These stock suspensions were diluted to approximately 1×10^7 CFU mL^{-1} and immediately used for the experiments.

2.4. Scanning electron microscopy (SEM)

The effect of the Chl–CHS complex on the morphology of *Salmonella* was examined by SEM. The bacterial suspension (approximately 1×10^7 CFU mL^{-1}) containing the 1.5×10^{-5} M Chl–0.1% CHS complex was incubated at 37 °C in the dark. In the next step, the samples consisting of 20 μL of the bacterial suspension were withdrawn, transferred to aluminum stubs, air-dried and sputter coated with 15 nm gold layer using Q150T ES sputter coater (Quorum Technologies, Lewes, England). The scanning was performed with an Apollo 300 (CamScan, Bingham, UK) scanning electron microscope at an accelerating voltage of 20 kV.

2.5. Light sources for inactivation of bacteria

An InGaN light emitting diode (LED) array (LED Engine, San Jose, USA; Inc. LZ1-00UA00) was used for the construction of the light source for the photoinactivation of bacteria. It consisted of an illumination chamber and a supply unit (Fig. 1A). A cooling system was integrated in the light prototype to dissipate heat from the source and minimize any heat transfer to the sample. LED emission maximum was at 405 nm with a

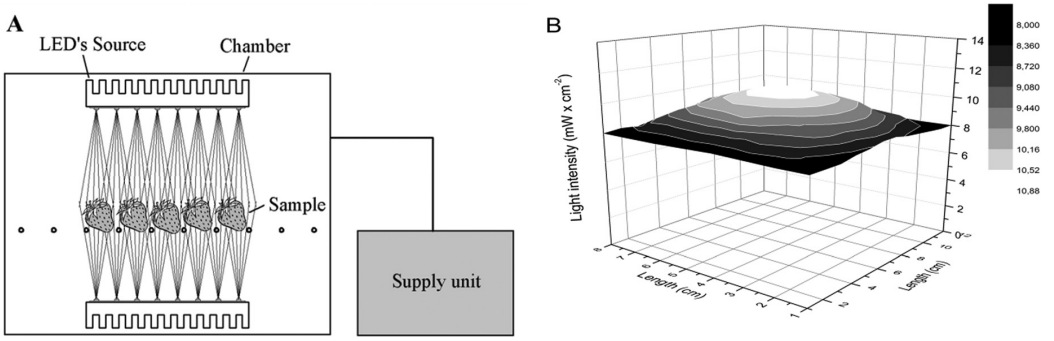

Fig. 1 Schematic presentation of LED-based light source prototype (A) and three-dimensional distribution of average light intensity from the top and bottom (B).

band width of 13 nm at full-width half maximum. Two rectangular 6 × 10 arrays (top and bottom), consisting of 60 LEDs, powered by a 20 V DC power supply were integrated in the chamber. The light intensity at the surface of the samples from top and bottom LED reached approximately 10 mW cm^{-2} (6 cm from the light source) and 11 mW cm^{-2} (3.5 cm from the light source), respectively. The light intensity was measured by using a 3 Sigma power and energy meter "Coherent" (California, USA) equipped with a piro-electrical detector J25LP04. The light dose was calculated as light intensity multiplied by irradiation time. The sample exposure time was adjusted according to the equation:

$$E = P \cdot t, \quad (1)$$

where E is the energy density (dose) in J cm^{-2}, P is the irradiance (light intensity) in W cm^{-2}, and t is the time in seconds. A three-dimensional model of distribution of power density of the emitted light from the top and bottom in the prototype is presented in Fig. 1B. Almost the same power density distribution was registered from LEDs in the bottom of the prototype; however, distributions from the top and from the bottom cannot be placed in one picture since it would overlap. The variation of light intensities on the illumination square was insignificant, since we use just its central part (±0.5 mW cm^{-2}).

2.6. Inactivation of *Salmonella* by different treatments

Aliquots of 20 mL of *S. enterica* suspension (~1 × 10^7 CFU mL^{-1} in 0.9% NaCl) containing 0.1% CHS (in 0.9% NaCl) (1), 1.5 × 10^{-5} M Chl (in 0.1 M PBS) (2), 1.5 × 10^{-5} M Chl–0.1% CHS complex in the dark (in 0.9% NaCl) (3), photoactivated 1.5 × 10^{-5} M Chl–0.1% CHS complex (in 0.9% NaCl) (4), just illuminated (5) and control (not treated at all) (6) were incubated in 50 mL flasks for cell culture cultivation in a shaker (130 rev min^{-1}) at 37 °C. The samples were removed after 1 min, 15 min, 30 min, 60 min and 120 min. 150 μL of the samples were placed in sterile flat bottom wells and then the samples (4–5) were exposed to light (light dose 38 J cm^{-2}). The antibacterial efficiency of treatments was evaluated by the spread plate method, comparing the viability of the treated and not treated bacteria. 100 μL of a diluted bacterial suspension after treatment was surface inoculated on a separate LB agar (LBA) plate. Afterwards the LBA plates were kept for 24 h at 37 °C. Bacterial populations were recalculated from CFU mL^{-1} to $\log_{10}$ mL^{-1}.

2.7. Evaluation of membrane integrity of treated bacteria

The bacterial cell membrane integrity was examined by the determination of the release of the intracellular material with absorption at 260 nm (OD_{260})[12] and 280 nm (OD_{280}).[13] The bacterial suspension (1 × 10^7 CFU mL^{-1}) containing the 1.5 × 10^{-5} M Chl–0.1% CHS complex (at 37 °C in the dark) was irradiated (doses of 25 and 38 J cm^{-2}). Aliquots of the 1.5 mL cell suspension were taken out and filtered to remove the bacteria. The UV absorbance of the cell supernatant at 260 nm and 280 nm was determined using a spectrophotometer (Helios Gamma & Delta ThermoSpectronic, Leicestershire, Great Britain).

2.8. Decontamination of strawberries from yeasts/molds by coating them with Chl–CHS and treating them with light

Strawberries (*Fragaria* × *ananassa* Duch.) in partially ripe stage were purchased from a local supermarket and used within 1 day. Some strawberries with natural microflora were soaked for 30 min in 0.1% CHS (1, chitosan coating), 1.5 × 10^{-5} M Chl–0.1% CHS (2, dark toxicity, Chl–CHS coating), 1.5 × 10^{-5} M Chl–0.1% CHS (3, photoactivated, Chl–CHS coating), and 1.5 × 10^{-5} M Chl (4, photoactivated), while other strawberries were just illuminated (5) or not treated (6, control). Samples 3, 4 and 5 were placed in the treatment chamber in sterile Petri dishes, dried and exposed to 405 nm light for 60 min (light dose 38 J cm^{-2}). The CHS (1), dark toxicity (2) and control (6) samples were not illuminated. 1 g of each strawberry and 9 ml of 0.9% NaCl solution was placed in sterile BagPage (Interscience, Saint-Nom-la-Bretèche, France) and homogenized using a BagMixer (Interscience, Saint-Nom-la-Bretèche, France) (in detail ref. 7 and 8). The antifungal activity of the photoactivated Chl–CHS complex against molds was evaluated

by the spread plate method.[7] 100 μL of a diluted bacterial suspension after treatment was surface inoculated on separate dichloran glycerol (DG18) agar (Liofilchem, Italy). Afterwards the plates were kept for 144 h at 30 °C. Fungal populations were recalculated from CFU g^{-1} to $\log_{10}$ g^{-1}. Every sample consisted of 1 berry, and the experiments were repeated 3–6 times.

The visual quality of strawberries was evaluated by checking spots, induced by the growth of spoilage microorganisms according to methods described in ref. 7.

2.9 Inactivation of inoculated *S. enterica* on the surface of strawberries by coating them with Chl–CHS complex and light

The target bacterium, *Salmonella enterica* was cultivated and prepared as mentioned above in section 2.3. The berries were soaked in a bacterial suspension for 30 min and incubated in the dark at 37 °C. After inoculation, the strawberries were soaked in 0.1% CHS (1, chitosan coating), 1.5×10^{-5} M Chl–0.1% CHS (2, dark toxicity, Chl–CHS coating), in 1.5×10^{-5} M Chl–0.1% CHS (3, photoactivated, Chl–CHS coating), in 1.5×10^{-5} M Chl (4, photoactivated using 19 J cm^{-2}), in 1.5×10^{-5} M Chl (5, photoactivated using 38 J cm^{-2}), in 0.9% NaCl solution (6, only illuminated) or in 0.9% NaCl solution (7, control) for another 30 min. Then samples 3–6 were placed in the treatment chamber on a sterile quartz glass plate, dried and exposed to 405 nm light for 60 min (light dose 38 J cm^{-2}) or 30 min (light dose 19 J cm^{-2}). The control (neither treated with the complex nor with light) samples and samples treated with 0.1% CHS were not illuminated. The following preparation of samples was the same as described in section 2.8, except for the growth medium for *Salmonella* which was selective Brilliant Green Lactose Sucrose Agar (Roth, Karlsruhe, Germany), and the growth conditions were 24 h at 37 °C. Every sample consisted of 1 berry. The experiment was repeated 3–4 times.

2.10. Electron spin resonance, EPR

The EPR spectra were recorded with a Bruker Elexsys E580 FT-EPR spectrometer (Billerica, USA) working in the X-band.

Before recording the spectra the surface of the treated and not treated strawberries was peeled, and the peelings were homogenized. The capillaries (BLAUBRAND micropipettes, intraMark, Hinckley, Great Britain) were filled with 1 g of treated strawberry. After that capillaries were put into the standard EPR tube.

2.11. Statistical analysis

The experiments were triplicated for each set of exposure. A standard error was calculated for every experimental point and marked in the figure as an error bar. The bars were sometimes too small to be visible. The data were analyzed using Origin 7.5 software (OriginLab Corporation, Northampton, MA 01060, USA). The significance of the results was assessed by analysis of variance (ANOVA). A value of $p < 0.005$ was considered as significant.

3. Results

3.1. Absorption and fluorescence spectra of Chl–CHS complex

In order to confirm the structure of the chlorophyllin–chitosan complex the absorption and fluorescence spectra were analyzed. Fig. 2 indicates that the absorption spectrum of the Chl–CHS complex in solution had peaks at λ = 405 nm and at λ = 652 nm. The fluorescence spectra presented in the same figure indicated very low (100 a.u.) fluorescence intensity (peak at 648 nm) of the complex. Just by adding 0.001% Triton to the complex solution monomerized chlorophyllin and increased the fluorescence intensity to 660 a.u. (peak at 674 nm). Taking into account the structure of both compounds, the interaction between the positively charged chitosan NH_3^+ group and negatively charged chlorophyllin COO^- group is most probable (Fig. 2).

3.2. Inactivation of *Salmonella enterica*

Multiple experimental data confirmed that the light alone at 38 J cm^{-2} dose did not diminish the viability of bacteria (Luksiene *et al.*, 2010,[34] Buchovec *et al.*, 2009[44]). The dark toxicity of Chl to *S. enterica* was negligible, since the cell viability after 120 min incubation reduced only by 0.12 log. Incubation of the cells with Chl (0–120 min) and subsequent illumination with visible light (405 nm, light dose 38 J cm^{-2}) decreased the viability of the cells more considerably: in this case the photosensitization treatment led to 1.8 log reduction (Fig. 3).

The antimicrobial properties of the Chl–CHS complex were assessed comparing its antimicrobial efficiency with that of CHS alone.

Thus, dark toxicity of the Chl–CHS complex slightly depends on the incubation time. The viability of *Salmonella* incubated with CHS alone (0–120 min) was diminished by 2.15 log. It indicated that the Chl–CHS complex exhibited some antibacterial action which was close to that of CHS alone

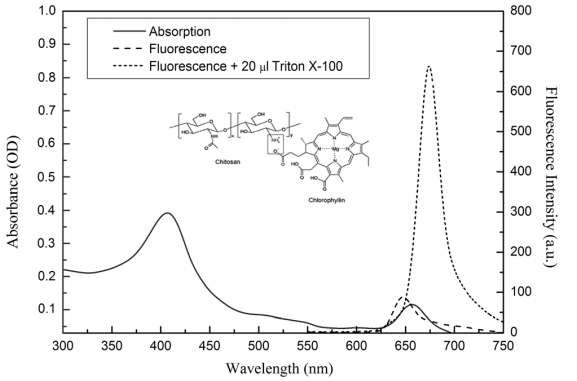

Fig. 2 Chemical formula, absorption and fluorescence spectra of 1.5×10^{-5} M Chl–0.1% CHS solution in 1×10^{-1} M PBS (pH 6.9) and fluorescence spectra of 0.001% Triton X-100 solution in 1.5×10^{-5} M Chl–0.1% CHS.

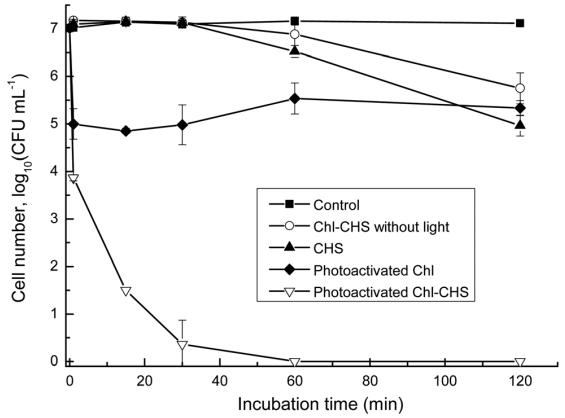

Fig. 3 Inactivation of *Salmonella enterica* as a function of incubation time when bacteria were treated with: photoactivated 1.5×10^{-5} M Chl (light dose 38 J cm^{-2}), 0.1% CHS, and photoactivated 1.5×10^{-5} M Chl–0.1% CHS complex (light dose 38 J cm^{-2}) in saline. Every point is the average of 3–6 experiments, and error bars sometimes are too small to be clearly visible.

(2.15 log). Just photoactivation of this complex drastically reduced the viability of *Salmonella* by 7.01 log at 2 times shorter incubation time.

Since different experimental conditions (6 samples) may change the pH of the bacterial suspension and hence affect the viability of bacteria it was necessary to measure the pH values in all the samples. It was determined that the pH value of the bacterial suspension in 10^{-1} M PBS shifted from 7.4 to 6.8 when the cell suspension was mixed with Chl or the Chl–CHS complex. However, when PBS was replaced by 0.9% NaCl, the pH value after mixing with Chl or the Chl–CHS complex decreased from 7.3 to 3.95.

In the next step it was important to assess the interaction of Chl–CHS–*Salmonella*. SEM images, presented in Fig. 4 indicate that the Chl–CHS biopolymer covered the entire surface of this Gram (–) bacterium.

3.3. Evaluation of cell membrane integrity in *S. enterica* after treatment with photoactive Chl–CHS complex

The effects of photoactivated Chl–CHS on bacterial membrane integrity were assessed by measuring the optical density at 260 nm (OD_{260}) (DNA absorption peak) and 280 nm (OD_{280}) (protein absorption peak) of cell free filtrates (supernatant) in the control and treated samples (Fig. 5). The results indicated that the release of intracellular material absorbing at $\lambda_{260\ nm}$ and $\lambda_{280\ nm}$ in the control supernatant was insignificant and did not depend on the light dose (light dose 0–46.8 J cm^{-2}). On the contrary, the release of intracellular components (both absorbing at $\lambda_{260\ nm}$ and $\lambda_{280\ nm}$) increased while increasing the light dose. For instance, absorption at $\lambda_{260\ nm}$ increased from 0.01 OD to 0.14 OD and absorption at $\lambda_{280\ nm}$ increased from 0.01 OD to 0.1 OD, when *S. enterica* was treated with the photoactivated Chl–CHS complex (60 min incubation with Chl and afterwards illuminated, light dose 38 J cm^{-2}).

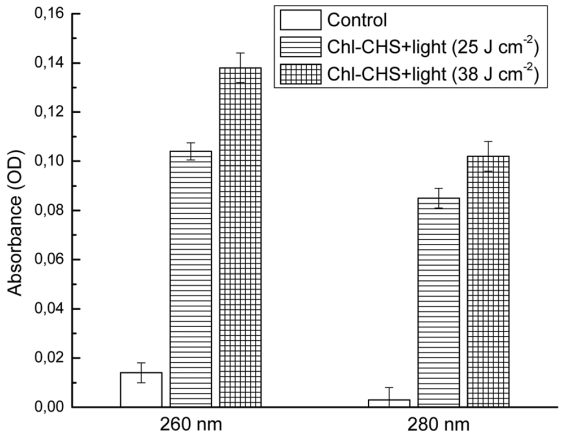

Fig. 5 Effects of photoactivated 1.5×10^{-5} M Chl–0.1% CHS complex on the leakage of UV-absorbing materials at 260 and 280 nm of *S. enterica* (20 min and 30 min illumination, light doses 25 J cm^{-2} and 38 J cm^{-2} respectively). Every point is the average of 3 experiments.

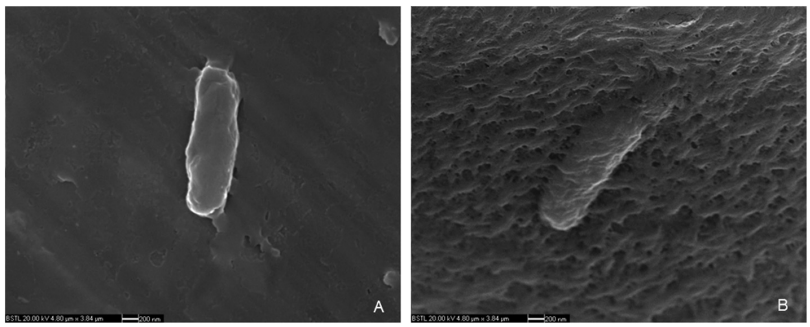

Fig. 4 Scanning electron microscopy image of *Salmonella enterica* Serovar *Typhimurium* strain DS88 (SL 5676 Smr pLM2) cells after treatment by 1.5×10^{-5} M Chl–0.1% CHS complex: not treated control (A), treated bacteria (B).

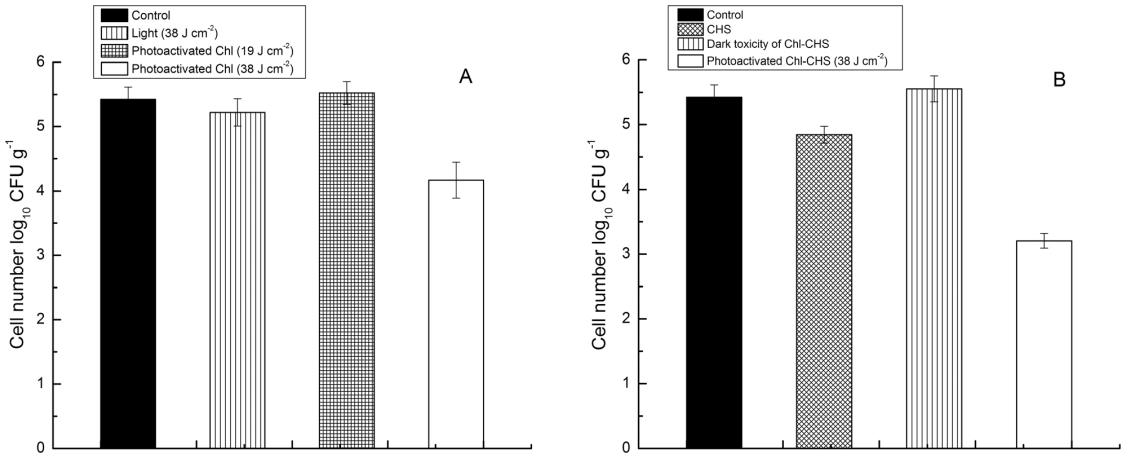

Fig. 6 (A) Inactivation of *Salmonella enterica* Serovar *Typhimurium* strain DS88 inoculated on the surface of strawberries by Chl-based photosensitization: 405 nm light (light dose 38 J cm^{-2}) and photoactivated 1.5×10^{-5} M Chl (light doses 19 and 38 J cm^{-2}); (B) inactivation of *Salmonella enterica* Serovar *Typhimurium* strain DS88 inoculated on the surface of strawberries by 1.5×10^{-5} M Chl–0.1% CHS complex: 0.1% CHS (incubation time 30 min), Chl–CHS dark toxicity (incubation time 30 min), and photoactivated 1.5×10^{-5} M Chl–0.1% CHS complex (light dose 38 J cm^{-2}, incubation time 30 min). Every point is the average of 3 experiments, dark toxicity and 405 nm light show no significant difference from control ($p > 0.005$).

3.4. Microbial decontamination of strawberries by different treatments

It is clear from Fig. 3 that *S. enterica* has low susceptibility to Chl-based photosensitization, and just photoactivated Chl–CHS reduced the pathogen population by 7 log. It was important to test whether *S. enterica* inoculated on the strawberries can be effectively inactivated by photoactivated Chl–CHS. Thus, data presented in Fig. 6A indicate that in control strawberries 5.4 log per g *Salmonella* counts have been found. Exposure to light (405 nm) alone even at a higher dose (38 J cm^{-2}) did not kill the cells. The treatment of strawberries with Chl-based photosensitization (1.5×10^{-5} M Chl) using a higher light dose (38 J cm^{-2}) reduced *S. enterica* viability by 1.3 log. Afterwards the effects of chitosan and the photoactivated Chl–CHS complex on the decontamination of strawberries have been evaluated (Fig. 6B). It is clear that the antimicrobial activity of 0.1% CHS alone (incubation time 30 min) against *S. enterica* was rather low, since it reduced the microbial load from 5.4 log to 4.8 log. The dark toxicity of Chl–CHS at 30 min incubation was insignificant and did not differ from the control (5.5 log). But a remarkable decrease of viable pathogens was observed (from 5.4 log to 3.2 log) after the illumination of strawberries coated with Chl–CHS (1.5×10^{-5} M Chl–0.1% CHS, 30 min, λ = 405 nm, dose – 38 J cm^{-2}).

As it was mentioned above strawberries are highly contaminated with molds and yeasts which is the main reason for their fast spoilage. Thus, naturally contaminated berries (4 log) were coated with Chl–CHS for 30 min and afterwards illuminated with a higher dose of visible light (38 J cm^{-2}) since molds exhibited a lower susceptibility to photosensitization than bacteria. Data presented in Fig. 7 allowed us to compare the antimicrobial efficiencies of different treatments.

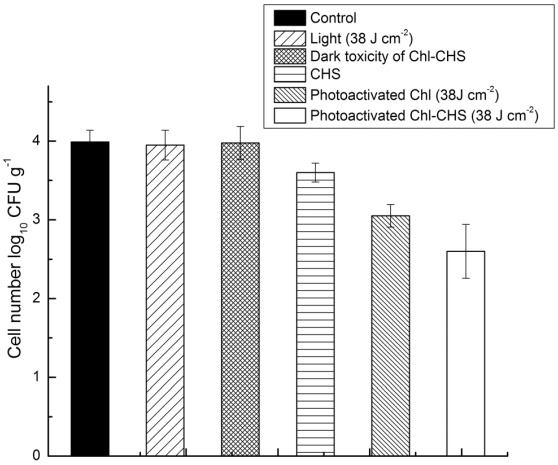

Fig. 7 Comparative analysis of different antimicrobial tools: efficiencies of inactivation of yeasts/molds on the surface of strawberries (405 nm light (light dose 38 J cm^{-2}), dark toxicity of 1.5×10^{-5} M Chl–0.1% CHS complex (incubation time 30 min), 0.1% CHS (incubation time 30 min), photoactivated 1.5×10^{-5} M Chl (light dose 38 J cm^{-2}) and photoactivated 1.5×10^{-5} M Chl–0.1% CHS complex (light dose 38 J cm^{-2}, incubation time 30 min). Every point is the average of 3–6 experiments, dark toxicity and 405 nm light show no significant difference from control, ($p > 0.005$).

The obtained data indicated that light alone or Chl–CHS without light had no effect on natural contamination of strawberries. Chitosan alone diminished the contamination of strawberries by 0.4 log, whereas Chl-based photosensitization reduced yeasts and molds up to 0.9 log. But the highest inacti-

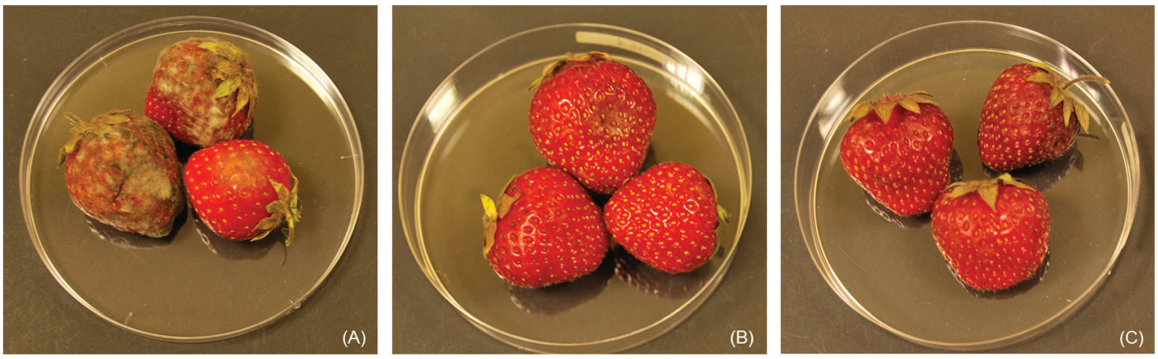

Fig. 8 Visual quality of strawberries 4 days after treatment: control berries (A); berries coated with chlorophyllin–chitosan (dark toxicity) (B), and berries treated with photoactivated chlorophyllin–chitosan (C).

vation of yeasts and molds was found when strawberries were treated with the photoactivated Chl–CHS coating (1.4 log).

3.5. Visual quality of treated strawberries

The overall appearance of the treated strawberries during storage at 22 ± 2 °C for 4 days was examined. Generally, the visual overall quality of strawberries gradually decreased over storage time. Our data on the visual quality of the control, strawberries coated with Chl–CHS without illumination, and strawberries treated with photoactivated Chl–CHS (in every case 60 strawberries have been used) indicate that it is possible to achieve some delay of spoilage when berries are coated with Chl–CHS and illuminated. For instance, as shown in Fig. 8A, 4 days after treatment control strawberries were totally infected (visually detected spots of infection), whereas the coating of strawberries with chlorophyllin–chitosan (Fig. 8B, dark toxicity) reduced the natural spoilage. It is obvious that the photoactivated Chl–CHS complex (Fig. 8C) was the most effective tool in delaying strawberry spoilage.

3.6. Detection of free radicals in treated strawberries by electron paramagnetic resonance (EPR)

The data presented in Fig. 9 show the EPR spectra of the control and treated by Chl–CHS strawberries in a wide field range (from 10 mT to 600 mT). The spectrum consists of typically 6 signals which were separated from each other by ~9 mT. According to Raffi and Stocker[14] these signals from 320 mT to 380 mT belong to Mn^{2+} which is normally present in strawberries in measurable amounts. The number of lines from the hyperfine interaction was determined by using the formula:

$$n = 2\mathrm{NI} + 1,$$

where n is a number of spectral lines, N is the number of equivalent nuclei, and I is the spin.

In our case, $N = 1$ and $I = 5/2$ from the manganese nucleus, thus the number of spectral lines was 6.

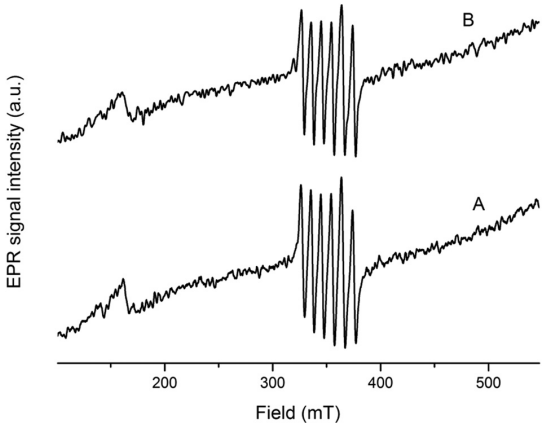

Fig. 9 EPR spectra of strawberries: control (A) and (B) treated with photoactivated Chl–CHS strawberries. Mn^{2+} lines (g = 1.87608, 1.92801, 1.98124, 2.03521, 2.09085 and 2.14451).

According to P. Leveque *et al.*[15] the signal in the lower field (170 mT) belongs to Fe^{3+}. Comparison of the spectra of the control and treated strawberries indicated that this treatment did not induce additional free radicals in the matrix of the strawberry.

4. Discussion

In order to increase the susceptibility of Gram (−) pathogens to Chl-based photosensitization the complexation of Chl with CHS was performed. Chitosan (polyβ-(1,4)-acetyl-D-glucosamine) is a cationic linear polysaccharide, obtained from a deacetylated derivative of chitin – the most abundant polysaccharide in nature after cellulose.[16] It is a tasteless fiber, non-toxic and biodegradable. Moreover, chitosan is a nutritional supplement which exhibits film-forming properties.

These features enable us to apply it as an edible coating for different types of foods.[17]

The results presented in this study clearly indicate that inactivation of the Gram (–) pathogen *S. enterica* can be enhanced by combining the antimicrobial properties of Chl-based photosensitization with that of chitosan (Fig. 3). It is clear that the antimicrobial properties of CHS alone are insignificant at short incubation times (0–30 min)(0 log). At a longer incubation time (120 min) it reduced the viability of *S. enterica* by 2 log. Chl-based photosensitization inactivated *Salmonella* by 2 log but not more, and the inactivation efficiency did not depend on the incubation time (Fig. 3). This enables us to presume that Chl interacts with the bacterium just superficially. A remarkable and very fast decrease of *Salmonella* viability (7 log) was observed when the bacteria were treated with the photoactivated Chl–CHS complex (light dose 38 J cm^{-2}). By no means, a question arises, whether low pH (in final Chl–CHS cell suspension in 0.9% NaCl, pH = 3.95) or light alone (405 nm) can diminish the *Salmonella* population. Data, published in our previous studies,[18,19] indicated that the *Salmonella* cells preserved their 100% viability when suspended and incubated for a rather long time (120 min) in 0.9% NaCl acidified by HCl to pH 4.6. Thus, just a minor impact of pH on the viability of Chl–CHS treated *Salmonella* can be anticipated. In 2012 Murdoch *et al.*[20] published data about the possibility to inactivate *Escherichia*, *Salmonella*, *Shigella*, *Listeria*, and *Mycobacterium* in suspension by LED-based light (405 nm). Meanwhile, statistically significant inactivation of *Salmonella* was achieved at a light dose of 150 J cm^{-2}, whereas in our experiments just 38 J cm^{-2} light dose has been used.

In order to understand whether Chl–CHS interacts with the bacterial surface the analysis of SEM images was performed. Data clearly indicate that *Salmonella*, incubated with 1.5×10^{-5} M Chl–0.1% CHS is fully covered by it (Fig. 4). Thus, the main target of the photoactivated Chl–CHS complex might be the cell membrane. As evidence, the intensive release of intracellular components absorbing at $\lambda_{260\ nm}$ and $\lambda_{280\ nm}$ (DNA and proteins) was detected after this treatment. Moreover, the release of intracellular components to some extent depended on the light dose. This might be addressed to the intensive membrane disintegration induced by the photoactivated Chl–CHS complex (Fig. 5). Chl being negatively-charged has weak interactions with the negatively charged *Salmonella*. But immobilization of Chl into a positively-charged CHS polymer enhanced the electrostatic interaction of Chl with the bacterium. After the illumination of the cells incubated with the complex multiple cell membrane injuries and effective killing were triggered.

The conventional treatment to reduce the microbial load on the surface of fruits is based on preharvest disease control by fungicides. As a result, multiple fungicide residues were found in more than 60% of strawberries.[21] Moreover, some aggressive compounds are harmful to humans and the environment.[22] Most important is the fact that all harmful microbes have developed high resistance to the fungicides. To combat the microbes conventional water-based sanitizers are not enough effective.[23] The widely accepted hypochlorite solution (NaOCl) (200 μg mL^{-1}, 2 min incubation) reduced the microbial contamination of strawberries just by 0.45 log.[24] Moreover, hypochlorous acid interacts with organic matter and releases chlorine, which eventually causes the formation of highly mutagenic compounds like trihalomethanes.[25,26] Ultrasound takes a short time (5–10 min), meanwhile, a power higher than 60 W diminished significantly the quality of berries.[27] An emerging approach to control strawberry microbial contamination is atmospheric pressure cold plasma (ACP). Misra *et al.*[28] observed that the total mesophiles and yeasts/molds on strawberries treated for 5 min with ACP were reduced by 2 log within 24 h after treatment. Meanwhile it is difficult to control this process. According to Alexandre *et al.*[24] UV treatment reduced the spoilage by 1 log when strawberries were treated at 4 °C. The International Consultative Group on Food Irradiation[29] allows irradiation of strawberries with the maximum dose of 3 kGy. Yu *et al.*[30] approved that this dose extended the shelf-life of berries by a factor of 2, but induced significant changes in texture and color. Moreover, the irradiated fruits are not popular among consumers.[31,32]

Photosensitization seems to offer a promising alternative as an effective non-thermal antimicrobial treatment which is environmentally friendly, saves water and energy at very reasonable costs.[33] After the spraying of the photosensitizer on the surface of the fruit most surface-distributed pathogens, harmful bacteria, viruses and molds bind to the photosensitizer.[34–37] The following illumination of fruits with light induced photocytotoxic reactions and death in surface-attached microorganisms without any harmful effects on the environment.[38–40]

It is obvious that not every photosensitizer which is of high chemical purity and exhibits high killing efficiency can be used for food safety purposes. In this case the photosensitizer must fulfil additional mandatory requirements, such as low cost, status of food additive or food component, which works at a very low concentration with no effects on the nutritional as well as organoleptic properties of the foods.[33,36] Hence, Chl is a copper-free water-soluble food additive (E140) used as a food colorant in dietary supplements and in cosmetics.[41] According to our data Chl interacted with the bacterial wall/outer membrane and just after the necessary light dose destroyed its integrity.[33,36] Thus, most important is the fact that this treatment has low mutagenicity, and microbes did not develop resistance to it.[42] The main disadvantage of photosensitization is the lower susceptibility of Gram (–) pathogens to neutral or negatively-charged photosensitizer-based photoinactivation.[43,44]

It must be mentioned that over the last decade interest has been rapidly growing in the development of bio-based packaging of fruits. It can enhance the safety and preserve nutritional/sensory attributes of fruits. Moreover, it reduces environmental pollution by non-biodegradable packaging. Such an edible coating can control water migration both in and out of the fruit in order to maintain the desired moisture content. In addition, it protects fruits from contamination, inhibits microbial proliferation and extends the shelf-life of

products.[11,16,45] For instance, chitosan coating prevented mechanical injury to perishable berries, reduced moisture losses, controlled gas (CO_2, O^{-2}) and extended the shelf-life of strawberries.[46] Thus, in the next step it was important to evaluate whether *Salmonella* inoculated on the surface of strawberries can be controlled by a photoactivated Chl–CHS coating. Data presented in Fig. 6 reveal that this treatment diminished *Salmonella* on strawberries by 2.2 log, whereas CHS alone by just 0.6 log. It is obvious that a photoactivated Chl–CHS coating is really an effective tool against Gram (–) *S. enterica* distributed on the surface of the strawberry. Moreover, the antimicrobial activity of the coating at longer incubation times must be stronger, since the antimicrobial effects of chitosan depended on the time (Fig. 3).

In the next step the efficiency of the photoactivated Chl–CHS coating against naturally distributed yeasts/molds on the surface of strawberries was evaluated. The data presented in Fig. 7 indicate that Chl-based photosensitization (1.5×10^{-5} M) reduced the naturally surface-attached microbes by 0.9 log. The antimicrobial effect of chitosan coating at a short incubation time is very mild (0.4 log). The highest inactivation of yeasts/molds was found when strawberries were treated with the photoactivated Chl–CHS coating (1.4 log). By no means, the irregularity and the different light reflecting properties of the strawberry surface can possibly account for the lower antimicrobial efficiency of the Chl–CHS coating in comparison with the data *in vitro*.[8] It must be emphasized that the antimicrobial efficiency of this treatment can be enhanced by more powerful LEDs.

The visual quality of the treated strawberries is a key parameter for consumers. It is important to note that under this experimental set up no effects on the color of strawberries have been found. Data presented in Fig. 8 indicate that the berry's visual texture was not damaged after coating with Chl–CHS and following illumination. Just a significant delay of spoilage of the treated berries in comparison with the control was observed during storage (Fig. 8A–C).

Leveque *et al.*[15] found out that EPR imaging could be applied for the monitoring of free radicals in various food samples. Other authors used EPR spectroscopy to evaluate the antioxidant activity of spices and herbs.[47–50] Moreover, the EPR method was successfully applied to distinguish between irradiated and not irradiated fruits and vegetables. Raffi and Stocker claimed that it is possible to detect irradiated berries (due to free radicals) as long as 25 days (stored at 4–5 °C).[14] As photosensitization treatment involves radical reactions it was important to check whether the photoactivated Chl–CHS coating induced additional long lasting reactive oxygen species in strawberries. Data indicated that both the recorded spectra (control and treated strawberries) (Fig. 9) exhibited strong signals due to the 6 lines of Mn^{2+} (which is a transition metal ion linked to enzymes in the strawberry).[14] Comparison of EPR spectra in the control and treated strawberries revealed that no radical-based fundamental changes occurred 1 hour after treatment. It means that despite the high antioxidant activity of these berries this treatment does not induce long-lasting free radicals in the strawberries, as for instance was observed in the case of 2 kGy ionizing radiation.[14] Hence, the obtained data indicate that the photoactivated Chl–CHS coating has potential to combat harmful and pathogenic microorganisms distributed on the surface of strawberries and can serve in the future for the development of a photoactive biodegradable edible coating with more pronounced antimicrobial properties.

5. Conclusions

In conclusion, the photoactive chlorophyllin–chitosan complex exhibits a high antimicrobial capacity against the Gram (–) food pathogen *S. enterica* and *in vitro*. It is able to cover the *Salmonella* surface and after the photoactivation it induced intensive membrane disintegration and total destruction of pathogens.

Moreover, our data indicated that the application of edible and active visible light Chl–CHS coating preserved strawberries much better in comparison with chitosan coating, since it reduced fruit contamination by *S. enterica* and yeasts/molds to desirable levels. Experimental data support the idea that the Chl–CHS coating photoactivated with visible light can be a useful tool for the preservation of strawberries according to the requirements of "clean green technology concept".

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Inactivation of Gram (−) bacteria *Salmonella enterica* by chlorophyllin-based photosensitization: Mechanism of action and new strategies to enhance the inactivation efficiency

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A B S T R A C T

This study is focused on the enhancement of susceptibility of Gram (−) bacteria *S. enterica* to chlorophyllin-based (Chl) photosensitization combining it with other antimicrobial tools. In order to find best combinations, the mechanism by which Chl-based photosensitization inactivates bacteria must be identified.

Data confirmed that photosensitization (Chl 1.5×10^{-5} M, for 1–120 min, 405 nm, 0–46.1 J/cm^2) reduced *S. enterica* population, just by 2.05 log (CFU/ml). Fluorimetric measurements indicated that just minor part of Chl was bound to *Salmonella* in suspension. Addition of sodium azide (NaN_3) (10 mM) protected bacteria from killing, what means that $^1O_2$ took place in photochemical reactions. Gene expression data confirmed that Chl-based photosensitization induced oxidative stress in bacteria cells, since mostly genes responsible for detoxification of ROS (*OxyR*, *AhpC*, *GrxA*) have been expressed in *Salmonella*. Moreover, the expression of genes, responsible for the inhibition of oxidative respiration (*AtpC*), cell division and down-regulation of metabolism (*SulA*) have been detected. In addition, Chl-based photosensitization induced significant release of intracellular components (absorbing at λ_{260} nm and λ_{280} nm) in bacteria that indicated increased membrane permeability. Thus, the combination of two antimicrobials (Chl-based photosensitization and chitosan (CHS)) with the same target (cellular membrane) in the presence of light drastically reduced viable *Salmonella* population (by 7.28 log). Combined treatment of photosensitization and high power pulsed UV light (HPPL) was also very effective, since reduced viable *Salmonella* by 7.5 log. Bacterial regrowth experiments clearly indicated that after both combined treatments *Salmonella* lost its ability to proliferate, and SEM images confirmed that after both treatments no viable bacteria have been found at all.

1. Introduction

Despite tremendous progress in technology and biomedical science the number of reported food-borne infections continues to rise. Health experts estimate that every year food-borne illnesses in the USA cost 77.7 billion US dollars in direct medical expenses and lost productivity [1]. The center of Disease Control and Prevention (CDC) reported 48 million illnesses, 128.000 hospitalizations, and 3.000 deaths every year due to foodborne illness caused by pathogenic microorganisms [2]. Actually, about 1 million people were infected with non-typhoidal *Salmonella*, resulting in 19,336 hospitalizations and 378 deaths in the US yearly [3], whereas 96,883 cases of human salmonellosis in 2011 were reported in the EU [4]. The main reason for such statistics is increasing resistance of pathogens to existing antimicrobial technologies.

Thus, considerable efforts are underway to develop novel strategies and find more effective and environmentally friendly decontamination treatment. Experimental studies conducted over the last decade in our laboratory confirmed that photosensitization using plant-based photosensitizers (hypericin) or photoactive food additive (chlorophyllin, E140ii), might serve as a promising tool to prevent infectious foodborne diseases, since it is able to decontaminate different fruits and vegetables [5,6]. It is important to note that such decontamination expanded the shelf-life of fruits and vegetables without any negative effects on their organoleptic properties or nutritional value. Naturally distributed mesophylic bacteria, spoilage fungi and food pathogens can be reduced

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by 1.5–2.5 log, depending on surface irregularity [6–8]. Therefore, photosensitization-based phenomenon might open a new avenue for the development of non-thermal, effective and ecologically friendly antimicrobial technology which would prevent infectious foodborne diseases [9]. However, differential susceptibility of Gram (+) and Gram (−) bacteria to Chl-based photosensitization limits its application [10–12]. Thus, to achieve equal inactivation of naturally distributed surface-attached Gram (+) and Gram (−) bacteria is complicated task. Hence, concept of hurdle antimicrobial technologies might be applied. It is known that combination of several antimicrobial tools can enhance the microbial inactivation and enable the use of lower individual treatment intensities, which do not cause destructive changes of food matrix [12]. Combined treatment is more effective because a number of sublethal stresses on a microbial cell press microorganism to expend energy to overcome the stressful environment, potentially leading to metabolic exhaustion and death [13]. In order to avoid microbial resistance and achieve synergy/additivity in the combined treatment basic knowledge about the inactivation mechanism are necessary. Nothing is published concerning mechanism of inactivation of bacteria by Chl-based photosensitization.

This study is focused on the mechanism of inactivation of Gram (−) bacteria by chlorophyllin-based photosensitization and evaluation of novel approaches to enhance it combining with other antimicrobial treatments.

2. Materials and Methods

2.1. Chemicals

Not coperized chlorophyll sodium salt (Chl) (MW 684.909; E/1% 401 nm: 6805–780 (pH 9)) was obtained from *ROTH*, Karlsruhe, Germany. Low molecular weight chitosan (CHS, Brookfield viscosity of 1% solution in 1% acetic acid at 20 °C 140 cP, M 70000) was obtained from *Aldrich*, Saint Louis, USA. Triton X-100 was purchased from *Merck*, Darmstadt, Germany. Deionized water used in all experiments had specific conductivity $< 1 \times 10^{-6}$ S/cm. Aqueous stock solution of CHS (pH 2.4 at 20 °C) containing 1% of CHS and 0.18% of HCl (*Aldrich*, Saint Louis, USA) was prepared dissolving in water appropriate amounts of HCl and then CHS. Aqueous stock solution of $1{,}5 \cdot 10^{-4}$ M Chl was prepared by dissolving Chl in water [8,14]. Aqueous stock solution of chlorophyllin–chitosan complex (Chl-CHS) (pH 2.4 at 20 °C) containing 1% of CHS, $1{,}5 \cdot 10^{-4}$ M of Chl and 0.18% of HCl was prepared by drop wise addition of aqueous 0.05% Chl solution into rapidly spinning aqueous solution containing 1.25% of CHS and 0.23% of HCl. Sodium azide (NaN_3) was obtained from *Acros Organics*, New Jersey, USA. After addition of the Chl-CHS to the bacterial suspension in NaCl (*Oxoid*, Basingstoke, UK), the pH of the final bacterial suspension was 3.95.

2.2. Cultivation of the Microorganism

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).

S. enterica was grown in Luria-Bertani medium (LB) (*Liofilchem*, Roseto Degli Abruzzi, Italy; pH = 7.1) incubated overnight at 37 °C. The overnight culture was 20-times diluted with fresh LB medium (optical density at 540 nm (OD_{540}) was 0.164) and grown at 37 °C to the mid-log phase (5×10^8 colony forming units (CFU)/ml, OD_{540} = 1.3). Cells were then harvested by centrifugation (10 min, 6 °C, 3574 × *g*) (MPW-260R, *MWP Med. instruments*, Warsaw, Poland) and resuspended in a two buffers: 0.01 M PBS (pH = 7.4) and 0.9% NaCl (pH = 7.3), to give about 2.5×10^9 CFU/ml. These stocks suspensions were diluted to about 1×10^7 CFU/ml and immediately used for the experiments.

2.3. Spectrophotometric and Fluorimetric Measurements

Absorption spectra of Chl solutions were recorded by spectrophotometer Heλios Gamma & Delta spectrophotometers, *ThermoSpectronic* (Leicestershire, Great Britain). Fluorescence spectra were obtained by *Perkin Elmer* fluorescence spectrophotometer LS-55 (Rodgau, Germany). Scan range parameters are as follows: excitation wavelength – 405 nm; emission – 550–750 nm; ex Slit – 10 nm; em Slit – 4 nm; scan speed (nm/min) – 200. All measurements were performed at 20 ± 2 °C.

Cell-chlorophyllin interaction was evaluated fluorimetrically. Cells ($\sim 1 \times 10^7$ CFU/ml in 0.01 M PBS) were incubated with 1.5×10^{-5} M Chl [8,14] in the dark at 37 °C for the 60 min. To evaluate the amount of cell-attached Chl, 3 ml aliquots of bacterial suspension after incubation were centrifugated (10 min, 6 °C, 3574 × *g*) (MPW-260R, *MWP Med. instruments*, Warsaw, Poland) and resuspended in 0.01 M PBS. Supernatant and cells in PBS were used immediately for fluorescence measurements.

2.4. Light Sources for Inactivation of Bacteria

LED based light source for the photoactivation was constructed at the Institute of Applied Sciences of Vilnius University. The emission maximum of the light was 405 nm and the intensity at surface of samples (at 6 cm from the light source) reached 9.6 mW/cm^2 was calculated as light intensity multiplied by irradiation time. Light intensity was measured by *3 Sigma* power and energy meter "Coherent" (California, USA) equipped with a piro-electrical detector J25LP04.

The high power pulsed UV light (HPPL) device constructed in our laboratory consisted of a chamber, a reflector with a flash lamp, and a power supply unit. The illumination spectrum of the xenon lamp was broad (200–1000 nm) with maximum at 260 nm. Pulse duration was τ = 112 μs, frequency 5 Hz. UV increments were obtained by increasing irradiation time [15].

2.5. Inactivation of Salmonella by Chl-based Photosensitization and Photoactivated Chl-CHS Conjugate

Aliquots of bacterial suspensions ($\sim 1 \times 10^7$ CFU/ml) with 10 times diluted Chl or Chl-CHS stocks solutions were incubated for cell culture cultivation in the shaker (120 rev/min) in the dark at 37 °C for different periods (0–120 min). For the photoactivation treatment, 150 μl of the samples were withdrawn, placed into sterile flat bottom wells and then exposed to the light (λ = 405 nm) for different fluence (0–46.1 J/cm^2). Control cells were not treated at all.

2.6. Combined Treatment of Chl-based Photosensitization and HPPL

For combined treatment, 100 μl of Chl-based photosensitization treated (46.1 J/cm^2) bacterial cells were surface inoculated on the LBA plates and exposed to 900 pulses of UV light (0.29 J/cm^2). UV light energy density was 0.325 mJ/cm^2, duration = 70 s. After 24 h colony forming units were counted by spread plate method.

2.7. Evaluation of Salmonella Regrowth After Different Treatments

Bacterial regrowth after treatments was evaluated by Heλios Gamma & Delta spectrophotometer (ThermoSpectronic, Great Britain) measuring optical density of cell growth. Bacterial cells after 1.5×10^{-5} M Chl-based photosensitization (23.0 and 46.1 J/cm^2), treatment with photoactivated Chl–CHS (17.3 J/cm^2), HPPL (0.29 J/cm^2), and combined treatment of Chl-based photosensitization (46.1 J/cm^2) and HPPL (0.29 J/cm^2) were chosen for regrowth experiments. After treatments (also control samples), *Salmonella* suspensions were moved to a fresh LB medium and cultivated in the shaker (120 rev/min)

Table 1
S. enterica primers and their sequences.

Gene	Forward primer	Reverse primer
GyrA (Gyrase subunit A)	5′GATGAGCGTCGTACCGAAAT	5′TCGTAATCTGTCAGCGGTTG
Tsf (Elongation factor TS)	5′TTGCTGGCAAAATCACTGAC	5′ACGCGACGAATGTTGATGT
RpoS (RNA polymerase sigma factor)	5′ATACGCAACCTGGTGGATTC	5′CGCAGGTATACGTTCAGCTC
OxyR (DNA-binding transcriptional regulator)	5′CCTATTTGCTGCCGCTTATC	5′CGAGTTGCGCTAACAACTGA
Gor (Glutathione oxidoreductase)	5′CCGACGGCAGTCTGACTCT	5′TTAATATTGTCGGTCGACGGTTC
GrxA (Glutaredoxin)	5′AGAACGCGACGATTTCAACT	5′TCTGCGGTACGGTTTCTACA
Fur (Ferric uptake regulator)	5′CAACCGTATACCGTGTGCTG	5′GATGATGCTGTTGCGTCAGT
SodA (Superoxide dismutase)	5′GCTAACCACAGCCTGTTCTG	5′AGTTGTCAACGGAACCGAAG
AhpC (Alkyl hydroperoxide reductase subunit C)	5′GACTGAACTGGGTGACGTTG	5′ATGCTTTGTGCGTGAAGTGA
AhpF (Alkyl hydroperoxide reductase subunit F)	5′TGCTGAACCCGCGTATTAAA	5′TTAACAAATACCGCCGGAAC
Tpx (Probably Thiol-peroxidase)	5′TGCTGAATATTTTCCCAAGCA	5′AAAAGGCAGGTCAGCAGAAA
Dps (DNA starvation/stationary phase protection protein)	5′AACAGGCCCACTGGAACA	5′GCTCGGCCATAGTATCCAGA
KatG (Catalase-peroxidase)	5′GTTACATCGGACCGGAAGTG	5′GCCGCTTTCAGGTTGATAAT
KatE (Catalase)	5′AGTTATCGGACGATGGAAGG	5′CATACCAGCGAGGCTTTACC
STM1731 (Catalase)	5′CCCGGCGTTGACAAACTC	5′GGCGTTCATAAATGATCTTCG
SodC (Superoxide dismutase)	5′CGGGAATGAAAGACGGTAAA	5′CCAAATGCCCTTTGTCATTA
SpoT (Bifunctional (p)ppGpp synthetase II/guanosine-3′,5′-bis pyrophosphate 3′-pyrophosphohydrolase)	5′GCGTAAAATCCGATCTCTTCC	5′TGCACTGCATAGGCAAAATC
DnaK (Chaperone protein)	5′CTATCGCGGTTTACGACCTC	5′TTGGTTGCCAGAACTTCAAA
CadA (Lysine decarboxylase 1)	5′AAAGAGACGCCTAACGCAAC	5′TGGACTTCACATCCAGAGTTTT
AtpC (AtpC F0F1 ATP synthase subunit epsilon)	5′TAAGCAGCACGGTCATGAAG	5′GAGATCCTGACCGCGAATC
OmpR (Osmolarity response regulator OmpR)	5′TTAAACTGAACCTCGGTACGCa	5′GGATGGCTGACTAACGCTTTC
OmpC (Outer membrane porin protein C)	5′GACTTATGCAATCGGCGAAG	5′ACCATACAGGCGAGCGTTAG
OtsA (Alpha,alpha-trehalose-phosphate synthase)	5′ATCAGGATATTCGCCACCAG	5′CGGTCGAAATGTTGATTCAG
OtsB (trehalose-phosphate phosphatase)	5′GGGAACCAATAAAGGCGAAG	5′CAGCCTCATCGGTCAAATC
KdpE (Response regulator in two-component regulatory system)	5′AATACCGGTGATTGTCCTTTC	5 AATGCCGAATGGCTTGCT
HtrA (Serine endoprotease)	5′AACCTGAACGGTGAGCTGAT	5′CGTCAGGTTTTTCACCATGT
SurA (Chaperone)	5′GGCAAACTGGCGATTACCTA	5′GACAATGTCGCCTTTCTTCG
FkpA (fkpA FKBP-type peptidyl-prolyl cis-trans isomerase)	5′GGCATCAAACTGGATAAAGATCA	5′GTCTGCAGCGTTTGCTCAAT
PspA (Phage shock protein PspA)	5′TGACCGATTTAATTGCTACGC	5′TTCGCTGAGTTTGTTTTCCA
STM0225 (Chaperone protein Skp)	5′GCAATCCATGAAAGCAGGTA	5′GAGCGCGATCTTTCTCAAAA
DnaJ (Chaperone protein)	5′ACATGGATCTCACCCTGGAA	5′GGTTGCGTGCCAGCTTTC
GroES (Co-chaperonin GroES)	5′GACGTGAAAGTTGGCGACAT	5′GCCAGAATGTCGCTTTCAGA
GroEL (Chaperonin GroEL)	5′AGACACCACCACCATCATCG	5′GTAGTCGGAGGTCGCTTCTTC
ClpX (ATP-dependent protease ATP-binding subunit ClpX)	5′GATCGCTAACCGTGTTGAAA	5′TGATCAAATCTTCCGGTTCA
Lon (Lon protease)	5′CGTAGTCTGGAGCGTGAAATC	5′ACCGAGGTAGTCGTGCAGAT
SulA (Cell division inhibitor)	5′GCCGTGAATGGGTACAGTCT	5′CGCTGTAATTTCCTGTACGC

in the dark at 37 °C.

2.8. Evaluation of Antimicrobial Activity

The antibacterial effects of all treatments on *S. enterica* were evaluated by the spread plate method. Thus, 100 µl of appropriate dilutions of bacterial test culture after treatment in vitro was surface inoculated on the separate LBA plate. Afterwards, the bacteria were kept in the thermostat for 24 h at 37 °C. Bacterial counts were transformed from CFU/ml into $\log_{10}$/ml. Detection limit of spread plate method-one colony forming unit.

2.9. Detection of $^1O_2$ in Chl-based Photosensitization Treated Bacteria

In order to identify the singlet oxygen ($^1O_2$) participation in Chl-based photosensitization induced cell damage and asses its impact on bacterial survival, bacterial cell suspension ($\sim 1 \times 10^7$ CFU/ml) was amended with the exogenous scavenger sodium azide (NaN_3) (specific $^1O_2$ scavenger) [16]. Stock solution of NaN_3 was prepared in PBS and used at a final concentration of 10 mM. The bacterial cells were incubated with 1.5×10^{-5} M Chl in the dark at 37 °C for 60 min, followed or not by addition of NaN_3 and illumination up to 38 and 50.7 J/cm^2 of 405 nm light.

2.10. Evaluation of Cell Membrane Integrity after Chl-based Photosensitization

The bacterial cell membrane integrity was examined spectrophotometricaly evaluating the cell release material which has absorption at $\lambda = 260$ nm [17] and $\lambda = 280$ nm [18]. The bacterial suspension after 1 h incubation with Chl at 37 °C in the dark was illuminated and aliquots of 3 ml cell suspension were taken out at different illumination time points corresponding to illumination of 5.8, 11.5, 17.3, 23, 40.3 and 46.1 J/cm^2. The samples (control (Control) and treated (Chl)) were filtered (0.20 µm filter) to remove the bacteria [17]. The UV absorbance of cell supernatant at $\lambda = 260$ and $\lambda = 280$ nm was determined using spectrophotometer (*Helios Gamma & Delta ThermoSpectronic*, Leicestershire, Great Britain).

2.11. Scanning Electron Microscopy (SEM)

The effect of Chl-based photosensitization, HPPL and combined treatment on the morphology of *Salmonella* was examined by SEM. After appropriate treatment the samples consisting of 20 µl of bacterial suspension were withdrawn, transferred to aluminum stubs, air-dried and sputter coated with 15-nm gold layer using Q150T ES sputter coater (*Quorum Technologies*, Lewes, England). The scanning was performed with an Apollo 300 (*CamScan*, Bingham, UK) scanning electron microscope at an accelerating voltage of 20 kV.

2.12. Real-time Quantitative PCR (RT-qPCR)

S. enterica strain was cultured at 37 °C to midlog phase. Then 1.5×10^{-5} M Chl was added and after 60 min incubation bacteria were illuminated with visible light (23 J/cm^2). Total RNA was extracted from these and control cells using the GeneJET RNA Purification kit (*Thermo scientific*, Vilnius, Lithuania). Complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis kit for

RT-qPCR (*Thermo scientific*, Vilnius, Lithuania). The cDNA synthesized was used as the template for RT-qPCR. The amplification product was detected using SYBR Green reagents (Maxima SYBR Green qPCR Master Mix (2 ×) (Thermo scientific)). Relative gene expression was determined using the comparative critical threshold (Ct) value method using a StepOnePlius Real-Time PCR machine (*Applied Biosystems*, Carlsbad, CA), and expressed as fold change in expression relative to control. Data were normalized to the housekeeping genes (*tsf* and *gyrA*) and the level of candidate gene expression between control and photoactivated Chl treatment was compared. The primers of *S. enterica* genes and their sequences are provided in Table 1.

Transcripts of the selected genes were quantified by RT-qPCR, and data were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method. A red line represents the minimum value at which the expression is considered significant. A ratio > 0 indicates upregulation of gene expression and a ratio < 0 indicates downregulation. Genes are grouped according the [25].

2.13. Statistical Analysis

The experiments were triplicated 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. The data were analyzed using Origin 7.5 software (*OriginLab Corporation*, Northampton, MA 01060, USA). The significance of the results was assessed by the analysis of variance (ANOVA). A value of $p < 0.005$ was considered as significant.

3. Results

3.1. Susceptibility of S. enterica to Chl-based Photosensitization In Vitro

Data presented in Fig. 1a indicate, that chlorophyllin at the concentration 1.5×10^{-5} M has absorption maximum at $\lambda = 405$ nm and lower absorption at 660 nm. In addition Chl is highly fluorescing compound and its fluorescence maximum is about 663 nm (Fig. 1a). The absorption and fluorescence spectra of Chl indicate that at this concentration the compound is mostly monomeric. So, LED-based light source used in experiments emitted light $\lambda = 405$ nm for the optimal excitation of chlorophyllin (Fig. 1b).

The susceptibility of *Salmonella* to Chl-based photosensitization is illustrated in Fig. 2a and b. Experiments confirmed clearly that the light alone (46.1 J/cm^2) had insignificant effects on the viability of bacteria (data not shown). The dark toxicity of Chl to *Salmonella* was negligible as well, since the cell number after 120 min incubation in the dark with Chl was reduced only by 0.12 log. Just incubation of cells with Chl and subsequent illumination with visible light (405 nm, 0–46.1 J/cm^2 fluence) decreased the number of bacterial cells more considerably: in this case the photosensitization treatment (15 min incubation with Chl in 0.01 M PBS and following illumination, 17.3 J/cm^2) led to 0.79 log reduction of *S. enterica* (Fig. 2a). An extension of incubation time from 15 to 120 min enhanced the inactivation of *Salmonella* to 2.05 log. Meanwhile, prolongation of illumination time from 30 min (fluence 17.3 J/cm^2) to 80 min (fluence 46.1 J/cm^2) has negligible effect (0.7 log) on the inactivation of *Salmonella* (Fig. 2b). Rather low antibacterial efficiency of Chl-based photosensitization was confirmed by the measurements of bacterial regrowth after the treatment (Fig. 2c). Some delay of regrowth took place when bacteria have been treated with 23.0 and 46.1 J/cm^2 illumination: ($t_{50(1)} = 2.5$ h and $t_{50(2)} = 6.0$ h respectively) what means that some cell injuries have been induced by the treatment. Meanwhile, it must be noticed that after some delay time *Salmonella* treated by photosensitization tend to regrowth (Fig. 2c).

Thus, to understand the cell-photosensitizer interaction we measured fluorescence spectra of Chl when cells were incubated with 1.5×10^{-5} M Chl and compared with Chl spectrum in supernatant and Chl spectrum in cells alone (without supernatant) (Fig. 3). Data indicated that most of the Chl remained in the supernatant and just minor part was bound to *Salmonella*. So far, this small fraction of Chl-attached *Salmonella* was damaged after photosensitization treatment.

3.2. Evaluation of Damages Induced in S. enterica after Chl-based Photosensitization

In order to find new effective combination of two antimicrobial treatments it is important to know cell injuries which are produced by each of them.

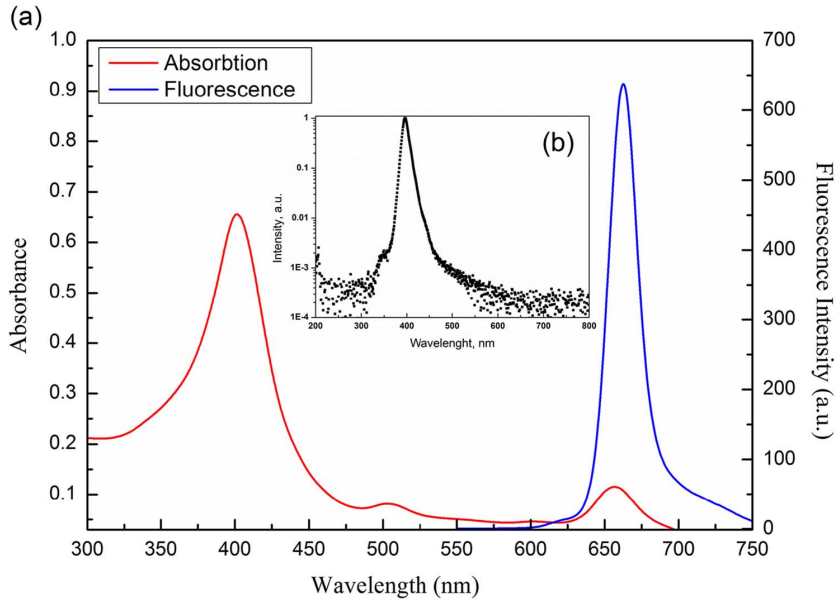

Fig. 1. Absorption and fluorescence spectra (a) of 1.5×10^{-5} M Chl (in 0.01 M PBS, at 20 ± 2 °C) and light emitted from LED-based light source for excitation (405 nm) of chlorophyllin (b).

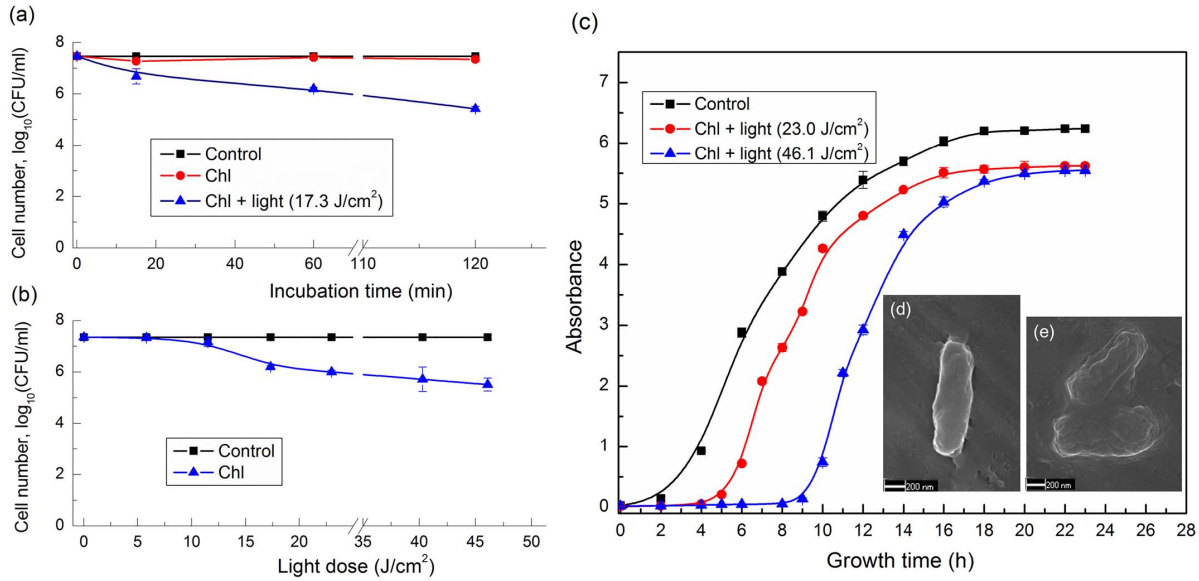

Fig. 2. Inactivation of *S. enterica* by 1.5×10^{-5} M Chl-based photosensitization as function of incubation time (a), fluence (b) (incub. time 60 min) and bacterial regrowth (c) (incub. time 60 min; 23–46.1 J/cm^2); SEM images of control (d) and cells after 1.5×10^{-5} M Chl-based photosensitization (incub. time 60 min; 23 J/cm^2) (e); every point is the average of 3–6 experiments, and error bars sometimes are too small to be more visible.

So, in the next step it was interesting to assess whether singlet oxygen participate in the photosensitization induced damage of bacteria. Data indicated that the presence of widely accepted singlet oxygen quencher NaN_3 at nontoxic concentration (10 mM) reduced the killing efficiency of photosensitization in statistically significant way ($p < 0.05$) and most bacteria survived (Fig. 4).

To reveal the molecular targets of Chl-based photosensitization in Gram (−) *Salmonella* the expression of 33 stress- related genes by RT-qRNA was quantified (Fig. 5). As expected, the expression levels of two housekeeping genes (*GyrA* and *Tsf*) were unchanged regardless of the treatment. The expressed genes (*OxyR*, *AhpC*, *GrxA*, *SulA*, *AtpC*, *groEL*, *STM0225*) according to their respective functions mostly belong to gene systems responsible for the adaptation and protection against oxidative stress (*OxyR*, *AhpC*, *GrxA*, *AtpC*). It is important to note, that the transcription of SOS response gene *SulA* encoding inhibitor of cell division, was slightly up-regulated as-well.

Since Chl is attached mostly to the surface of bacterium, we examinated the effects of Chl-based photosensitization treatment on

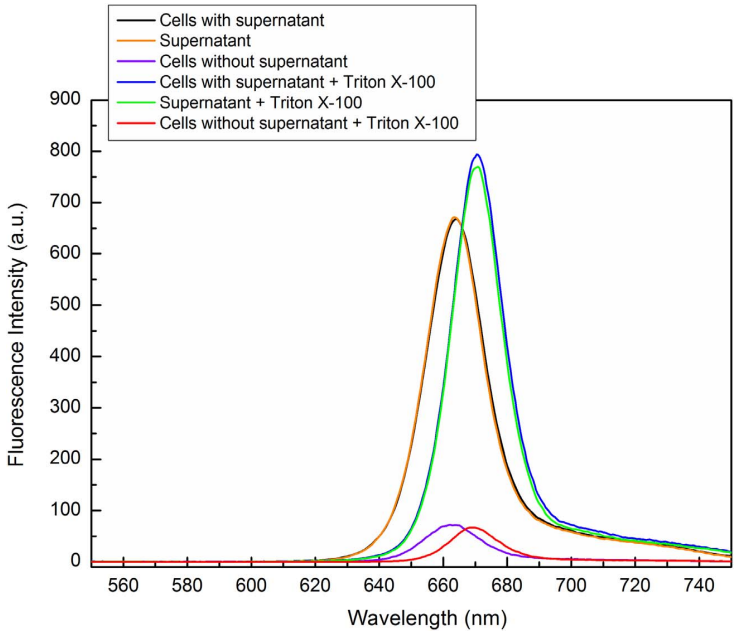

Fig. 3. Detection of cell-attached chlorophyllin in *S. enterica* from fluorescence spectra: 1.5×10^{-5} M chlorophyllin, 60 min incub., 0.01 M PBS (at 20 ± 2 °C).

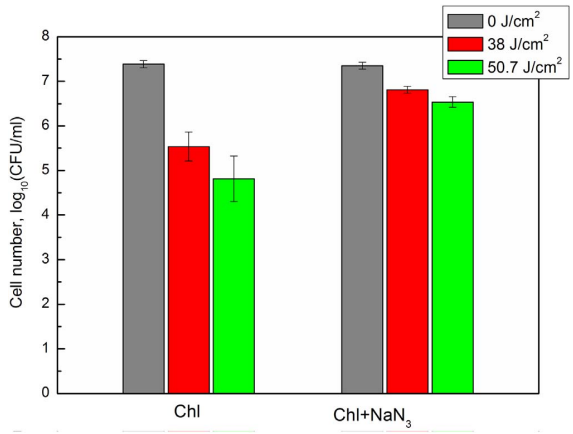

Fig. 4. Effect of NaN_3 on viability of *S. enterica* after Chl-based photosensitization (incub. with $1.5 \cdot 10^{-5}$ M Chl, 60 min followed or not by addition of NaN_3 (10 mM) and illumination (38 and 50.7 J/cm^2). Every point is the average of 3 experiments, Chl-based photosensitization show significant difference from Chl-based photosensitization + NaN_3 ($p < 0.0005$).

S. enterica membrane integrity. It was assessed by measuring the absorption at 260 nm and 280 nm of cell free filtrates in control and treated samples (Fig. 6). The results indicated that the release of intracellular material absorbing at λ_{260nm} and $\lambda_{280\ nm}$ in control samples was insignificant and did not depend on light fluence (0–46.8 J/cm^2). On the contrary, the release of intracellular components (both absorbing at λ_{260} nm and λ_{280} nm) in treated bacteria increased since absorption changed from 0.01 to 0.146–0.164 (when *S. enterica* was 60 min incubated with Chl and illuminated, 46.1 J/cm^2). It means that some cell membrane injuries occurred in *Salmonella* after Chl-based photosensitization. Scanning electron microscopy images presented in Fig. 2d, e indicated some shrinkage in *Salmonella* cells induced immediately after photosensitization treatment (1.5×10^{-5} M Chl, 60 min. incub., 23 J/cm^2). As a rule, such morphological changes are the consequence of increased membrane permeability. Thus, these images confirmed our previous data about membrane disintegration and leakage of some intracellular components in *Salmonella* after photosensitization (Fig. 6).

These data prompted us to search for more effective inactivation combining several antimicrobial treatments with main target-cell membrane.

3.3. Inactivation of Salmonella by Photoactivated Chl–CHS Conjugate In Vitro

In order to increase susceptibility of *Salmonella* to photosensitization we used photoactive chlorophyllin-chitosan (Chl–CHS) conjugate. The structural formula and absorption spectrum of this conjugate was presented in our previous study [14]. The antimicrobial properties of Chl–CHS conjugate were assessed comparing its antimicrobial efficiency in the dark and in the light CHS (Fig. 7a). Previous experiments indicated that just insignificant reduction of *Salmonella* counts was found after 0–20 min incubation with CHS [14]. The increase of incubation time from 20 min to 120 min enhanced the bacterial inactivation to 2.32 log. Dark toxicity of Chl–CHS conjugate depended on incubation time, and just after 120 min of incubation it reached 1.04 log reduction. It indicated that Chl–CHS conjugate exhibited some antibacterial action which was close to that of CHS alone. However, photoactivation of this conjugate drastically reduced the number of viable *Salmonella* (from 7.28 log to 0 log) at much shorter incubation time. Bacterial regrowth curves presented in Fig. 7b clearly indicated that at these experimental conditions *Salmonella* exhibited lethal destructions without any possibility to survive and regrowth (Fig. 7b). SEM images, presented in Fig. 7c and d indicated that biopolymeric conjugate covered all surface of bacterium, and after photoactivation cells underwent shrinkage.

3.4. Inactivation of Salmonella Combining Chl-based Photosensitization With High Power Pulsed UV Light

Results presented in Fig. 8a indicated that *S. enterica* after 60 min

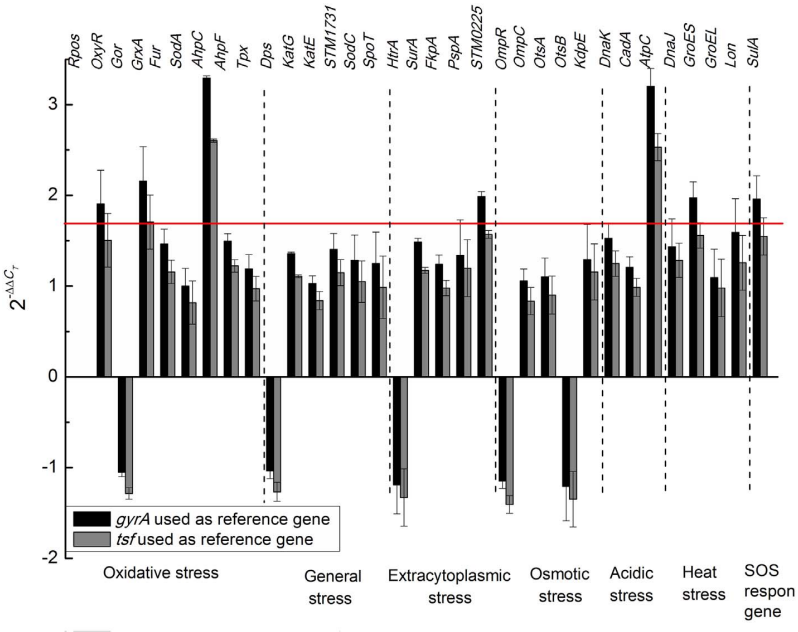

Fig. 5. Relative gene expression levels in *S. enterica*, treated by Chl-based photosensitization (incub. $1.5 \cdot 10^{-5}$ M Chl, 60 min, 38 J/cm^2).

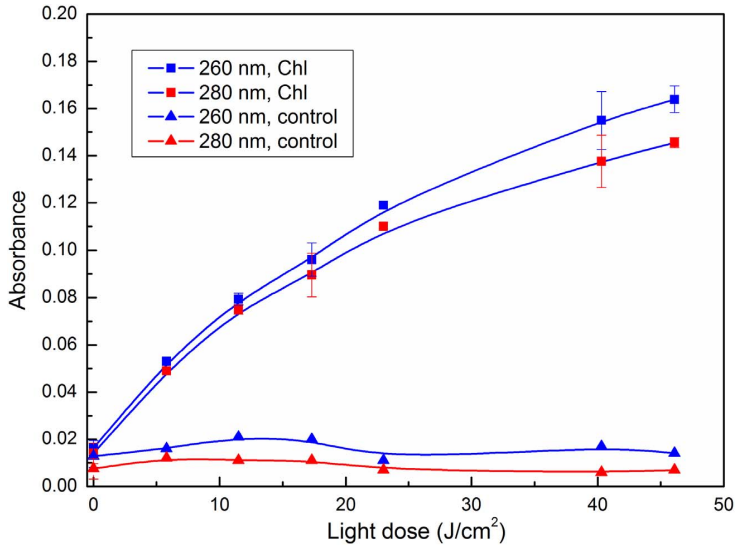

Fig. 6. Release of intracellular components (at 260 nm and 280 nm) from *S. enterica* after 1.5×10^{-5} M Chl-based photosensitization. Every point is the average of 3 experiments, and error bars sometimes are too small to be more visible.

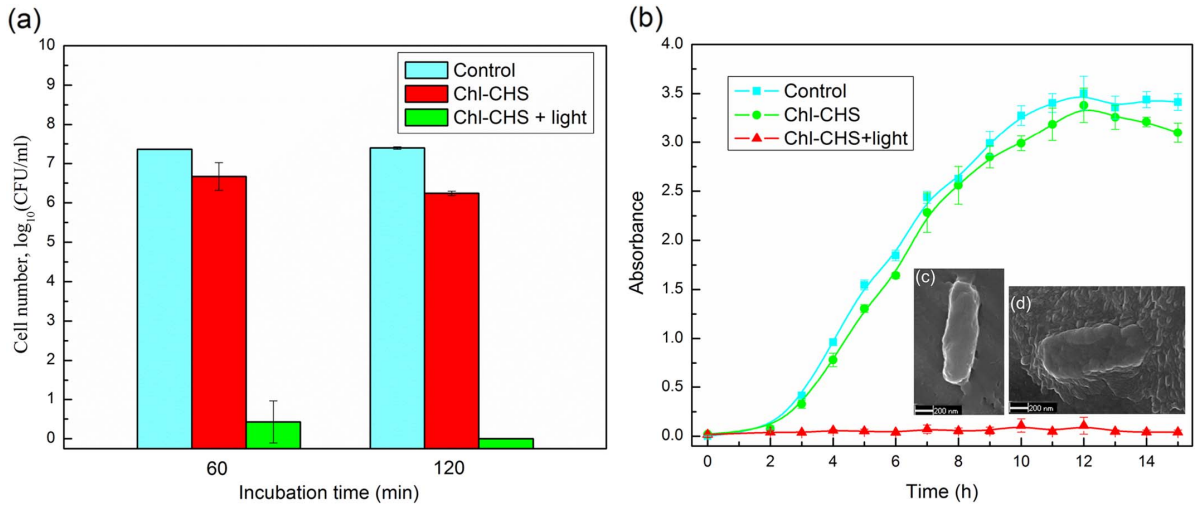

Fig. 7. Inactivation of *S. enterica* by photoactivated $1{,}5 \cdot 10^{-5}$ M Chl–0.1% CHS in 0.9% NaCl (17.3 J/cm^2) (a); regrowth of bacteria after treatment (b); SEM images of control (c) and treated (d) *Salmonella* (incub. time 30 min; 12.7 J/cm^2); every point is the average of 3–6 experiments, and error bars sometimes are too small to be more visible.

incubation with 1.5×10^{-5} M Chl and following illumination (46.1 J/cm^2) was inactivated by 1.9 log. HPPL treatment (UV, 0.29 J/cm^2) reduced the cell population by 6.4 log and with 9 h delay the regrowth of bacteria was observed (Fig. 8b). Combined treatment of photosensitization and HPPL reduced the growth of *Salmonella* to undetectable level. SEM images presented in Fig. 8c clearly indicated that high power pulsed UV light at this dose was able to destroy *Salmonella* cells. Data presented in Fig. 8a and b clearly revealed that combining these two antimicrobial treatments it was possible to achieve *Salmonella* inactivation without any bacterial regrowth detected (Fig. 8b). SEM images presented in Fig. 8c and d confirmed the idea, that after combined treatment no viable bacteria were found in the sample at all.

4. Discussion

It is well documented, that photosensitization-based inactivation is not enough effective to kill Gram (−) bacteria when neutral or anionic photosensitizers have been used. Neutral, anionic, or cationic photosensitizers can inactivate mostly Gram (+) bacteria [19], whereas Gram (−) bacteria due to more complex cell wall structure and additional negatively charged outer membrane are less susceptible to this treatment and need higher doses of photosensitizer and light [20]. Several attempts have been made to achieve identical and equal inactivation of naturally distributed microorganisms, consisting of both Gram (−) and Gram (+) bacteria.

One of the most ordinary approaches to kill Gram (−) bacteria is the use of cationic photosensitizers. The photoinactivation of *S. enterica* using cationic dye Malachite green resulted in 2.3 log reduction of bacterial population [21]. The photosensitizing properties of cationic phenothiazine dyes have been employed for sterilization of blood plasma in German and Swiss centers since 1992 [22].

Photosensitization of Gram (−) bacteria by endogenously produced

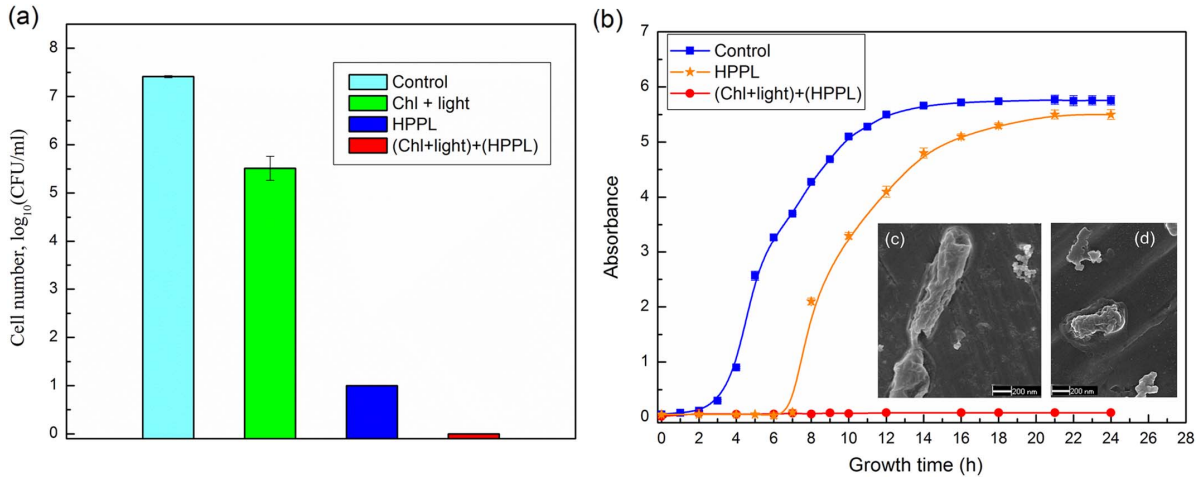

Fig. 8. Inactivation of *S. enterica* by 1.5×10^{-5} M Chl-based photosensitization in combination with pulsed light (HPPL) (a); regrowth of bacteria after different treatments (b); SEM analysis of *Salmonella* after combined treatment: (c) cells after HPPL treatment; (d) cells after combined treatment of Chl-based photosensitization (46.1 J/cm^2) and HPPL (0.29 J/cm^2). Every point is the average of 3–6 experiments, and error bars sometimes are too small to be more visible.

porphyrins is another effective approach to their eradication. Enhanced production of endogenous porphyrins by 5-aminolevulinic acid (ALA) has been observed in such food pathogens as *S. enterica*, *B. cereus*, *L. monocytogenes* [10,21,23]. Our previous results revealed, that it was possible to kill both Gram (−) and Gram (+) bacteria in uniform way by 7 orders of magnitude combining Chl-based photosensitization with ALA-mediated photosensitization [9].

To increase susceptibility of Gram (−) bacteria to photosensitization with negatively charged photosensitizers, polycationic agent polymixin B nanopeptide (PMNP) as non-toxic bacterial membrane disintegrator has been applied. It enabled the penetration of negatively-charged photosensitizers into Gram (−) bacteria. After photosensitization treatment complete inhibition of bacterial growth was observed [19].

The absorption and fluorescence spectra of used photosensitizer chlorophyllin indicated that at selected concentration the compound was mostly monomeric, since adding Triton X-100 just slightly changed the fluorescence intensity [14]. So, LED-based light source used in experiments emitted light $\lambda = 405$ nm for the optimal excitation of Chl (Fig. 1b).

Data obtained on inactivation of Gram (−) *S. enterica* by negatively-charged chlorophyllin-based photosensitization (Fig. 2a and b) indicated that just 2.05 log reduction of microbial population can be achieved when cells were incubated with Chl for 0–120 min and exposed to light (0–46.1 J/cm^2). Just short delay of *Salmonella* regrowth (7 h) after illumination (46.1 J/cm^2) was observed; afterwards fast regrowth has been found (Fig. 2c). Mostly anionic photosensitizers interact with negatively-charged bacteria by electrostatic interaction between cell wall divalent cations (Ca^{2+}, Mg^{2+}) and photosensitizer [24]. Actually, there are three main ways for cell-photosensitizer interaction:

1. Photosensitizer does not bind to the bacterium, remains outside and damages just cell wall;
2. It binds the bacterium externally and damages extracellular structures.
3. Photosensitizer accumulates inside the bacterium. In this case the photosensitizer is transported inside the cell, where it associates with the key structures and irreversibly damages them after photosensitization [25]. It is important to note, that the increase of incubation time from 2 to 60 min with Chl did not change neither the amount of cell-bound photosensitizer (data not shown), nor cell inactivation level (Fig. 2) what means that probably Chl bound the cell from outside and did not need longer incubation time to accumulate inside the cell. Moreover, just minor part of all Chl in suspension was bound to *Salmonella*, since most of the Chl left in the supernatant (Fig. 3). It is important to note that the main (but not only) prerequisite for effective inactivation of bacteria by photosensitization is interaction with photosensitizer (external or internal). Obtained data indicate that low (2 log) inactivation level of *Salmonella* can be explained by rather low cell interaction with chlorophyllin.

In order to find new effective combination of two antimicrobial treatments it is important to know cell injuries which are produced by each of them. The question arise what is the mechanism of cell damage after Chl-based photosensitization. Usually after light excitation the triplet-state photosensitizer interacts with molecular oxygen, electron donors or acceptors and can produce reactive oxygen species (ROS). This interaction may proceed in two distinctly different ways: type 1 mechanism involves electron transfer and leads to free radicals, such as superoxide anion, hydroxyl radical. The type 2 mechanism leads to the formation of singlet oxygen ($^1O_2$), which is reactive and directly destroys bacteria. Both reaction types can occur simultaneously and the ratio between them depends on the structure of photosensitizer and microenvironment [26]. Data presented in Fig. 4 indicated that $^1O_2$ quencher NaN_3 protected bacteria from photosensitization induced killing what means that type 2 mechanism took place in photochemical reactions.

Bacterial genome contains a number of stress-response gene systems which enable them to survive under various environmental conditions [27,28]. Thus, it was important to find gene systems which respond to photochemical reactions induced by photosensitization in bacteria. The expressed genes (*OxyR*, *AhpC*, *GrxA*, *SulA*, *AtpC*, *groEL*, *STM0225*) according to their respective functions mostly belong to gene systems responsible for adaptation to oxidative stress and defense, for extracytoplasmic and acidic stress. RT-qPCR data confirmed that cellular homeostasis in *Salmonella* after Chl-based photosensitization was unbalanced, and bacteria had to adapt to the emerging ROS (Fig. 5). One of key actors to regulate bacterial defense against photosensitization-induced injuries is *OxyR* [27,28] which in turn up-regulate the expression of *GrxA* and *AhpC* genes. Both of them have clear antioxidant roles: alkyl hydroperoxide reductase (ahpC) protects against the toxic effects of peroxides [29,30], glutaredoxin I (grxA) can restore the

impaired cellular thiol-disulfide balance [30]. The up-regulated *AtpC* gene is encoding the epsilon subunit of the F_oF_1 membrane ATPase. Enhanced expression of ATPase could increase the transfer of electrons through the electron transport chain, pumping protons out of the cell to help maintain the intracellular pH homeostasis [31]. Importantly, the transcription of SOS response gene *SulA* encoding cell division inhibition [32] was slightly up-regulated as-well what was confirmed by photosensitization-induced inactivation of bacteria (Fig. 2).

In the next step it was important to identify damages, produced by Chl-based photosensitization. Thus, examination of SEM images of damaged bacterial population indicated intensive shrinkage of bacterial cells which usually is a result of leakage of intracellular components. To confirm the increase of membrane permeability in *Salmonella* after Chl-based photosensitization the leakage of some protein (λ = 280 nm) and DNA components (λ = 260 nm) has been found (Fig. 6). Thus, these data confirmed the idea that photosensitization treatment induced significant cell membrane disintegration which enabled extensive leakage of DNA and protein components.

Obtained results on *Salmonella* gene-response to Chl-based photosensitization and afterwards induced damages prompted us to combine this treatment with antimicrobial effects of chitosan (CHS). Chitosan, being natural cationic linear positively-charged polysaccharide due to electrostatic interaction of protonated NH_3^+ groups with negatively-charged phosphate and pyrophosphate groups of membrane lipopolisaccharides increased membrane permeability and provoke internal osmotic imbalance inhibited the growth of microorganisms [33]. Chl-chitosan conjugate can mediate the interaction of negatively charged bacterial cell wall and negatively charged photosensitizer, and enhance the possibility of cell-photosensitizer interaction. Chl–CHS conjugate in the dark exhibited some antimicrobial activity against *Salmonella* (1 log after 2 h incubation) and it was comparable with that of CHS alone [2]. It is important to note that antimicrobial efficiency of chitosan depended on the incubation time, and these data are in line with results published in [34]. As development of novel antimicrobial technology mostly requires short treatment time we paid more attention on inactivation level at shorter incubation time (0–30 min.). Photoactivation of chlorophyllin-chitosan conjugate enhanced drastically the killing efficiency and very sharp reduction of viable cell population (~7 log) was achieved even after 30 min incubation. The analysis of SEM images of *Salmonella* treated by photoactivated chitosan-chlorophyllin complex indicated the clear covering of bacteria by this biopolymer, shrinkage and reduction of bacterial volume and size (Fig. 7c and d). Taking into account that chitosan-chlorophyllin conjugate is able to form polymer, such “photoactive” antimicrobial film can serve as carrier of wide range of various antimicrobials in the future [35].

In order to increase the inactivation of Gram (−) bacteria it seems promising to combine photosensitization with high power pulsed light, which in 2000 year was approved by the USA Food and Drug Administration as save technology for decontamination of food or food-related surfaces [36]. High power pulsed UV light (HPPL) is a non-thermal antimicrobial technology that proposes decontamination of surfaces by intense and short duration (microseconds) pulses of broad spectrum light (200–1000 nm), is mercury-free, has limited energy costs and short exposure time [37]. It is well documented, that the main cellular target for high power pulsed light (“working” wavelength, λ = 260 nm) is DNA. Thus, combining membrane injuries induced by Chl-based photosensitization with DNA damages produced by pulsed light it is possible to increase the lethal destruction of Gram (−) bacteria. In our previous study combining photosensitization and HPPL treatment a remarkable reduction of viable counts (6–7 log) of Gram (−) *Salmonella* and Gram (+) *Listeria* have been observed [38]. Data obtained in this study revealed that combined treatment of HPPL and photosensitization can be useful strategy to obtain significant inactivation of viable *Salmonella* cells (Fig. 8a) In order to confirm it, *Salmonella* regrowth experiments were performed and no viable cell was detected after combined treatment (Fig. 8b). SEM analysis indicated that there were no structurally undamaged bacteria after this treatment (Fig.8c, d). To this end, the obtained results confirmed the idea, that combined treatment of photosensitization and high power pulsed light might be an effective tool to combat both Gram-positive and Gram-negative bacteria in an effective and uniform way.

5. Conclusions

Results obtained indicated that *S. enterica* was less susceptible to negatively-charged chlorophyllin-based photosensitization due to more complex cell wall structure, additional negatively charged outer membrane and low chlorophyllin-cell membrane interaction. Meanwhile, in the photosensitization-based destruction of these bacteria $^1O_2$ took place. Moreover, the expression of genes after photosensitization (*OxyR*, *AhpC*, *GrxA*, *SulA*, *AtpC*, *groEL*, *STM0225*) confirmed that cells had to survive oxidative stress induced by emerging ROS. Eventually it was found that photosensitization treatment induced significant cell membrane disintegration which enabled extensive leakage of DNA and protein components and induced shrinkage of cells.

So far, applying the concept of hurdle antimicrobial technologies, based on the mechanism of action of every antimicrobial treatment, enabled us to combine several antimicrobial tools. Combining antimicrobial activity of chlorophyllin-based photosensitization with that of chitosan (both have the same target cell membrane) the drastic reduction of *S. enterica* viability without any regrowth has been observed. Combination of high power pulsed UV light with Chl-based photosensitization (two different targets) induced lethal destructions in bacteria without any detectable regrowth of bacterial population.

To this end, the obtained results confirmed the idea that combined treatment of photosensitization with chitosan or high power pulsed UV light might be an effective strategy to combat both Gram-positive and Gram-negative bacteria in an effective and uniform way.

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VILNIAUS UNIVERSITETAS

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MAISTO PATOGENŲ INAKTYVACIJA FOTOAKTYVUOTU CHLOROFILINU: POVEIKIO MECHANIZMAS, OPTIMIZAVIMAS IR PRITAIKYMO GALIMYBĖS

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VILNIUS UNIVERSITY

IRINA BUCHOVEC

INACTIVATION OF FOOD PATHOGENS BY PHOTOACTIVATED CHLOROPHYLLIN: MECHANISM OF ACTION, OPTIMIZATION AND POSSIBLE APPLICATIONS

II part of doctoral dissertation
Summary of doctoral dissertation
Biomedical Science, Biophysics (02 B)

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Scientific consultant – Prof. habil. Dr. Živilė Lukšienė (Vilnius University, Biomedical Sciences, biophysics – 02B).

Dissertation will be defended at Vilnius University Biophysics council:

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SANTRUMPOS

$^1O_2$ – singuletinis deguonis

ALA – δ-aminolevulininė rūgštis

aps./min – apsisukimų per minutę skaičius

CDC – ligos kontrolės ir prevencijos centras

Chl–CHS – chlorofilino chitozano konjugatas

Chl – natrio chlorofilinas

CHS – chitozanas

DG18 – dichlorano glicerolio agaras

EPR – elektronų paramagnetinis rezonansas

ES – Europos Sąjunga

FS – fotosensibilizatorius

HCl – druskos rūgštis

JAV – Jungtinės Amerikos Valstijos

kfv – kolonijas formuojantys vienetai

LB – Luria-Bertani terpė

LBA – Luria-Bertani agaras

NaN_3 – natrio azidas

NaOCl – natrio hipochloritas

OT – optinis tankis

PBS – fosfatinė buferinė terpė

PDA – bulvių dekstrozės agaras

PSO – pasaulinė sveikatos organizacija

SEM – skenuojanti elektroninė mikroskopija

TSYE – Triptono sojos sultinis su 0,6% mielių ekstrakto

TSYEA –Triptono sojos sultinio agaras su 0,6% mielių ekstrakto

TURINYS

ĮVADAS

Pasaulinės Sveikatos Organizacijos (PSO) duomenimis, vis augantis su maistu susijusių ligų plitimas, yra viena didžiausių ne tik besivystančių, bet ir išsivysčiusių šalių sveikatos problemų (1). Jungtinėse Amerikos Valstijose esantis Ligų Kontrolės ir Prevencijos Centras (LKPC) kasmet praneša apie 48 milijonus ligų, 128000 hospitalizacijų ir 3000 mirčių atvejų, kurių priežastis yra patogeninių mikroorganizmų sukeltos su maistu susijusios ligos (2). Ekspertų apskaičiuota, kad su maistu susijusių ligų sukelti ekonominiai nuostoliai JAV kasmet siekia 77,7 mlrd. dolerių (3).

Gram-neigiama bakterija *Salmonella enterica* yra vienas pagrindinių maisto patogenų pasaulyje (4). Kiekvienais metais JAV salmonelioze suserga 10 mln. žmonių, iš kurių 19336 yra hospitalizuojami ir net 378 miršta (5). Europos Sąjungos (ES) šalyse 2011 metais užregistruoti 96883 susirgimai salmonelioze. Daugiausiai susirgimų buvo užregistruota Čekijos Respublikoje (80,69 atvejai 100 000 gyventojų), Slovakijoje (71,70 atvejai) ir Lietuvoje (70,70 atvejai) (6). Gram-teigiama *Listeria monocytogenes* yra pagrindinis mirtį sukeliantis su maistu susijęs patogenas (mirtingumas siekia 30%) (7). Pasak CDC, kasmet JAV yra registruojama apie 1600 ligų ir 260 mirčių atvejų, susijusių su listerioze (6). Tuo tarpu ES 2011 metais buvo registruoti 1524 listeriozės atvejai (5). Dėl Gram-teigiamo, sporas formuojančio *Bacillus cereus* patogeno JAV užregistruoti 2050 ligų atvejų (8).

Braškių (Fragaria × ananassa Duch.) derlius visame pasaulyje siekia 4÷5 milijonus tonų, o ES – 1,1 megatonų per metus (9). Pilkasis kekeras (*Botrytis cinerea*) yra plačiai paplitęs patogeninis grybas, sukeliantis braškių kekerinį (arba pilkąjį) puviną. Jei netaikoma cheminė apsauga, dėl šio fitopatogeninio pelėsio netenkama net 30÷40% braškių derliaus (10). Naudojant cheminę apsaugą ant 63% braškių uogų aptinkama pesticidų likučių (11).

Egzistuojančios tradicinės vaisių ir daržovių saugos technologijos ne tik neužtikrina saugaus maisto, bet ir sukelia visą eilę pašalinių poveikių. Tai skatina mokslininkus ieškoti naujų, efektyvesnių ir saugesnių technologijų.

Vienas iš tokių modernių, neterminių vaisių saugos metodų gali būti fotosensibilizacija (infekcinių ligų gydyme - fotodinaminė terapija) (12,13).

Fotosensibilizacijos esmę sudaro 3 komponentų – fotosensibilizatoriaus (FS), regimosios šviesos ir deguonies sąveika. Deguonies turinčioje aplinkoje sukeliamos radikalinės citotoksinės reakcijos, dėl kurių sutrinka ląstelių veikla ir jos žūna (14,15). Vienas iš plačiai nagrinėjamų ir jau naudojamų medžiagų mikroorganizmų fotoinaktyvacijai sukelti yra endogeninių porfirinų pirmtakas δ-aminolevulininė rūgštis (ALA). Tačiau ALA yra pakankamai brangus junginys, reikalaujantis nemažai laiko endogeninių porfirinų sintezei, todėl jo panaudojimas padidina technologijos sąnaudas (14).

Natrio chlorofilinas (Chl) yra vandenyje tirpus, neigiamai įkrautas maistinis dažiklis, žinomas kaip maisto priedas E140ii (16). Mūsų atlikti tyrimai rodo, kad Chl indukuota fotosensibilizacija yra efektyvi prieš Gram-teigiamas bakterijas, bet Gram-neigiamos bakterijos ir mikromicetai yra mažiau jautrūs šiam poveikiui. Tolygiai visų mikroorganizmų inaktyvacijai galima Chl indukuotą fotosensibilizaciją kombinuoti su kitomis antibakterinėmis technologijomis.

Siekiant padidinti Gram-neigiamų maisto patogenų ir mikromicetų jautrumą Chl indukuotai fotosensibilizacijai, buvo pradėtas taikyti chlorofilino–chitozano (Chl–CHS) konjugatas. Taip pat ši technologija buvo pritaikyta braškių mikrobiologiniam užterštumui mažinti.

Darbo tikslas:

Padidinti maisto patogenų Gram-neigiamos *S. enterica*, Gram-teigiamų *L. monocytogenes*, *B. cereus* ir augalų patogeno pelėsinio grybo *B. cinerea* jautrumą fotosensibilizacijos poveikiui. Ištirti galimus Chl indukuotos fotosensibilizacijos ir fotoaktyvuoto Chl–CHS konjugato poveikio mechanizmus, bei pritaikyti šį antimikrobinį poveikį su maistu susijusių paviršių ir kai kurių vaisių paviršių nukenksminimui.

Uždaviniai:

1. Ištirti ir įvertinti ALA-indukuotos fotosensibilizacijos poveikį Gram-teigiamų (*L. monocytogenes*, *B. cereus*) bei Gram-neigiamų (*S. enterica*) bakterijų ląstelėms *in vitro* ir ant maisto pakuočių paviršiaus.
2. Ištirti ir įvertinti Chl indukuotos fotosensibilizacijos poveikį maisto patogenų *L. monocytogenes*, *B. cereus*, *S. enterica* bei augalų patogeno pelėsinio grybo *B. cinerea* ląstelėms *in vitro* ir ant maisto pakuočių paviršiaus.
3. Įvertinti neigiamai įkrauto Chl indukuotos fotosensibilizacijos antimikrobinį efektyvumą kombinuojant jos poveikį su teigiamai įkrauto CHS antimikrobinėm savybėmis, imobilizuojant Chl į CHS polimerą.
4. Įvertinti Chl indukuotos fotosensibilizacijos ir fotoaktyvuoto Chl–CHS konjugato sukeltas pažaidas bakterijose.
5. Įvertinti fotoaktyvuoto Chl–CHS konjugato poveikį braškių paviršiuje esančioms mielėms ir mikromicetams, jo įtaką braškių mikrobiologiniam užkrėstumui ir organoleptinėms savybėms.

Aktualumas ir naujumas:

1. Pirmą kartą maisto patogenai buvo inaktyvuojami iki šiol fotosensibilizacijoje nenaudotu fotosensibilizatoriumi Chl, kuris yra žinomas kaip maisto priedas (E140ii).
2. Pirmą kartą panaudojus ALA ir Chl indukuotą fotosensibilizaciją, buvo efektyviai inaktyvuojami maisto patogenai (3,7÷4 log) ant maisto pakuočių (poliolefino).
3. Pirmą kartą buvo atliktas ir įvertintas patogenų inaktyvacijos efektyvumas, kombinuojant antimikrobines Chl indukuotos fotosensibilizacijos ir CHS savybes.
4. Pirmą kartą buvo įvertintas fotoaktyvuoto Chl–CHS konjugato poveikis braškių mikrobiologinio nukenksminimo efektyvumui. Buvo nustatyta, kad braškių galiojimo trukmė pailgėja 40% be neigiamo poveikio maistinei vertei ar organoleptinėms savybėms.

Ginamieji teiginiai:

1. ALA indukuota fotosensibilizacija gali efektyviai inaktyvuoti maisto patogenus *in vitro* ir ant maisto pakuočių paviršių.
2. Gram-neigiamos bakterijos ir mikromicetai yra atsparesni Chl indukuotos fotosensibilizacijos poveikiui nei Gram-teigiamos bakterijos.
3. Gram-neigiamų bakterijų jautrumą Chl indukuotai fotosensibilizacijai galima padidinti kombinuojant jos poveikį su teigiamai įkrauto CHS poveikiu, imobilizuojant neigiamai įkrautą Chl į CHS polimerą.
4. Fotoaktyvuotas Chl–CHS gali būti ateityje naudojamas gaminant valgomas fotoaktyvias antimikrobines plėveles, kurios gali išsaugoti braškes ir prailginti jų vartojimo trukmę be neigiamo poveikio šių uogų bendram antioksidaciniam aktyvumui, išvaizdai, spalvai, svoriui ir kitoms savybėms.

2. MEDŽIAGOS IR METODIKA

2.1. Medžiagos

2.1.1. Reagentai

Reagentai darbui atlikti buvo gauti iš kompanijų **Carl Roth GmbH&Co**: Natrio chlorofilinas, KCl, KH_2PO_4; **Fluka-Sigma-Aldrich**: chitozanas, δ - Aminolevulininė rūgštis, metanolis, acetato buferis, 2,4,6,-tripyridyl-s-triazine (TPTZ, $FeCl_3 \times 6H_2O$); **ACROS**: natrio azidas, natrio hipochloritas (13%); **CHEMAPUR**: druskos rūgštis 35–38% (HCl), glicerolis; **Liofilchem**: Luria-Bertani terpė, agaras, DG18 terpė - dichlorano glicerolio agaras, Triptono sojos 0,6% mielių ekstrakto terpė, Triptono sojos 0,6% mielių ekstrakto agaras; Chloramfenikolis; **Difco**: Bulvių-dekstrozės agaro terpė; **OXOID**: NaCl; **REACHEM**: $Na_2HPO_4x12H_2O$; **Sigma**: 97% acetonas; **MERCK**: Tritonas X-100; **Vilniaus degtinė**: 96% etanolis; **B/Braun**: dezinfikantas Meliseptol.

2.1.2. Terpės

Bakterijų kultūros buvo auginamos skystose ir agarizuotose Luria-Bertani (LB) ir Triptono sojos sultinio su 0,6% mielių ekstraktu (TSYEA) terpėse. Priešgrybelinis aktyvumas buvo įvertintas ant bulvių dekstrozės agaro (PDA) ir dichlorano glicerolio (DG18) agaro (17–25).

2.1.3. Bakterijų ir grybo kamienai

Darbe naudotų bakterijų ir grybo kamienų sąrašas pateiktas 2.1 lentelėje. *Salmonella enterica* - Gram-neigiamos, judrios, sporų neformuojančios, fakultatyvinės anaerobinės 0,7÷1,5 / 2,0÷5,0 μm dydžio lazdelės sukeliančios salmoneliozę (26). *Salmonella* gali augti labai plačiame pH intervale: 3,8÷9,5 (27). *S. enterica* aptinkama įvairiuose maisto produktuose: šviežiuose

vaisiuose, mėsos produktuose, kiaušiniuose (28). *Listeria monocytogenes* - Gram-teigiamos sporų neformuojančios 0,5÷2 μm diametro lazdelės formos labai patogeninės bakterijos, sukeliančios listeriozę (29,30). *L. monocytogenes* puikiai prisitaiko prie aplinkos sąlygų – gali išgyventi esant įvairiai temperatūrai (-0,4 ÷ +45 ºC), pH (4,3÷9,6), aukštai druskų koncentracijai (10% NaCl) ir žemam vandens aktyvumui (*Aw* iki 0,90). Aplinkoje dažniausiai formuoja atsparias bioplėveles. *L. monocytogenes* bakterijos aptinkamos įvairiuose produktuose: daržovėse, piene, įvairių rūšių sūriuose, žuvies ir mėsos produktuose (31). *Bacillus cereus* – Gram-teigiamos fakultatyvinės anaerobinės, sporas formuojančios, judrios, 1,4 μm ilgio lazdelės. *B. cereus* yra chemoorganotrofai, galintys augti nuo +10 ºC iki +45 ºC temperatūroje (32). *B. cereus* bakterijos aptinkamos įvairiuose maisto produktuose: mėsoje, javuose, šviežiose daržovėse, vaisiuose ir uogose. Pilkojo puvinio sukėlėjas pilkasis kekeras *Botrytis cinerea* yra didelį ekonominį nuostolį darantis patogenas, kuris sukelia daugiau negu 200 augalų rūšių puvinių jų vegetacijos metu bei sandėliuojant produkciją (33).

2.1 lentelė Bakterijų ir grybo kamienai.

		Kamienas	Šaltinis
Bakterija	*S. enterica* serovar Typhimurium	DS88 (SL5676 Sm^2 pLM32)	Prof. D. H. Bamford (Helsenkio Universitetas, Suomija).
	L. monocytogenes	$ATC_{L3}C$ 7644	Nacionalinė Veterinarijos Laboratorija (Vilnius, Lietuva)
	B. cereus	ATCC 12826	Nacionalinis visuomenės sveikatos centras (Vilnius, Lietuva)
Grybas	*B. cinerea*	Sclerotiniaceae šeima	Vilniaus Universiteto kolekcija

2.1.4. Tirpalai

ALA tirpalas: pradinis ALA tirpalas buvo gaminamas tirpinant ALA 0,01 M PBS buferyje (18). **CHS tirpalas:** CHS ištirpinamas druskos rūgštyje (HCl), maišant magnetine maišykle. Gaunamas tirpalas (prie 20 ºC: pH 2,4), kurio 100 g yra 1,25 g CHS ir 0,23 g HCl (24,34). **Chl tirpalas:** pradinis Chl tirpalas

buvo ruošiamas tirpinant medžiagą autoklavuotame distiliuotame vandenyje. **Chl–CHS konjugatas:** paruošiamas 0,01 Chl–1% CHS konjugato tirpalas, kurio 100 g yra 1 g CHS, 0,18 g HCl ir 0,01 g Chl. Pradinio tirpalo pH 2,4, o įnešus jį į 0,9% NaCl, jis padidėja iki 3,95 (24,34). Tyrimuose naudota 0,001 Chl–0,1% CHS konjugato koncentracija atitinka $1{,}5 \cdot 10^{-5}$ M Chl koncentracijai. **0,01 M PBS tirpalas (pH 7,4):** 2,7 mM KCl, 1,8 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4.

2.1.5. Prietaisai

Visi tyrimai atlikti Vilniaus Universiteto Fotonikos ir nanotechnologijų instituto moderniose laboratorijose naudojant naujai įsigytą mikrobiologinio darbo, biocheminės ir spektrofotometrinės analizės bei skenuojančios mikroskopijos įrangą (17–25).

2.1.6. Šviesos šaltinis naudojamas eksperimentuose

Švitinimui skirtas prototipas buvo sukonstruotas Vilniaus universiteto Fotonikos ir Nanotechnologijų institute (24). Kaip šviesos šaltiniai naudoti InGaN šviestukai (LED), (LED Engine, Inc. LZ1-00UA00, JAV). LED emisijos maksimumas – 405 nm, o intensyvumas mėginių paviršiuje siekia 10 mW/cm^2 iš viršaus (6 cm atstumu nuo šviestukų) ir 11 mW/cm^2 iš apačios (3,5 cm atstumu nuo šviestukų). Bandiniams tenkanti šviesos dozė D apskaičiuojama, švitinimo intensyvumą I dauginant iš švitinimo laiko t ($D = I \cdot t$). Prototipo viršuje sumontuota efektyvi prietaiso vėdinimo sistema, mažinanti galimą šilumos poveikį mėginiams.

2.1.7. Programinė įranga ir duomenų bazės internete

OriginPro 8 (OriginLab Corporation, JAV), Microsoft Excel 2010 (Microsoft, JAV), StatSoft Statistica 10 (JAV), Primer3 (v. 0.4.0) duomenų bazė internete (http://frodo.wi.mit.edu/Primer3).

2.2. Tyrimų metodika

2.2.1. Spektrofotometriniai ir fluorimetriniai matavimai

Absorbcijos spektrai buvo matuojami PerkinElmer UV/VIS Lambda 25 spektrofotometru, fluorescencijos spektrai – fluorescenciniu spektrofotometru PerkinElmer LS-55. Matavimai buvo atliekami naudojant kvarcines kiuvetes (spindulio kelio ilgis 1cm), į jas įpilant 3 ml tiriamojo tirpalo.

Endogeninių porfirinų fluorescenciniai matavimai bakterijų ląstelių suspensijoje ($1 \cdot 10^7$ kfm/ml 0,01M PBS) buvo atliekami po inkubacijos tamsoje, esant $3 \cdot 10^{-3}$ ir $7{,}5 \cdot 10^{-3}$ M ALA (37 °C). Matavimo parametrai: sužadinimo bangos ilgis (Ex) 390nm; matavimo spektrinis intervalas (Em) 590÷750nm; spektrų užrašymo sparta: 200 nm/min. Ląstelėse gaminamų endogeninių porfirinų analizė buvo atliekama pagal Lukšienės (35) darbe aprašytą metodiką.

10 kartų praskiestų Chl ir Chl–CHS konjugato tirpalų sugerties ir fluorescencijos spektrų matavimai buvo atliekami tamsoje 0,01 M PBS ir 0,9% NaCl. Sugerties spektrai matuoti 300÷710 nm bangos ilgio intervale. Fluorescencijos matavimo parametrai: Ex = 390nm; Em = 590÷750nm; spektrų užrašymo sparta: 200 nm/min. Taip pat spektrai užregistruojami į tirpalą įlašinus 20 µl detergento Tritono X–100.

Ląstelių ir Chl tarpusavio sąveika taip pat buvo įvertinta fluorimetriškai. *S. enterica* ir *L. monocytogenes* bakterijų ląstelės ($1 \cdot 10^7$ kfv/ml 0,01 M PBS) po atitinkamos inkubacijos su $1{,}5 \cdot 10^{-5}$ M ir $1{,}5 \cdot 10^{-7}$ M Chl buvo 10 min centrifuguojamos (6 °C, 3574 g) ir resuspenduojamos 0,01 M PBS (25).

1.2.2. Mikroorganizmų auginimas

Bakterijų kultūros palaikomos ant agarizuotos terpės. Eksperimentams bakterijų kultūros persėjamos į 20 ml sterilią terpę ir auginamos per naktį (~16 val.) inkubacijos termostate, esant 37 ºC temperatūrai, aeruojant 120 aps./min greičiu. Tiriamų bakterijų kultūros auginamos, kol pasiekiama eksponentinio augimo fazė (Lentelė 2.2).

2.2 lentelė Bakterijų augimo sąlygos.

Kamienas	Terpė	OT_{540}	kfv/ml
S. enterica serovar *Typhimurium* DS88 (SL5676 Sm^2 pLM32)	LB	1,3	$5 \cdot 10^8$
L. monocytogenes $ATC_{L3}C$ 7644	TSYE	0,9	$1{,}16 \cdot 10^9$
B. cereus ATCC 12826	LB	1	$6 \cdot 10^7$

1 cm diametro kiuvetėse paruoštų bakterijų mėginių optinis tankis (OT) matuotas λ=540 nm spektrinėje srityje. Atitinkamo OT bakterijų ląstelių suspensijos buvo centrifuguojamos 10 min (6 °C, 3574 g). Supernatantas nusiurbiamas, o centrifuguotos ląstelės, priklausomai nuo tolimesnės bandymo eigos, resuspenduojamos naudojant 0,01 M PBS buferį arba 0,9% NaCl (17–25).

B. cinerea mikromiceto kultūra buvo išskirta iš braškių ir palaikoma persėjant ją ant agarizuotos PDA terpės 24 °C temperatūroje.

2.2.3. ALA indukuota fotosensibilizacija *in vitro*

Ląstelių suspensija (~$1 \cdot 10^7$ kfv/ml) kartu su atitinkamos koncentracijos ALA ($3 \cdot 10^{-3}$ ir $7{,}5 \cdot 10^{-3}$ M) buvo inkubuojama (120 aps./min) tamsoje, 37 °C temperatūroje 0,01 M PBS buferyje. Kontrolinis mėginys inkubuojamas tomis pačiomis sąlygomis, tačiau vietoj ALA pridedamas toks pats kiekis PBS buferio. Po atitinkamo inkubacijos laiko (0÷60 min) imami mėginiai fluorescencijai matuoti arba švitinimui (λ=405 nm, šviesos dozė 0÷24 J/cm^2) (18–20).

2.2.4. Chl indukuotos fotosensibilizacijos ir fotoaktyvuoto Chl–CHS konjugato poveikis bakterijų gyvybingumui *in vitro*

Ląstelių suspensija (~$1 \cdot 10^7$ ir $1 \cdot 10^8$ kfv/ml) su atitinkamos koncentracijos Chl ($7{,}5 \cdot 10^{-8}$ M÷$1{,}5 \cdot 10^{-5}$ M) ir Chl–CHS (0,00001/0,001 %÷0,001/0,1%) konjugatu buvo inkubuojama (120 aps./min) tamsoje (37 °C) 0,01 M PBS buferyje arba 0,9 % NaCl. Po atitinkamo inkubacijos laiko (0÷120 min) imami mėginiai fluorescencijai matuoti arba švitinimui (λ=405 nm, šviesos dozė 0÷51 J/cm^2). Tiriant Chl ir ląstelės sąveikos intensyvumą, prieš švitinimą (šviesos dozė 0÷88,7 J/cm^2) buvo atliktas supernatantinio Chl atskyrimas. Bakterijos su atitinkamos koncentracijos Chl po inkubacijos tamsoje (2 min *Listeria* ir 60 min *Salmonella*) buvo 10 min centrifuguojamos (3574 g, +6 °C), nuosėdos resuspenduojamos tokiame pačiame distiliuoto ir autoklavuoto vandens kiekyje.

2.2.5. Chl indukuotos fotosensibilizacijos ir fotoaktyvuoto Chl–CHS konjugato poveikis *B. cinerea* gyvybingumui

Eksperimentams paruošta, autoklavuota PDA terpė su $1{,}5 \cdot 10^{-4}$ M Chl ir 0,001 Chl–0,1% CHS buvo išpilstyta į lėkšteles. Į lėkštelės vidurį buvo patalpinamas 5 mm diametro 4 dienų senumo *B. cinerea* micelio. Dalis lėkštelių po 24 h inkubacijos tamsoje termostate (24 °C) buvo švitinama regimąja šviesa (šviesos dozės 35 J/cm^2 (Chl) ir 76 J/cm^2 (Chl–CHS)). Kita dalis lėkštelių nebuvo apšvitinta (tamsinis mėginys). Kontroliniams mėginiams buvo naudojama PDA terpė be Chl ir Chl–CHS. Poveikis *B. cinerea in vitro* įvertinamas vizualiai ir pamatavus kolonijos diametrą (cm). Augimo slopinimo laipsnis procentais (%) apskaičiuotas pagal formulę $x = R - r/R \times 100$ (R – kolonijos diametras kontrolinėje lėkštelėje; r – kolonijos diametras lėkštelėse su Chl ar Chl–CHS)

2.2.6. CHS ir Chl–CHS konjugato antibakterinio aktyvumo tyrimai (tamsoje)

Ląstelių suspensija (~$1 \cdot 10^7$ kfv/ml) su atitinkamos koncentracijos CHS (0,1%) ir Chl–CHS konjugatu (0,00001÷0,001% ir 0,001÷0,1%) buvo inkubuojama (130 aps./min) tamsoje (37 °C) 0,9% NaCl tirpale. Po atitinkamo inkubacijos laiko (1 min, 15 min, 30 min, 60 min ir 120 min) imami mėginiai ląstelių gyvybingumui nustatyti.

2.2.7. Skenuojanti elektroninė mikroskopija (SEM)

Chl indukuotos fotosensibilizacijos ir fotoaktyvuoto Chl–CHS konjugato poveikis *S. enterica* ir *L. monocytogenes* ląstelių morfologijai buvo tiriamas naudojant SEM (modelis CamScan Apollo 300). 20 µl skirtingai paveiktų bakterijų suspensijos mėginiai buvo užlašinti ant varinės plokštelės, išdžiovinti ore ir padengti 15 nm aukso sluoksniu naudojant plonų metalinių sluoksnių joninio dulkinimo įrangą (modelis Q150T ES). Skenavimas buvo atliktas esant 20 kV elektronų pluoštelio įtampai.

2.2.8. Bakterijų ląstelių membranos pralaidumo įvertinimas po poveikio

Chl indukuotos fotosensibilizacijos ir fotoaktyvuoto Chl–CHS konjugato poveikių įtaka *S. enterica* (~$1 \cdot 10^7$ kfv/ml) ir *L. monocytogenes* (~$1 \cdot 10^8$ kfv/ml) ląstelių membranos pralaidumui buvo tikrinama spektrofotometriniu metodu, matuojant tirpalo sugertį ties λ=260 nm (36) ir λ=280 nm (37). Matavimams buvo naudojami 3 ml pro sterilų 0,20 µm baktericidinį filtrą prafiltruoti suspensijų mėginiai po atitinkamo švitinimo:

1. $1,5 \cdot 10^{-5}$ M Chl *Salmonella*: 0; 5,8; 11,5; 17,3; 23; 40,3 ir 46,1 J/cm^2.
2. 0,001 Chl–0,1% CHS konjugatas *Salmonella*: 25 ir 38 J/cm^2 .
3. $1,5 \cdot 10^{-6}$ M Chl *Listeria*: 0; 0,29; 0,58; 1,15; 11,5 J/cm^2.

2.2.9. Singuletinio deguonies ($^1O_2$) įtakos nustatymas Chl indukuotų fotoaktyvuotų bakterijų gyvybingumui

Bakterijų suspensijos su Chl ir atitinkamos koncentracijos (0÷40 mM) natrio azidu (NaN_3) (fizikiniu $^1O_2$ gesikliu) (38,39) buvo inkubuojamos tamsoje 37 ºC temperatūroje :

1. *Salmonella*: ~$1 \cdot 10^7$ kfv/ml; $1{,}5 \cdot 10^{-5}$ M Chl; inkubacijos laikas 60 min.
2. *Listeria*: ~$1 \cdot 10^8$ kfv/ml; $1{,}5 \cdot 10^{-7}$ M Chl; inkubacijos laikas 2 min.

Po inkubacijos buvo imami mėginiai švitinimui (λ=405 nm, šviesos dozė 0÷88,7 J/cm^2). Dalis mėginių prieš švitinimą buvo centrifuguojami (10 min, 3574 g, +6 °C). Pradinis NaN_3 tirpalas buvo paruoštas ištirpinus atitinkamą kiekį NaN_3 autoklavuotame distiliuotame vandenyje.

2.2.10. Bakterijų, prisitvirtinusių prie maisto pakuočių paviršiaus, fotoinaktyvacija

Maistui pakuoti naudojamos poliolefino pakuotės buvo gautos iš LINPAC (West Yorks, Didžioji Britanija). Eksperimentams buvo naudojami 4 × 8 cm dydžio pakuočių pavyzdžiai, kurie buvo užpilami 50 ml atitinkamos bakterijų suspensijos (~$1 \cdot 10^7$ kfv/ml) ir 30 min laikomi laminare (geresniam ląstelių prisitvirtinimui). Tuomet pakuočių pavyzdžiai buvo inkubuojami tamsoje (5, 10 ir 20 min) su $3 \cdot 10^{-3}$ M (*B. cereus*) ir $7{,}5 \cdot 10^{-3}$ M ALA (*S. enterica*) bei $1{,}5 \cdot 10^{-5}$ M Chl (*B. cereus* ir *L. monocytogenes*). Po inkubacijos pakuočių pavyzdžiai buvo paliekami išdžiūti laminare 20 min ir švitinami (5÷20 min). Nešvitinti kontroliniai pavyzdžiai buvo inkubuojami PBS buferyje be FS.

2.2.11. Braškių nukenksminimas Chl, Chl–CHS konjugatu ir šviesa

2.2.11.1. Įvairių braškes nukenksminančių poveikių palyginamoji analizė

Braškių (Fragaria × ananassa Duch.) uogos buvo įsigyjamos prekybos centre tą pačią dieną ar dieną prieš tyrimų pradžią. Buvo ištirti 0,1 % CHS (30 min mirkymas), 0,001 Chl–0,1% CHS (30 min mirkymas), fotoaktyvuoto 0,001 Chl–0,1% CHS konjugato (šviesos dozė 76 J/cm^2), fotoaktyvuotų 0,001 % ir 0,1 % Chl (šviesos dozė 76 J/cm^2) ir 200 ppm natrio hypochlorito (NaOCl) (1 min mirkymas) poveikiai braškių paviršiaus nukenksminimui (24).

2.2.11.2. Apdorotų braškių galiojimo trukmės įvertinimas

Apdorotų braškių uogų galiojimo laikas įvertintas pritaikius Kaplan-Meier išgyvenimo kreives (40). Po poveikio mėginiai buvo laikomi 22 ± 2 ºC temperatūroje. Taip pat lygiagrečiai buvo vertinamas apdorotų uogų sugedimo laipsnis panaudojus Kittemann metodiką (41), kai uogų užkrėstumo lygis buvo vertinamas 1÷6 skalėje. Braškių išvaizda įvertinta naudojant Mahmoud darbe aprašytą metodiką (42), kai vizualiai buvo stebimas pelėsinių grybų išplitimas uogų paviršiuje.

2.2.12. Braškių organoleptinių savybių įvertinimas po apdorojimo fotoaktyvuotu Chl–CHS konjugatu

2.2.12.1. Apdorotų braškių bendro antioksidacinio aktyvumo matavimai

Braškių uogų antioksidacinės savybės po fotoaktyvuoto Chl–CHS konjugato poveikio buvo ištirtos taikant geležies (II) jonų redukcijos antioksidacinės galios nustatymo (FRAP) metodą (43). FRAP metodu buvo įvertintas antioksidantų gebėjimas rūgštinėje terpėje redukuoti geležies 2,4,6-tripyridyl-s-triazino $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ kompleksą į intensyviai mėlynos spalvos $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ kompleksą. Matavimams naudoto ekstrakto paruošimas: 1 g uogų homogenizuojamas su 50 ml metanolio. Darbinio FRAP reagento paruošimas: 0,3 M/l acetato buferis (pH 3,6), 0,01 M/l 2,4,6,-

tripyridyl-s-triazino (TPTZ) ištirpinamas 0,04 M/l HCl ir 0,02 M/l $FeCl_3 \times 6H_2O$ ištirpintame distiliuotame vandenyje.

Matavimams sumaišomi 3 ml darbinio FRAP reagento ir 100 µl tiriamo braškių ekstrakto. Mišinys laikomas kambario temperatūroje ir po 4 min išmatuojama jo absorbcija esant λ=593 nm bangos ilgiui. Gautos reikšmės palyginamos su etaloniniu dvivalentės geležies jonų tirpalu (100÷1000 µM). Redukcinio aktyvumo galia išreiškiama mM Fe^{2+}/kg, t.y. vienam sausos maisto medžiagos kilogramui (angl. dry weight of food material).

2.2.12.2. Braškių uogų spalvos ir svorio kitimo matavimai

Braškių uogų spalvos pokyčiai po fotoaktyvuoto Chl–CHS konjugato poveikio buvo įvertinti spektrofotometriškai. Išmatuoti spektrai intervale 350÷650 nm naudojant spektrometrą Lamda 25 (PerkinElmer). Detalesnis eksperimento aprašymas pateiktas literatūroje (44).

Atliekant uogų svorio kitimo matavimus, braškės buvo padalintos į 2 grupes po 20 vienetų. Pirmosios, kontrolinės, grupės uogos nebuvo mirkomos ir švitinamos. Antrąją, eksperimentinę, grupę sudarė uogos po poveikio fotoaktyvuotu Chl–CHS (šviesos dozė 76 J/cm^2). Tolimesniam uogų laikymui buvo naudojamas šaldymo inkubatorius su drėgmės kontrole CLIMACELL 111. Laikymo aplinkos parametrai buvo šie: temperatūra ~6 ± 0,5°C, santykinė oro drėgmė (RH) 75 ± 5%, ventiliatoriaus apsukų lygis 80% nuo maksimalaus. Galimas uogų svorio praradimas išreiškiamas %.

Braškių uogų vandens kiekio pokyčiai po fotoaktyvuoto Chl–CHS konjugato poveikio buvo įvertinti registruojant FT-Raman spektrus. Išmatuoti spektrai intervale 3500÷500 cm^{-1} naudojant FT-Raman spektrometrą "Bruker MultiRam". Eksperimente naudota λ=1064 nm bangos ilgio ir 100 mW galios sužadinimo spindulioutė.

2.2.14. Braškių EPR spektrų registravimas

Elektronų paramagnetinio rezonanso (EPR) spektrai registruoti Bruker Elexsys E580 FT-EPR spektrometru. Spektrometro darbinė juosta – X, aukšto dažnio ateinančio iš generatoriaus slopinimas–17 dB, moduliacijos dažnis – 100 kHz, moduliacijos amplitudė - 3G. Braškės prieš EPR matavimus inkubuojamos 60 min su 0,001 Chl–0,1% CHS konjugatu, vėliau švitinamos iš abiejų pusių 405 nm bangos ilgio šviesa (šviesos dozė 76 J/cm^2) ir 2 min homogenizuojamos. Paskutiniame mėginio paruošimo spektro matavimui etape 1 g apdorotų braškių uogų substrato patalpinamas į stiklinį kapiliarą.

2.2.15. Gyvybingumo tyrimai

Antibakterinis ir antifungicidinis poveikių efektyvumas buvo įvertintas panaudojus išsėjimo metodiką („spread plate method“).

Antibakterinis aktyvumas *in vitro*

100 µl atitinkamo skiedimo bakterijų suspensijos po poveikio *in vitro* buvo išsėjami LBA lėkštelės paviršiuje. Lėkštelės laikomos 24 h 37 °C termostate. Bakterijų gyvybingumas buvo išreikštas dešimtainiu logaritmu – $\log_{10}$ (kfv/ml).

Antibakterinis aktyvumas *in vivo*

Po švitinimo maisto pakuočių pavyzdžiai buvo užpilami 30 ml sterilaus 0,01 M PBS buferio ir homogenizuojami 1 min Bagmixer aparatu. Toliau 100 µl atitinkamo mėginio suspensijos buvo išsėjami agarizuotos lėkštelės paviršiuje. Lėkštelės laikomos 24 h 37 °C termostate. Bakterijų gyvybingumas buvo išreikštas dešimtainiu logaritmu – log kfv/ cm^2.

Antifungicidinis aktyvumas *in vivo*:

1 g braškės ir 9 ml 0,9 % NaCl tirpalo buvo homogenizuojami 2 min BagMixer aparatu. Toliau 100 µl atitinkamo mėginio suspensijos buvo išsėjami agarizuotų TSYE (mezofilinių bakterijų nustatymui) ir DG18 (mielių ir mikromicetų nustatymui) lėkštelių paviršiuje. Lėkštelės laikomos 24 h 37 ir 30 °C temperatūroje termostate. Bakterijų ir grybų gyvybingumas buvo išreikštas dešimtainiu logaritmu – log kfv/g.

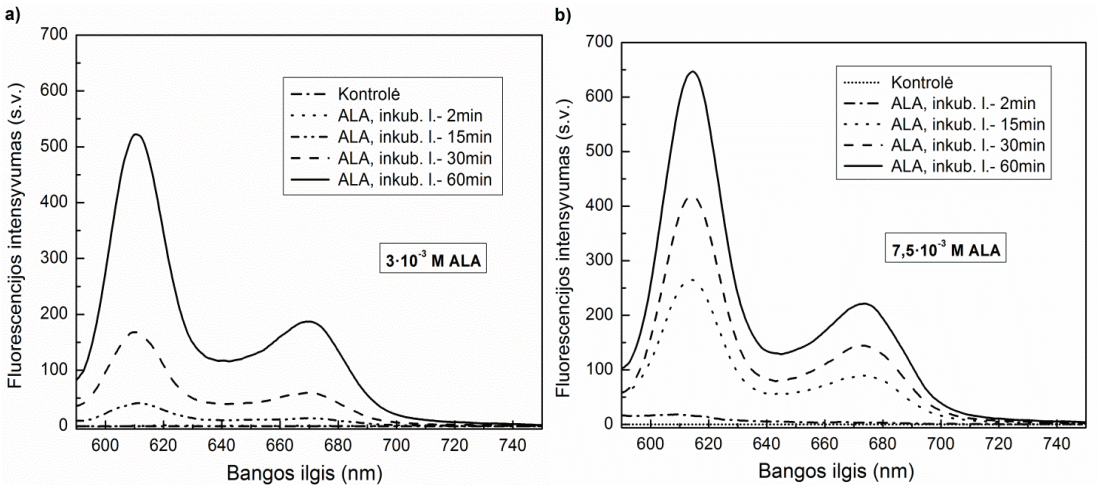
2.2.16. Statistinė analizė

Eksperimentai kartoti 3 kartus. Duomenys apdoroti su programine įranga “Origin 7.5“ ir pateikti su apskaičiuotais standartiniais nuokrypiais. Statistinė analizė atlikta StatSoft programa STATISTICA 10. Statistiškai reikšmingu skirtumu laikyta $P < 0,005$ (mikrobiologinei analizei) ir $P < 0,05$ (atliekant eksperimentus su braškėmis).

3. REZULTATAI

3.1. Iš ALA susintetintų endogeninių porfirinų nustatymas bakterijose

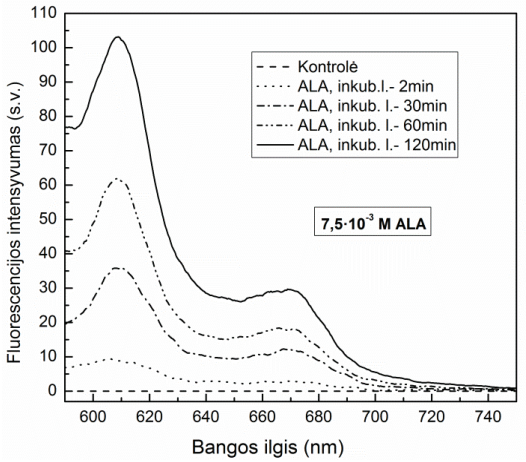
Atliekant mokslinius tyrimus endogeninių porfirinų biosintezė dažnai stimuliuojama egzgoneniais pirmtakais – ALA ar ALA esteriais (45). Ne kiekviena bakterija sugeba susintetinti iš ALA tokį kiekį endogeninių porfirinų, kurio užtektų fotosensibilizacija indukuotai inaktyvacijai (46,47). Todėl darbo pradžioje buvo įvertintas tiriamų Gram-teigiamų *B. cereus*, *L. monocytogenes* ir Gram-neigiamos *S. enterica* bakterijų sugebėjimas iš egzogeninio ALA susintetinti endogeninius porfirinus. Endogeninių porfirinų detekcijai bakterijų ląstelėse buvo naudojama fluorescencijos spektroskopija (35). Bakterijų ląstelių ir ALA ($3\cdot10^{-3}$ M ir $7{,}5\cdot10^{-3}$ M koncentracijos) suspensijos po inkubacijos tamsoje buvo tiriamos analizuojant jų fluorescencijos spektrus 590÷700 nm intervale. Pasak Szocs (48), šis spektrinis diapazonas su piku ribose λ=612÷615 nm yra siejamas su endogeniniais porfirinais, pvz., uroporfirinais ir koproporfirinais. Atsižvelgiant į kitų mokslininkų atliktus tyrimus buvo pasirinktos $3\cdot10^{-3}$ M ir $7{,}5\cdot10^{-3}$ M ALA koncentracijos (46,48,49).



3.1 paveikslas *B. cereus* endogeninių porfirinų fluorescencijos spektrai, išmatuoti po inkubacijos su $3\cdot10^{-3}$ M (a) ir $7{,}5\cdot10^{-3}$ M (b) ALA.

Atlikta fluorimetrinė analizė parodė, kad endogeninių porfirinų fluorescencijos intensyvumas (sužadinimas λ=390 nm) *Bacillus* ląstelėse po

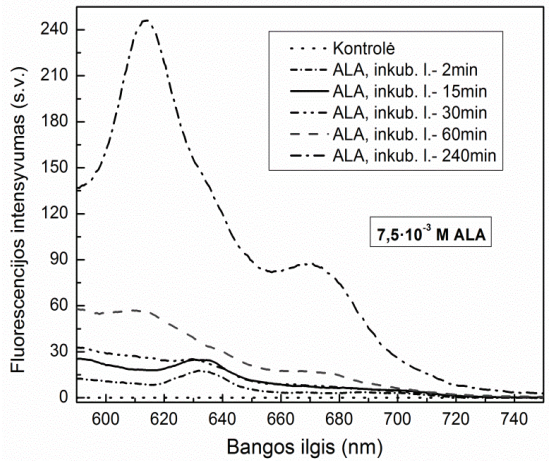
inkubacijos (0÷60 min) tamsoje su $3 \cdot 10^{-3}$ M ALA didėjo priklausomai nuo inkubacijos laiko (3.1a paveikslas). Endogeninių porfirinų fluorescencijos intensyvumas po 2 min inkubacijos su $3 \cdot 10^{-3}$ M ALA buvo nedidelis, tačiau jo vertė po 60 min inkubacijos padidėjo iki 500 s.v.. Siekiant įsitikinti, kad buvo panaudota tinkama ALA koncentracija, buvo padidinta ALA koncentracija iki $7{,}5 \cdot 10^{-3}$ M. Endogeninių FSs fluorescencijos spektrų analizė parodė, kad taikant $7{,}5 \cdot 10^{-3}$ M ALA koncentraciją ir tokį patį 60 min inkubacijos laiką endogeninių porfirinų fluorescencijos intensyvumo vertė išaugo iki 650 s.v. (3.2b paveikslas).



3.2 paveikslas *L. monocytogenes* endogeninių porfirinų fluorescencijos spektrai, išmatuoti po inkubacijos su $7{,}5 \cdot 10^{-3}$ M ALA.

Panaudojant tą pačią metodiką, buvo ištirtas Gram-teigiamos *L. monocytogenes* sugebėjimas iš egzogeninės ALA pagaminti endogeninius porfirinus (3.2 paveikslas). Bakterijų ląstelės tamsoje buvo inkubuojamos 0÷120 min su $7{,}5 \cdot 10^{-3}$ M ALA. Tuomet, siekiant nustatyti endogeninių porfirinų sintezę, buvo analizuojami fluorescencijos spektrai 590÷700 nm srityje (sužadinimas λ=390 nm). 3.2 paveiksle pateikti duomenys rodo, kad endogeninių porfirinų santykinis fluorescencijos intensyvumas auga didėjant inkubaciniam laikui su ALA. Fluorescencijos intensyvumas buvo labai mažas po 2 min inkubacijos su $7{,}5 \cdot 10^{-3}$ M ALA (11 s.v.), bet po 30 min reikšmingai padidėjo (39 s.v.). Padidinus *L. monocytogenes* ląstelių kiekį ir $7{,}5 \cdot 10^{-3}$ M ALA inkubacijos laiką iki 60 min, fluorescencijos intensyvumo vertė išaugo iki 65 s.v., o pailginus inkubacijos laiką iki 120 min, padidėjo iki 105 s.v..

Gram-neigiamos bakterijos *S. enterica* ląstelės buvo inkubuojamos tamsoje 0÷240 min su $7{,}5 \cdot 10^{-3}$ M ALA. Endogeninių porfirinų sintezė buvo analizuojama 590÷700 nm srityje (sužadinimas λ=390 nm) (3.3 paveikslas). Svarbu pažymėti, kad Gram-neigiamos *Salmonella* endogeninių porfirinų fluorescencijos intensyvumas, lygiai taip pat kaip ir prieš tai ištirtų Gram-teigiamų *Bacillus* ir *Listeria*, priklauso nuo inkubacijos laiko su egzogeniniu ALA. Iš 3.3 paveiksle pateiktų fluorescencijos spektrų matyti, kad po 2, 15 ir 30 min inkubacijos laikų bakterijų ląstelės tik pradeda gaminti endogeninius porfirinus (pikas ties 610÷630 nm). Padidinus *S.enterica* ląstelių kiekį ir $7{,}5 \cdot 10^{-3}$ M ALA inkubacijos laiką iki 240 min, fluorescencijos intensyvumo vertė išaugo iki 246 s.v..



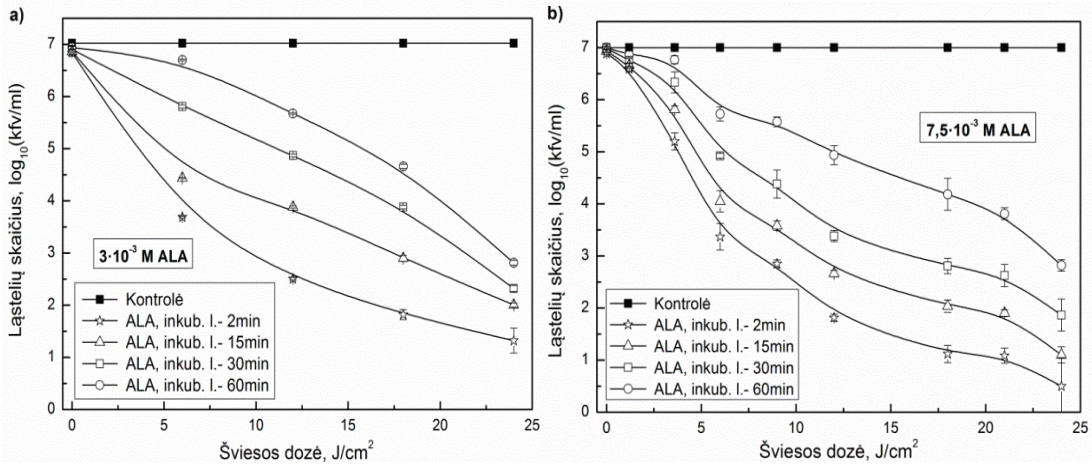
3.3 paveikslas *S. enterica* endogeninių porfirinų fluorescencijos spektrai išmatuoti po inkubacijos su $7{,}5 \cdot 10^{-3}$ M ALA.

Kadangi vienas iš darbo uždavinių buvo ištirti, ar *B. cereus* ATCC 12826, *L. monocytogenes* $ATC_{L3}C$ 7644 ir *S. enterica* DS88 (SL5676 Sm^2 pLM32) gali iš egzogeninio ALA gaminti endogeninius porfirinus, todėl nebuvo aiškinamasi, kokio tipo endogenius porfirinus gamina tiriamosios bakterijos. Tikslas buvo surasti tinkamą endogeninių porfirinų sintezės laiką, kuris būtų optimalus paviršius nukenksminančiai technologijai.

3.2. Bakterijų jautrumas ALA indukuotai fotosensibilizacijai *in vitro*

Endogeninių porfirinų fluorescencijos spektrų analizė parodė, kad Gram-teigiamos *B. cereus*, *L. monocytogenes* ir Gram-neigiama *S. enterica* efektyviai sintetina endogeninius FSs iš egzogeninės ALA. Tai leidžia daryti prielaidą, kad ALA-indukuota fotosensibilizacija gali būti puiki kovos priemonė prieš maisto patogenus. Gauti rezultatai paskatino įvertinti ALA-indukuotos fotosensibilizacijos poveikį *B. cereus*, *L. monocytogenes* ir *S. enterica* ląstelių gyvybingumui.

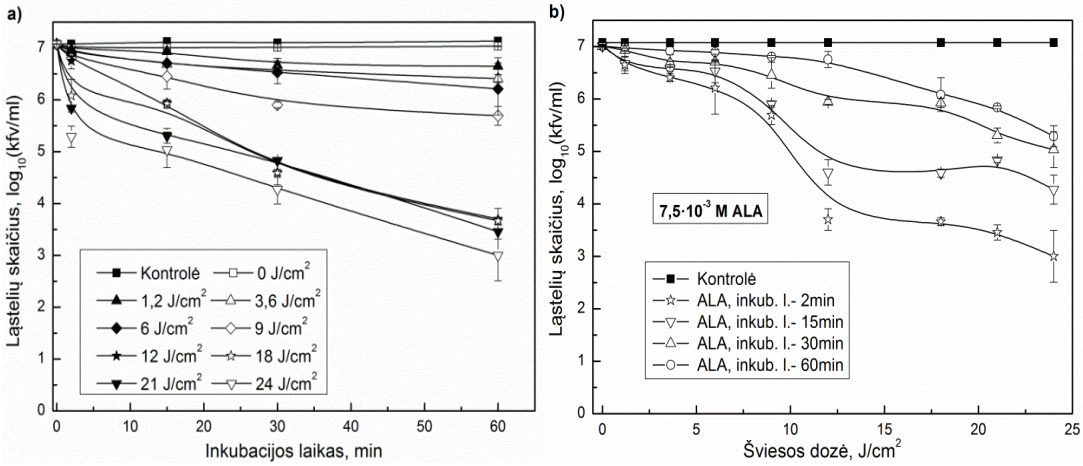
Iš gautų rezultatų matyti, kad ALA ($3\cdot10^{-3}$ M ir $7{,}5\cdot10^{-3}$ M koncentracijos) įtaka *Bacillus, Listeria* ir *Salmonella* gyvybingumui be šviesos poveikio yra labai nežymi. Tik po inkubacijos su ALA ir bakterijų ląstelių švitinimo ($\lambda = 405$nm; šviesos dozė 0÷24 J/cm^2) reikšmingai sumažėja jų gyvybingumas (3.4–3.6 paveikslai). Svarbu pažymėti, kad labai mažos šviesos dozės nebuvo efektyvios, kadangi ne visi endogeniniai porfirinai buvo sužadinti šviesa į S_1 būseną. Negavę reikiamų fotonų, jie nesugebėjo aktyvuoti tolimesnių citotoksinių reakcijų.



3.4 paveikslas $3\cdot10^{-3}$ M (a) ir $7{,}5\cdot10^{-3}$ M (b) ALA indukuotos fotosensibilizacijos poveikis *B. cereus* gyvybingumui esant skirtingoms šviesos dozėms. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Iš duomenų pateiktų 3.4a paveiksle matyti, kad pailginus *Bacillus* inkubaciją su $3\cdot10^{-5}$ M ALA nuo 2 min iki 60 min, jos ląstelių inaktyvacija žymiai padidėjo. Naudojant 60 min inkubacijos laiką ir 24 J/cm^2 šviesos dozę *B. cereus* buvo inaktyvuota 5,75 log lastelių. Fotosensibilizacijos efektyvumą galima išreikšti ląstelėje susintetinto FS ir bendros bakterijų ląsteles

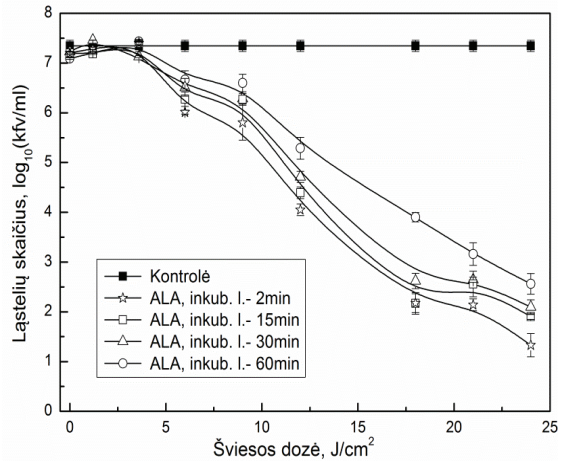
pasiekiančios šviesos energijos sandauga. Būtent todėl, tiriant *B. cereus* jautrumą ALA indukuotai fotosensibilizacijai, ypatingas dėmesys buvo skirtas inaktyvacijos priklausomybei nuo laiko ir naudotos ALA koncentracijos. 3.4b paveiksle pateikti duomenys rodo, kad didesnė $7{,}5\cdot10^{-3}$ M ALA koncentracija padidina *B. cereus* inaktyvaciją, tačiau šis padidėjimas ypač priklauso nuo inkubacijos laiko ir švitinimo dozės. Trumpas inkubacijos laikas (2 min) ir mažos šviesos dozės (1,2÷9 J/cm^2) nėra efektyvios, tuo tarpu 15÷60 min inkubacija ir 12÷24 J/cm^2 švitinimo dozės yra daug veiksmingesni ir labiau tinkantys antibakterinėms technologijoms.



3.5 paveikslas $7{,}5\cdot10^{-3}$ M ALA indukuotos fotosensibilizacijos poveikis *L. monocytogenes* gyvybingumui esant skirtingam inkubacijos laikui (a) ir šviesos dozei (b). Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Iš eksperimentinių duomenų pateiktų 3.5 paveiksle matyti, kad vien tik *L. monocytogenes* ląstelių inkubacijos su $7{,}5\cdot10^{-3}$ M ALA (tamsinis poveikis, šviesos dozė 0 J/cm^2) neužtenka tam, kad įvyktų ląstelių inaktyvacija. Taip pat vien tik šviesos poveikis neturi jokios įtakos *Listeria* ląstelių gyvybingumui (3.5b paveikslas). Tik ląstelių inkubacija su egzogeniniu ALA ir švitinimas (λ=405nm) ženkliai sumažina išgyvenusių ląstelių skaičių. Nors *Listeria* pasižymi mažesniu jautrumu ALA indukuotai fotosensibilizacijai negu kita mūsų tirta Gram-teigiama bakterija *Bacillus*, galima stebėti aiškią jos inaktyvacijos priklausomybę nuo inkubacijos laiko su ALA (arba pagamintų endogeninių porfirinų koncentracijos) (3.5a paveikslas) ir šviesos dozės (3.5b paveikslas). Žuvusių *L. monocytogenes* ląstelių skaičius siekė tik 4 log po 60

min inkubacijos su ALA ir 24 J/cm^2 šviesos dozės. Tačiau iš pateiktų grafikų galima padaryti prielaidą, kad taikant didesnę šviesos dozę ir modifikuojant inkubacijos laiką būtų galima pasiekti didesnį inaktyvacijos rezultatą.



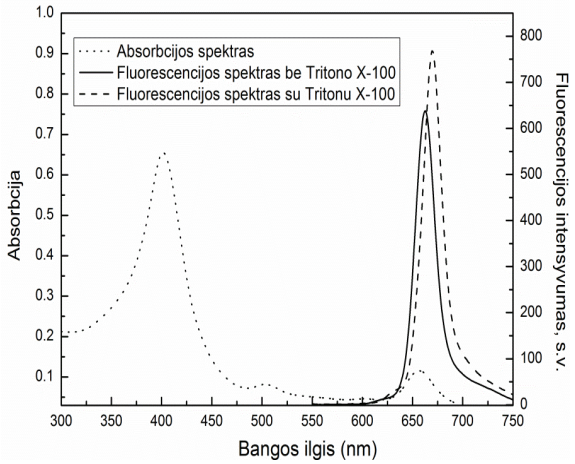
3.6 paveikslas 7,5·10^{-3} M ALA indukuotos fotosensibilizacijos poveikis *S. enterica* gyvybingumui esant skirtingoms šviesos dozėms. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

7,5·10^{-3} M ALA indukuotos fotosensibilizacijos poveikis *S. enterica* gyvybingumui buvo ištirtas taikant skirtingą inkubacijos laiką (0÷60 min) ir švitinimo dozę (0÷24 J/cm^2). Kaip matyti 3.6 paveiksle, *Salmonella* ląstelės išliko gyvybingos su 7,5·10^{-3} M ALA tamsoje ir apšvitinus kontrolines ląstelių suspensijas (ląstelės be ALA). Tik apšvitinus ląsteles po inkubacijos su ALA buvo gautas nuo šviesos dozės priklausomas ląstelių populiacijos sumažėjimas (3.6 paveikslas). Gauti rezultatai patvirtina, kad *Salmonella* inaktyvacija ALA-indukuota fotosensibilizacija priklauso nuo inkubacijos laiko su ALA ir ypač nuo taikomos švitinimo dozės. Panaudojus mažą (6 J/cm^2) švitinimo dozę, ląstelių gyvybingumas sumažėjo 0,5÷1,5 log, tuo tarpu padidinus švitinimo dozę iki 24 J/cm^2 ląstelių populiacija sumažėjo 4,5÷6 log.

Išanalizavus gautus rezultatus galima teigti, kad ALA indukuota fotosensibilizacija taikytomis eksperimentinėmis sąlygomis skystoje aplinkoje ženkliai inaktyvuoja Gram-neigiamą *S. enterica* (5,9 log), Gram-teigiamas *L. monocytogenes* (4 log) ir *B. cereus* (6,4 log). Tačiau reikia pažymėti, kad *Salmonella* ir *Bacillus* yra jautresnės ALA indukuotos fotosensibilizacijos poveikiui negu *Listeria*.

3.3. Maisto patogenų ir kenksmingų mikromicetų inaktyvacija po Chl indukuoto fotosensibilizacijos poveikio *in vitro*

Stipri tirtų bakterijų inaktyvacija po ALA indukuotos fotosensibilizacijos poveikio leido tikėtis tokio pat gero efekto eksperimentuose naudojant Chl.

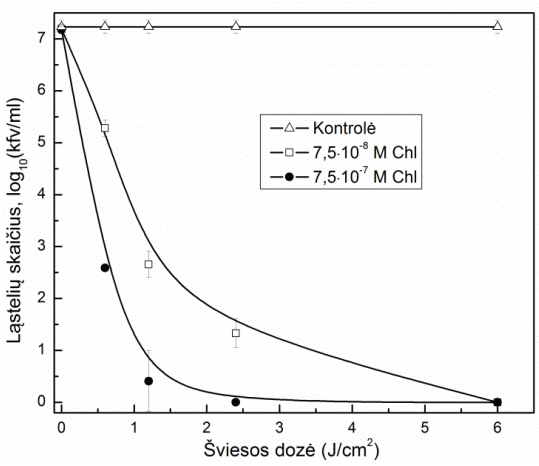


3.7 paveikslas $1{,}5\cdot10^{-5}$ M Chl (0,01 M PBS) sugerties ir fluorescencijos spektrai

Prieš atliekant inaktyvacijos tyrimus buvo išmatuoti $1{,}5\cdot10^{-5}$ M Chl sugerties ir fluorescencijos spektrai (0,01 M PBS), eksperimentų rezultatai pateikti 3.7 paveiksle. Išanalizavus absorbcijos spektrą matyti, kad Chl molekulė turi du absorbcijos maksimumus: vieną didesnį žadinant λ=405 nm bangos ilgio šviesa ir kitą, daug mažesnį, žadinant λ=655 nm bangos ilgio šviesa. Tolimesniems eksperimentams buvo pasirinktas λ=405 nm bangos ilgio šviesos šaltinis, optimaliai sužadinantis Chl. Pats Chl yra stipriai fluorescuojantis junginys. Chl esančio PBS buferyje fluorescencijos maksimumas yra apie 663 nm (3.7 paveikslas). Atlikta fluorescencijos spektrų analizė parodė, kad $1{,}5\cdot10^{-5}$ M Chl molekulės yra šiek tiek agreguotos, nes įmaišius į tirpalą 20 µl agregatų detergento Tritono-X100, fluorescencijos intensyvumo vertė padidėjo nuo 640 s.v iki 768 s.v.

Tyrimų pradžioje, atlikus literatūros analizę, nebuvo rasta duomenų apie Gram-teigiamų *B. cereus*, *L. monocytogenes* ir Gram-neigiamos *S. enterica* Chl indukuota fotoinaktyvaciją. Todėl eksperimentuose buvo išbandytos

įvairios Chl koncentracijos: nuo didžiausios $1.5 \cdot 10^{-5}$ M iki mažiausios $7.5 \cdot 10^{-8}$ M. (3.8–3.10 paveikslai). Pirmiausiai buvo įvertintas šviesa nepaveikto Chl toksiškumas ir atskirai galimas šviesos poveikis bakterijų ląstelėms. Eksperimentiniai duomenys parodė, kad vien tik šviesos įtaka bakterijų gyvybingumui yra labai nežymi. Iš pateiktų duomenų matyti, kad visų trijų bakterijų inkubacija tamsoje su skirtingomis Chl koncentracijomis nuo 2 min iki 120 min neturėjo jokios įtakos jų gyvybingumui. Tik po inkubacijos ir švitinimo žymiai sumažėjo bakterijų kiekis.

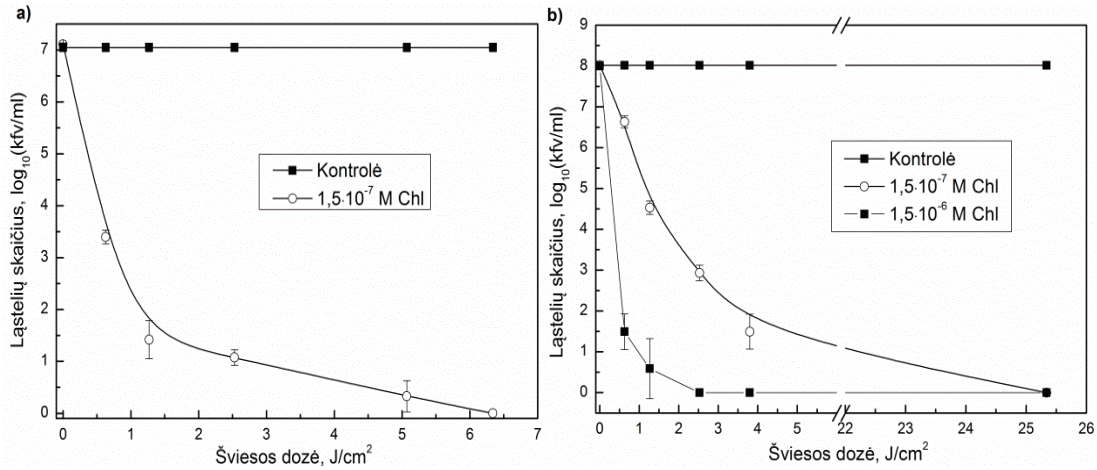


3.8 paveikslas *B. cereus* (~$1 \cdot 10^7$ kfv/ml) inaktyvacija $1{,}5 \cdot 10^{-8}$ M ir $1{,}5 \cdot 10^{-7}$ M Chl indukuota fotosensibilizacija esant skirtingoms šviesos dozėms. Inkubacijos laikas 2 min. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

B. cereus ląstelių inaktyvacijai buvo taikomas labai trumpas 2 min inkubacijos laikas. Kaip pavaizduota 3.8 paveiksle, po inkubacijos su $7{,}5 \cdot 10^{-8}$ M Chl ir švitinimo (šviesos dozė 6 J/cm^2) buvo gauta 7 log inaktyvacija. Toks pat geras inaktyvacijos efektas su didesne $7{,}5 \cdot 10^{-7}$ M Chl koncentracija buvo gautas panaudojus perpus mažesnę 2,4 J/cm^2 šviesos dozę. Išanalizavus gautus rezultatus, buvo nustatyta *Bacillus* ląstelių inaktyvacijos priklausomybė nuo Chl koncentracijos ir švitinimo dozės (3.8 paveikslas).

Remiantis gautais rezultatais su *Bacillus* ląstelėmis, Gram-teigiamo patogeno *Listeria* tyrimams buvo pasirinktas toks pats trumpas 2 min inkubacijos laikas. Po 0,5 min švitinimo (šviesos dozė 0,6 J/cm^2), išgyvenusių 10^8 tankio *Listeria* ląstelių skaičius sumažėjo 1,4 karto (3.9b paveikslas). Tuo tarpu esant tokiai pačiai švitinimo dozei, bet mažesniam 10^7 kfv/ml ląstelių

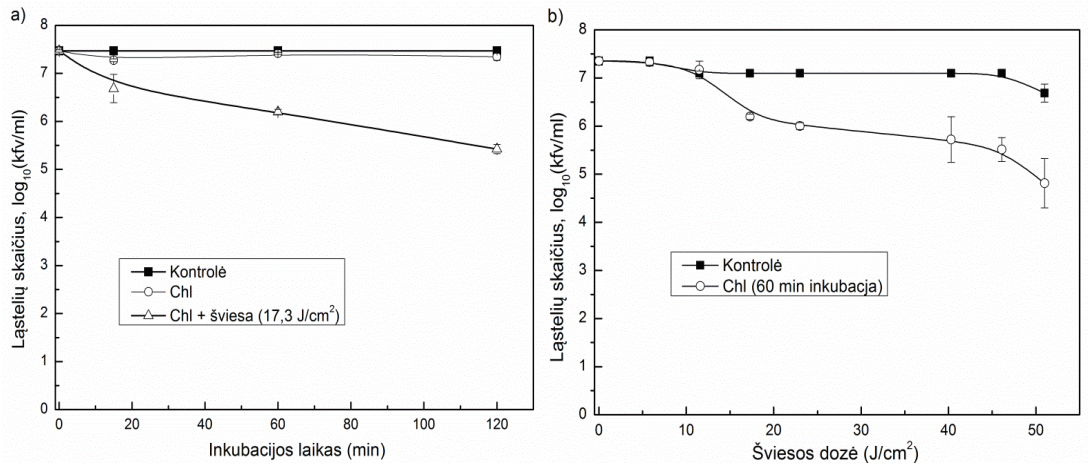
tankiui, poveikis išaugo iki 3,7 log (3.9b paveikslas). Po 1 min švitinimo (šviesos dozė 1,3 J/cm^2) 10^8 ir 10^7 tankio ląstelių skaičius atitinkamai sumažėjo 3,5 log ir 5,6 log. Iš 3.9a paveikslo matyti, kad 10^7 kfv/ml *L. monocytogenes* ląstelių inkubacija esant $1{,}5\cdot10^{-7}$ M Chl ir 5 min švitinimas (šviesos dozė 1,5 J/cm^2) lemia efektyvią bakterijų inaktyvaciją iki 7 log. 10^8 kfv/ml tankio *Listeria* ląstelės buvo visiškai (8 log) inaktyvuotos tik po inkubacijos esant $1{,}5\cdot10^{-7}$ M ir $1{,}5\cdot10^{-6}$ M Chl bei švitinimo (atitinkamai 25,3 J/cm^2 ir 2,5 J/cm^2 šviesos dozės).



3.9 paveikslas *L. monocytogenes* $1\cdot10^7$ kfv/ml (a) ir $1\cdot10^8$ kfv/ml (b) ląstelių inaktyvacija Chl indukuota fotosensibilizacija esant skirtingoms šviesos dozėms. Inkubacijos laikas 2 min (0,01 M PBS). Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

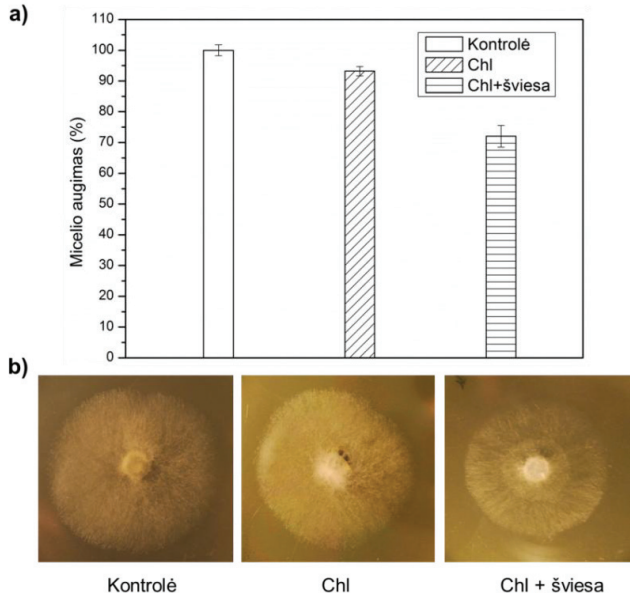
Yra žinoma, kad Gram-neigiamos bakterijos yra atsparesnės fotosensibilizacijos indukuotam poveikiui, o ypač dalyvaujant anijoniniams FS, tyrimų metu buvo panaudota didesnė neigiamai įkrauto Chl koncentracija. 3.10 paveiksle yra pateiktos *S. enterica* jautrumo priklausomybės nuo inkubacijos laiko ir šviesos dozės. Prieš pradedant eksperimentus buvo patikrintas atkirai šviesos (51 J/cm^2) ir $1{,}5\cdot10^{-5}$ M koncentracijos Chl galimas poveikis *Salmonella* ląstelių gyvybingumui. Po 120 min inkubacijos tamsoje ląstelių skaičius sumažėjo tik 0,12 log. Tik po ląstelių inkubacijos su Chl ir švitinimo (λ=405 nm, 0÷51 J/cm^2 šviesos dozė) pastebimai sumažėjo ląstelių skaičius. 15 min inkubacija 0,01 M PBS esant $1{,}5\cdot10^{-5}$ M Chl ir po jos atliktas švitinimas (šviesos dozė 17,3 J/cm^2) sumažino *S. enterica* gyvybingumą 0,79 log (3.10a paveikslas). Prailginus inkubacijos laiką nuo 15 min iki 120 min ir

naudojant tą pačią 17,3 J/cm^2 švitinimo dozę ląstelių inaktyvacija padidėja iki 2,05 log. Gauti rezultatai parodė, kad panaudojus 60 min inkubacijos laiką ir prailginus švitinimo laiką nuo 30 min (šviesos dozė 17,3 J/cm^2) iki 80 min (šviesos dozė 46,1 J/cm^2) buvo gautas nedidelis (0,7 log) inaktyvacijos augimas (3.10b paveikslas). Šviesos dozę padidinus iki 51 J/cm^2, išgyvenusių *Salmonella* ląstelių skaičius sumažėjo 2,5 log.



3.10 paveikslas *S. enterica* (~1·10^7 kfv/ml) inaktyvacija 1,5·10^{-5} M Chl indukuota fotosensibilizacija esant skirtingiems inkubacijos laikui (a) ir šviesos dozėms (b). Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Moksliniais tyrimais yra nustatyta, kad mikromicetiniai grybai yra daug atsparesni fotosensibilizacijos indukuotam poveikiui negu bakterijos (12,13). Todėl inaktyvuojant pelėsinį grybą *Botrytis* buvo pasirinktas ilgesnis inkubacijos laikas ir didesnė Chl koncentracija. Duomenys, pateikti 3.11 paveiksle, rodo *B. cinerea* micelio augimo slopinimą po poveikio 1,5·10^{-4} M Chl. Kontrolinio *B. cinerea* varianto monokolonijos diametras buvo 100%. Po 2 dienų augimo *Botrytis* kontrolinių monokolonijų diametras siekė 4,33 cm. Kai grybas buvo auginamas ant terpės su 1,5·10^{-4} M Chl po apšvitinimo (šviesos dozė 35 J/cm^2), jo monokolonijos diametras siekė 3,35 cm (28% slopinimas) (3.11b paveikslas). Šviesa neaktyvuoto Chl poveikis grybo augimui buvo labai nežymus.

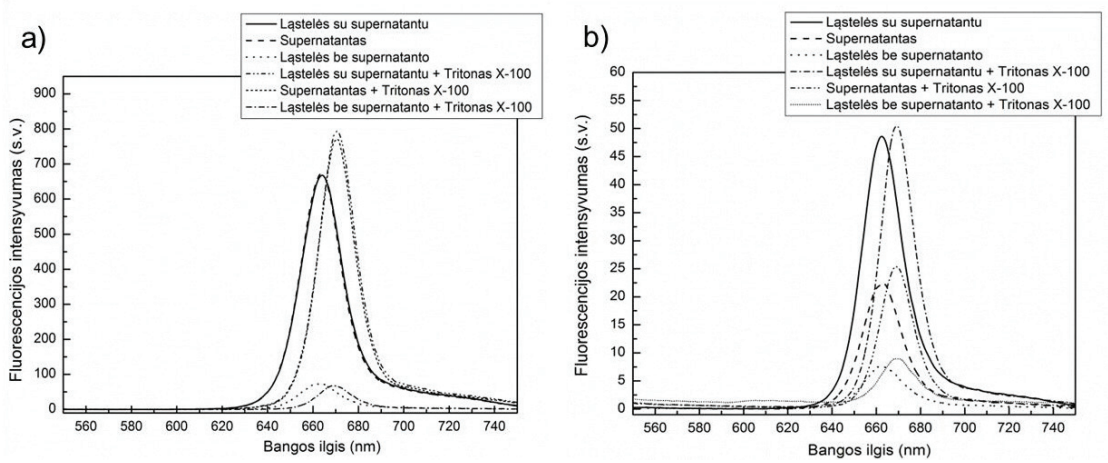


3.11 paveikslas *B. cinerea* 2-jų dienų kontrolinių ir paveiktų ($1,5 \cdot 10^{-4}$ M Chl, šviesos dozė 35 J/cm^2) monokolonijų augimo skirtumai: įvertintas slopinamo radialinio augimo sumžėjimas procentais (a) ir pavyzdinės monokolonijų nuotraukos esant vienodam masteliui (b)

3.4. Bakterijų ląstelių membranos pralaidumo ir struktūrinių pažeidimų įvertinimas po Chl indukuotos fotosensibilizacijos poveikio

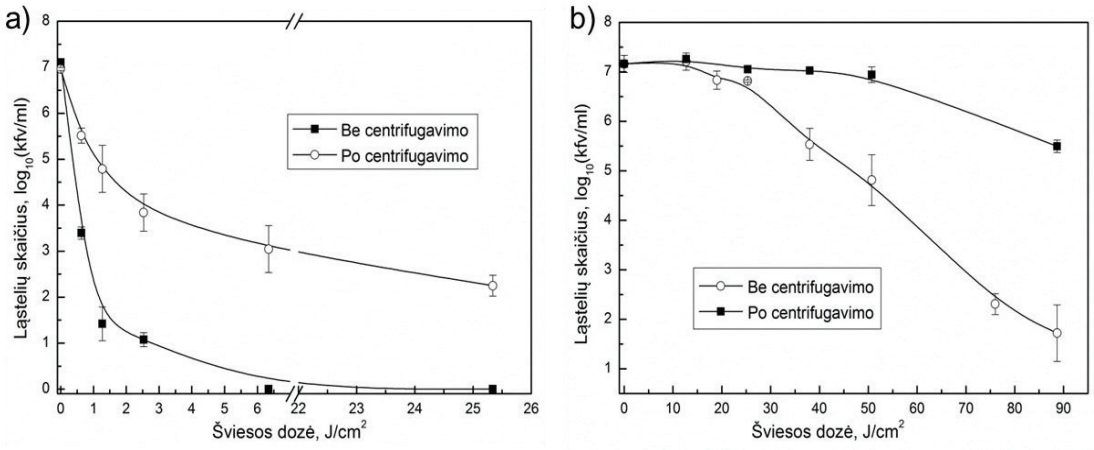
Siekiant nustatyti, kaip tvirtai Chl molekulės susiriša su ląstele taikiniu, buvo išmatuoti Chl fluorescencijos spektrai po inkubacijos su *S. enterica* (60 min) ir *L. monocytogenes* (2 min) ląstelėmis. Buvo lyginamas Chl fluorescencijos spektras supernatante ir vienų ląstelių suspensijoje (be supernatanto) (3.12 paveikslas). Iš duomenų pateiktų 3.12a paveiksle matyti, kad po centrifugavimo didesnė Chl dalis pasiliko supernatante ir tik nedidelis kiekis prisijungė prie Gram-neigiamos *Salmonella.* Atsižvelgiant į šiuos rezultatus, galima daryti prielaidą, kad būtent ši nedidelė *Salmonella* ląstelių dalis yra pažeidžiama po fotosensibilizacijos poveikio. 3.12b paveiksle pateikti Gram-teigiamos *Listeria* fluorescencijos grafikai rodo, kad ~50% Chl po centrifugavimo lieka supernatante ir ~20% prisijungia prie ląstelių. Tyrimų metu taip pat buvo atlikta fluorescencijos spektrų analizė, įmaišius į supernatantą ir į ląstelių suspensijas be supernatanto 20 µl detergento Tritono-X100. 3.12 paveiksle matyti, kad $1,5 \cdot 10^{-7}$ ir $1,5 \cdot 10^{-5}$ M Chl molekulės yra šiek

tiek agreguotos. Fluorescencijos intensyvumas įmaišius detergentą padidėjo nežymiai.



3.12 paveikslas Chl fluorescenciniai spektrai po inkubacijos 0,01 M PBS buferyje (20 ± 2 ºC) su *S. enterica* ($1{,}5\cdot10^{-5}$ M Chl, 60 min inkubacija) a) ir *L. monocytogenes* ($1{,}5\cdot10^{-7}$ M Chl, 2 min inkubacija) b) ląstelėmis.

Siekiant patikrinti, kiek inaktyvacijos efektyvumą įtakoja Chl susirišimas su *Salmonella* ir *Listeria* ląstelėmis, buvo atlikti inaktyvacijos tyrimai neatskirus ir atskirus supernatantinį Chl, taip pat po atskirimo įterpiant arba neįterpiant NaN_3. Neprisijungusio prie ląstelių Chl atskirimas buvo atliekamas naudojant centrifugą. Pastebėta, kad eksperimentuose po Chl pašalinimo iš ląstelių suspensijos įvyksta fotoinaktyvacijos poveikio sumažėjimas (3.14 paveikslas). Iš pateiktų grafikų matyti, kad ši tendencija yra būdinga abiem bakterijų kamienams: Gram-teigiamam *Listeria* ir Gram-neigiamam *Salmonella. L. monocytogenes* inaktyvacija Chl indukuota fotosensibilizacija ($1{,}5\cdot10^{-7}$ M Chl, šviesos dozė 0÷25,3 J/cm^2) po Chl atskirimo yra pateikta 3.14a paveiksle. Ląstelių inaktyvacija 7 log buvo gauta tik tuo atveju, kai iš suspensijos prieš švitinimą (šviesos dozė 6,3 J/cm^2) nebuvo pašalintas supernatantinis Chl. Atlikus prieš švitinimą (šviesos dozė 1.3 J/cm^2) supernatantinio Chl pašalinimą iš suspensijos, išgyvenusių bakterijų skaičius sumažėjo tik 3,9 log. Net padidinus šviesos dozę iki 25,3 J/cm^2, nebuvo gauta visiška *Listeria* inaktyvacija (3.14a paveikslas).

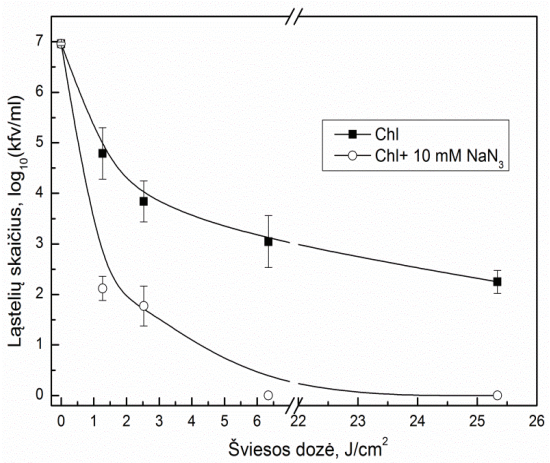


3.14 paveikslas Bakterijų inaktyvacija Chl indukuota fotosensibilizacija atskyrus ir neatskyrus supernatantinį Chl esant skirtingoms šviesos dozėms: *L. monocytogenes* - $1{,}5\cdot10^{-7}$ M Chl, inkubacijos laikas 2 min a) ir *S. enterica* - $1{,}5\cdot10^{-5}$ M Chl, inkubacijos laikas 60 min b). Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Identiški eksperimetai buvo atlikti ir su Gram-neigiama *S. enterica* ($1{,}5\cdot10^{-5}$ M Chl, šviesos dozė 0÷88,7 J/cm^2). Gauti rezultatai parodė, kad inaktyvacija buvo efektyvesnė prieš švitinimą nepašalinus supernatantinį Chl iš ląstelių suspensijos (3.14b paveikslas). 3.14b paveiksle matyti, kad išgyvenusiųjų *Salmonella* ląstelių skaičius po Chl atskirimo ir sekančio 70 min švitinimo (šviesos dozė 88,7 J/cm^2) sumažėjo tik 1,7 log. Tuo tarpu nepašalinant iš suspensijos supernatantinį Chl, inaktyvacija išaugo iki 5,4 log (3.14b paveikslas).

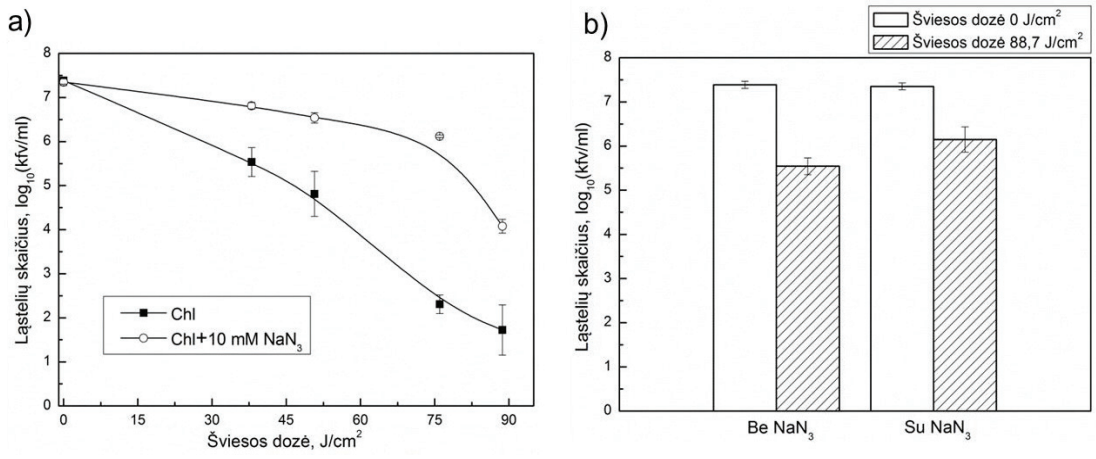
Siekiant nustatyti, ar singuletinis deguonis ($^1O_2$) dalyvauja tirtų bakterijų ląstelių Chl indukuotos fotosensibilizacijos sukeltuose pažeidimuose ir įtakoja šių ląstelių gyvybingumą, į bakterijų suspensijas (~$1\cdot10^8$ kfv/ml *Listeria* ir ~$1\cdot10^7$ kfv/ml *Salmonella*) buvo įmaišomas NaN_3 fizikinis $^1O_2$ gesiklis) (38,39). Tyrimų pradžioje buvo įsitikinta, kad darbe naudotos NaN_3 koncentracijos pačios neturi jokio neigiamo poveikio bakterijų ląstelių gyvybingumui. Tolimesniems tyrimams buvo pasirinkta kitų mokslininkų dažnai naudojama 10 mM NaN_3 koncentracija. NaN_3 įmaišius į *L. monocytogenes* ir $1{,}5\cdot10^{-7}$ M Chl suspensiją be Chl atskirimo, po švitinimo ląstelių gyvybingumas neatsistatė. Visai priešingi rezultatai buvo gauti įmaišius 10 mM NaN_3 į *Listeria* ir Chl suspensiją, kurioje pašalintas supernatantinis Chl. Ląstelių inaktyvacija, kuri dėl supernatantinio Chl

atskirimo buvo sumažėjusi, šiuo atveju žymiai padidėjo. Kaip rodo 3.16 paveiksle pateikti duomenys, visiška ląstelių inaktyvacija (7 log) buvo gauta apšvitinus ląsteles (šviesos dozė 6.3 J/cm^2) po supernatantinio Chl pašalinimo ir NaN_3 įnešimo. Panašius duomenis, kai NaN_3 skatina bakterijų inaktyvaciją gauna ir kiti mokslininkai (50). Yra manoma, kad inaktyvaciją skatina susiformavę NaN_3 radikalai.



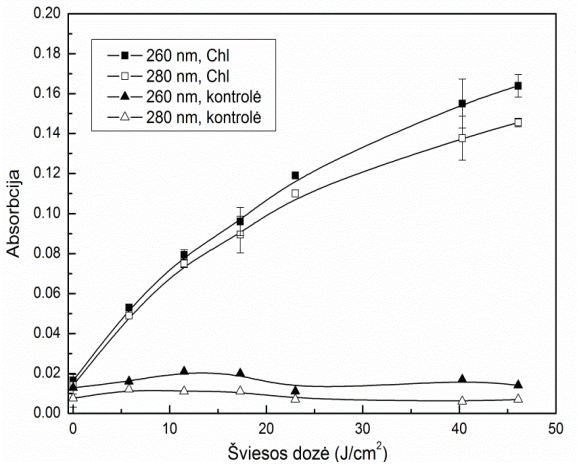
3.16 paveikslas NaN_3 įtaka *L. monocytogenes* 1,5·10^{-7} M Chl indukuotos fotosensibilizacijos efektyvumui atskyrus supernatantinį Chl esant skirtingoms šviesos dozėms. Inkubacijos laikas 2 min. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Statistiškai reikšmingas ($p < 0,05$) Chl indukuotos fotoinaktyvacijos efektyvumo sumažėjimas įmaišant į *Salmonella* ląstelių suspensiją 10mM NaN_3 buvo gautas neatskirus supernatantinį 1,5·10^{-5} M Chl (3.17a paveikslas). Neatliekant po inkubacijos švitinimo, Chl ir NaN_3 nepasižymėjo žudančiu poveikiu. Tačiau, 3.17a paveiksle pateikti duomenys rodo, kad pašalinus supernatantinį Chl ir įterpus NaN_3, bei apšvitinus 38 J/cm^2 ir 88,7 J/cm^2 šviesos dozėmis, *S. enterica* fotoinaktyvacija sumažėjo atitinkamai 1,3 log ir 2,4 log. 10 mM NaN_3 įtaka *Salmonella* ląstelių gyvybingumui atskirus supernatantinį Chl (1,5·10^{-5} M) buvo labai nedidelė (3.17b paveikslas). Pritaikius 60 min inkubaciją su Chl ir 88,7 J/cm^2 šviesos dozę, buvo gauta 1,84 log inaktyvacija. Kaip rodo 3.17 paveiksle pateikti duomenys, skirtumas tarp ląstelių gyvybingumų po šviesos poveikio su NaN_3 ir be NaN_3 buvo daug mažesnis (0,6 log) negu tuo atveju, kai supernatantinio Chl pašalinimas nebuvo atliekamas (2,4 log).



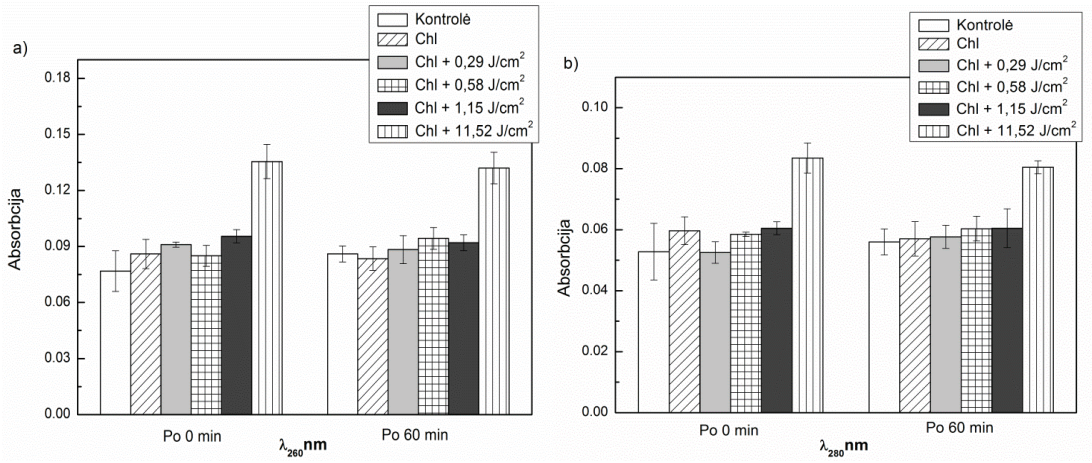
3.17 paveikslas NaN_3 įtaka *S. enterica* $1{,}5 \cdot 10^{-5}$ M Chl indukuotos fotosensibilizacijos efektyvumui neatskyrus (a) ir atskyrus (b) supernatantinį Chl esant skirtingoms šviesos dozėms. Inkubacijos laikas 60 min. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Siekiant padidinti Chl indukuotos fotosensibilizacijos efektyvumą prieš Gram-neigiamas bakterijas, buvo planuojama kombinuoti šį fotosensibilizacijos indukuotą poveikį su kita antimikrobine technologija. Būtent todėl buvo svarbu žinoti, kokias ląstelės pažaidas sukelia Chl indukuota fotosensibilizacija. Šiam tikslui, darbe buvo atlikti *L. monocytogenes* ir *S. enterica* ląstelių membranos integralumo tyrimai kontrolinėse ir fotoaktyvuotose mėginiuose. Ląstelių membranos integralumas buvo įvertintas spektrofotometriniu metodu, matuojant bakterijų ląstelių filtrato sugertį ties $\lambda = 260$ nm (DNR absorbcijos pikas) ir $\lambda = 280$ nm (baltymų absorbcijos pikas). Kontrolinėse *Salmonella* mėginiuose nebuvo aptiktas viduląstelinių komponentų išlaisvinimas (3.19 paveikslas). Kontrolinių mėginių filtratų absorbcija ties $\lambda = 260$ nm ir $\lambda = 280$ nm buvo labai nežymi. Ir priešingai, viduląstelinių komponentų išsiskirimas iš fotoaktyvuotų (60 min inkubacija su $1{,}5 \cdot 10^{-5}$ M Chl, šviesos dozė 0÷46,8 J/cm^2) bakterijų į aplinkinę terpę padidino absorbciją. Absorbcijos padidėjimas priklausė nuo šviesos dozės ir išaugo nuo 0,01 iki 0,146 ($\lambda = 280$ nm) ir nuo 0,01 iki 0,164 ($\lambda = 260$ nm). Šie rezultatai parodo, kad *Salmonella* ląstelėse po Chl indukuotos fotosensibilizacijos įvyksta tam tikros membraninės pažaidos.



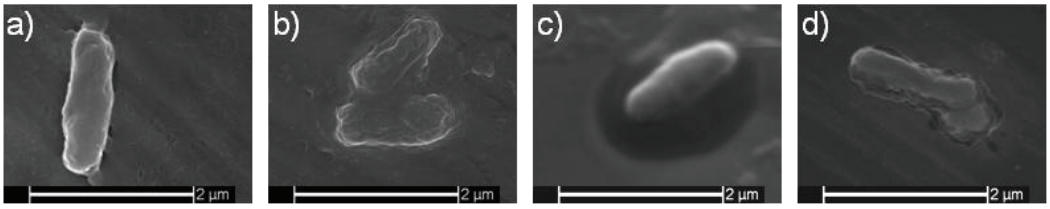
3.19 paveikslas $1{,}5\cdot10^{-5}$ M Chl indukuotos fotosensibilizacijos įtaka *S. enterica* ląstelių membranos integralumui: viduląstelinių komponentų absorbcijos ties λ=260 nm ir λ=280 nm priklausomybė nuo šviesos dozės. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

3.20 paveiksle yra pavaizduoti *L. monocytogenes* ląstelių membranų integralumo tyrimai. Buvo pamatuoti ląstelių filtratų absorbcijos signalai ties λ=260 nm ir λ=260 nm iš karto (0 min) ir 60 min po Chl indukuotos fotosensibilizacijos poveikio (2 min inkubacija su $1{,}5\cdot10^{-7}$ M Chl, šviesos dozė 0÷11,52 J/cm^2). Kontrolinių filtratų absorbcijos signalas buvo nežymus: ties λ=280 nm – 0,053 po 0 min ir 0,056 po 60 min; ties λ=260 nm – 0,077 po 0 min ir 0,086 po 60 min.



3.20 paveikslas $1{,}5\cdot10^{-6}$ M Chl indukuotos fotosensibilizacijos įtaka *L. monocytogenes* ląstelių membranos integralumui: viduląstelinių komponentų absorbcijos priklausomybė nuo šviesos dozės ties λ = 260 nm a) ir λ = 280 nm b). Matavimai atlikti 0 ir 60 min po poveikio. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Viduląstelinių komponentų išlaisvinimas po Chl indukuotos fotosensibilizacijos (2 min inkubacija su $1{,}5\cdot10^{-6}$ M Chl, šviesos dozė 0÷11,52 J/cm^2) poveikio suaktyvėjo žymiai padidinus šviesos dozę. 3.20 paveiksle matyti, kad iš karto po poveikio absorbcija ties λ=260 nm išaugo nuo 0,086 (šviesos dozė 0 J/cm^2) iki 0,136 (šviesos dozė 11,52 J/cm^2), o ties λ=280 nm – nuo 0,06 iki 0,084. Svarbu pažymėti, kad 60 min po poveikio jokio žymaus skirtumo nebuvo pastebėta.



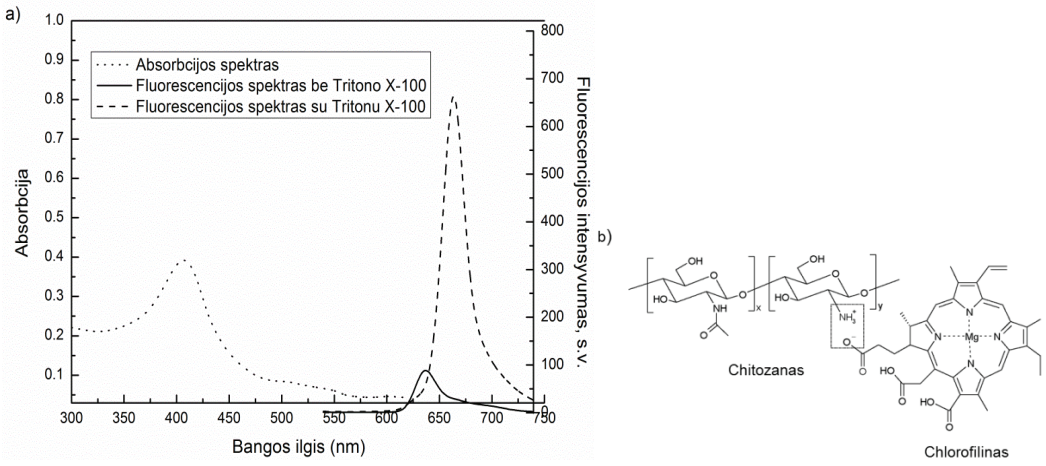
3.21 paveikslas *S. enterica* ir *L. monocytogenes* ląstelių SEM nuotraukos po Chl indukuotos fotosensibilizacijos poveikio: a) ir c) kontrolinės *Salmonella* ir *Listeria* ląstelės; b) *Salmonella* ląstelės po $1{,}5\cdot10^{-5}$ M Chl indukuotos fotosensibilizacijos poveikio (inkubacijos laikas 60 min; šviesos dozė 23 J/cm^2); d) *Listeria* ląstelės po $1{,}5\cdot10^{-7}$ M Chl indukuotos fotosensibilizacijos poveikio (inkubacijos laikas 2 min; šviesos dozė 1,3 J/cm^2).

Chl indukuotos fotosensibilizacijos poveikis Gram-neigiamos *Salmonella* ir Gram-teigiamos *Listeria* ląstelių morfologijai buvo ištirtas iš karto po poveikio naudojant SEM (3.21 paveikslas). SEM nuotraukose matyti, kad po Chl indukuotos fotosensibilizacijos poveikio įvyksta ląstelių susitraukimas ir viduląstelinių medžiagų išėjimas. Šie ląstelių morfologiniai pokyčiai, patvirtina mūsų prieš tai darytų membranų integralumo tyrimų metu gautus rezultatus.

3.5. Fotoaktyvuoto Chl–CHS konjugato poveikis patogenams ir mikromicetams *in vitro*

Pagrindinis Chl trūkumas – nedidelis efektyvumas prieš Gram-neigiamas bakterijas ir mikromicetus, būtent todėl mikroorganizmų fotoinaktyvacijai buvo pradėtas taikyti Chl–CHS konjugatas. Sekančiame tyrimų etape buvo atliekami eksperimentai su fotoaktyvuotu Chl–CHS konjugatu. Nagrinėjant Chl–CHS konjugato struktūrą, buvo išanalizuoti jo

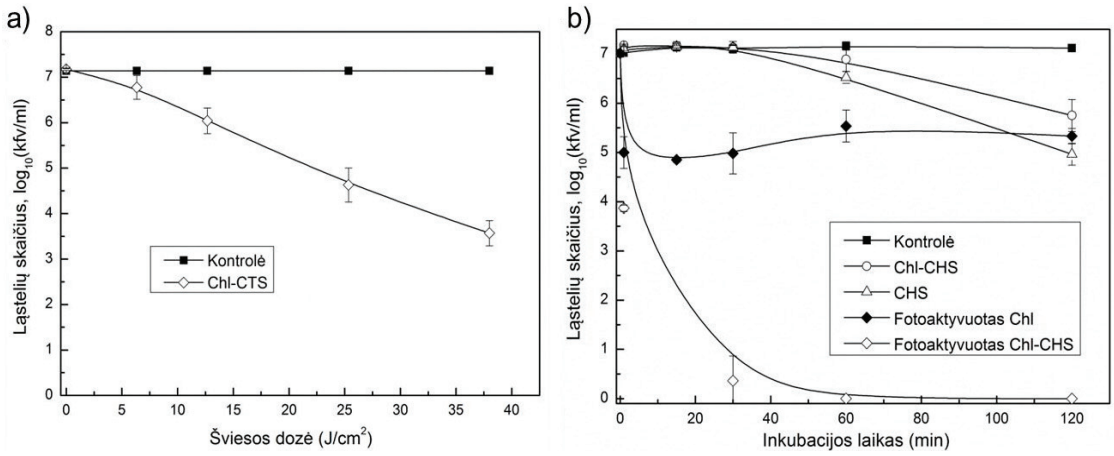
absorbcijos ir fluorescencijos spektrai. 3.22a paveiksle matyti, kad 0,9% NaCl tirlpale esančio Chl–CHS konjugato absorbcijos spektras turi 2 būdingus pikus ties λ=405 nm ir λ=652 nm. Konjugato fluorescencijos spektras, pateiktas tame pačiame paveiksle, leidžia manyti, kad 0,9% NaCl tirpale jis yra stipriai agreguotas. Chl–CHS konjugato fluorescencijos piko signalo vertė ties λ=647 nm buvo 88 s.v.. Tik detergento Tritono įterpimas išskaidė susidariusius agregatus: signalo vertė padidėjo iki 663 s.v., o fluorescencijos pikas pasislinko į λ=674 nm (3.22a paveikslas). 3.22 paveiksle yra parodyta teorinė Chl–CHS konjugato formulė, kuri buvo sukurta remiantis abiejų junginių struktūra. Labiausiai tikėtina, kad ryšys susidarė tarp teigiamai įkrauto chitozano NH_3^+ grupės ir neigiamai įkrauto chlorofilino COO^- grupės.



3.22 paveikslas 0,001 Chl–0,1% CHS konjugato esančio 0,9% NaCl tirpale (at 20 ± 2 ºC) absorbcijos ir fluorescencijos spektrai a) bei cheminė formulė b).

Antimikrobinis Chl–CHS konjugato aktyvumas buvo patikrintas prieš du patogenus: Gram-neigiamą *S. enterica* ir Gram-teigiamą *L. monocytogenes*. Kadangi prieš tai atlikti fotoinaktyvacijos tyrimai su Chl parodė didesni *S. enterica* ir *B. cinerea* atsparumą šiam poveikiui negu *L. monocytogenes* ir *B. cereus*, tolimesniuose eksperimentuose prieš Gram-neigiamą bakteriją *S. enterica* ir grybą *B. cinerea* buvo naudojama didesnė Chl–CHS konjugato koncentracija negu prieš Gram-teigiamą *L. monocytogenes*. 0,001–0,1% konjugato koncentracija, atitinkanti prieš tai tyrimuose naudotą $1{,}5 \cdot 10^{-5}$ M Chl koncentraciją, buvo pritaikyta prieš bakteriją *S. enterica* ir grybą *B. cinerea*, o 0,00001–0,001% koncentracija, atitinkanti $1{,}5 \cdot 10^{-7}$ M Chl koncentraciją, prieš

Gram-teigiamą *L. monocytogenes*. *S. enterica* jautrumas fotoaktyvuoto Chl–CHS konjugato poveikiui yra pavaizduotas 3.23 paveiksle. Eksperimentų metu buvo nustatyta, kad vien tik šviesa (šviesos dozė 0÷38 J/cm^2) ar vien tik Chl–CHS (šviesos dozė 0 J/cm^2) neturėjo žymaus efekto ląstelių gyvybingumui (Paveikslas 3.23a). Tik pritaikius po inkubacijos su Chl–CHS atitinkamą švitinimą, pavyko ženkliai sumažinti *Salmonella* populiaciją. Kaip matyti 3.23a paveiksle, panaudojus 1 min inkubaciją ir 38 J/cm^2 švitinimo dozę, buvo gauta 3,6 log inaktyvacija.

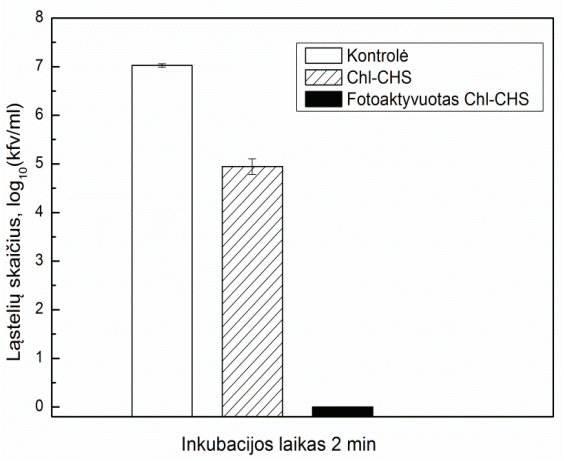


3.23 paveikslas *S. enterica* inaktyvacijos fotoaktyvuotu 0,001% Chl–0,1% CHS konjugatu (inkubacijos laikas–1 min) priklausomybė nuo šviesos dozės a) ir *S. enterica* inaktyvacijos priklausomybė nuo inkubacijos laiko naudojant 0,1% CHS (pH 3,46), fotoaktyvuotus 0,001% Chl (pH 6,96) ir 0,001% Chl–0,1 % CHS konjugatą (pH 3,95) (šviesos dozė–38 J/cm^2) b). Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

3.23b paveiksle yra pavaizduota CHS, fotoaktyvuoto Chl–CHS konjugato ir Chl poveikių priklausomybė nuo inkubacijos laiko. Visiška ląstelių inaktyvacija buvo gauta tik po fotoaktyvuoto 0,001% Chl–0,1% CHS konjugato poveikio, panaudojus 60 min inkubaciją ir 38 J/cm^2 šviesos dozę. Visais kitais atvejais (fotoaktyvuoto 0,001% Chl, 0,1% CHS, 0,001% Chl–0,1% CHS šviesa neaktyvuotas konjugatas) nepavyko gauti tokio žymaus fotoinaktyvacijos efekto net padidinus inkubacijos laiką iki 120 min.

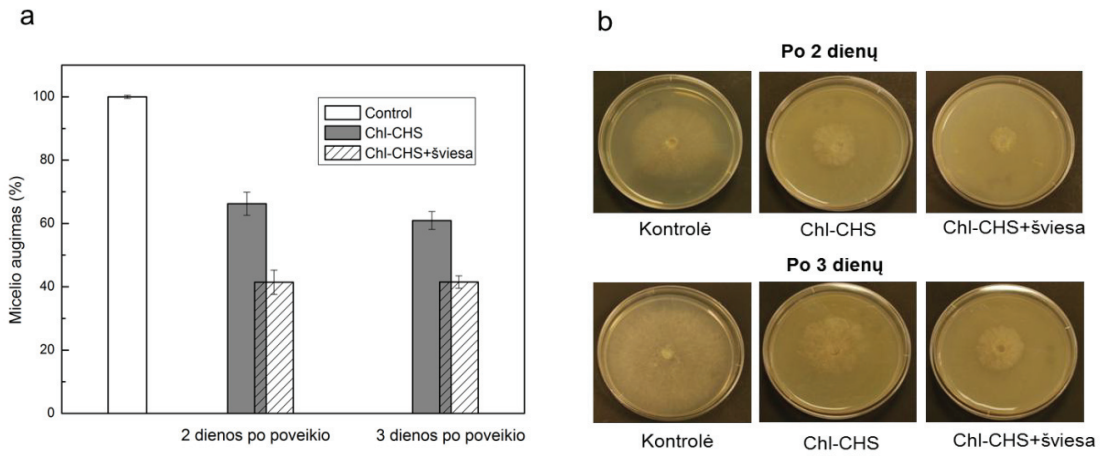
Kadangi skirtingos eksperimentinės sąlygos gali pakeisti bakterijų suspensijos pH, tyrimų metu buvo pamatuotas visų tirpalų pH. Buvo nustatyta, kad bakterijų suspensijų pH vertė 0,01 M PBS buferyje pasikeitė nuo 7,4 iki 6,8, kai į tirpalą buvo įmaišomas Chl ar Chl–CHS konjugatas. Tačiau, kai

vietoj PBS buferio buvo naudojamas 0,9% NaCl tirpalas, pH vertė sumaišius bakterijų suspensiją su Chl–CHS konjugatu, nukrisdavo iki 3,95.



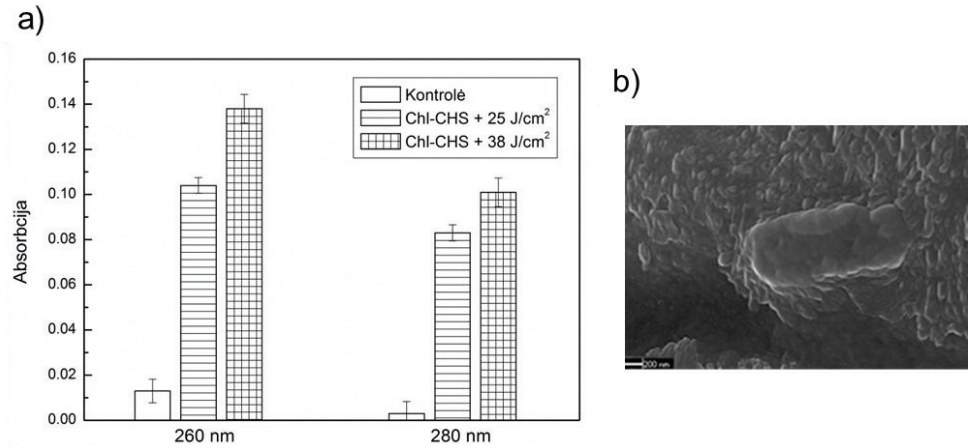
3.24 paveikslas *L. monocytogenes* inaktyvacija fotoaktyvuotu 0,00001% Chl – 0,001% CHS konjugatu, inkubacijos laikas – 2 min, šviesos dozė 2,9 J/cm^2. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Kaip parodyta 3.24 paveiksle, Chl–CHS konjugatas be šviesos poveikio pasižymėjo pastebimu antimikrobiniu efektu prieš Gram-teigiamą *Listeria.* Jau po 2 min inkubacijos buvo gauta 2,1 log inaktyvacija. Fotoaktyvuotas Chl–CHS konjugatas sumažino *Listeria* ląstelių gyvybingumą 7 log po 2 min inkubacijos ir 2,9 J/cm^2 šviesos dozės (3.24 paveikslas). Gauti rezultatai parodė, kad Gram-teigiamas maisto patogenas *L. monocytogenes* yra daug jautresnis fotoaktyvuoto ir nefotoaktyvuoto Chl–CHS konjugato poveikiui negu Gram-neigiama *S. enterica*.



3.25 paveikslas *B. cinerea* monokolonijų augimo slopinimas 2÷3 dienos po fotoaktyvuoto 0,001% Chl–0,1% CHS konjugato poveikio (šviesos dozė 76 J/cm^2) a); slopinimo įvertinimui naudotos monokolonijų nuotraukos b).

3.25 paveiksle pateikti duomenys rodo pelėsinio grybo *B. cinerea* micelio augimo slopinimą po poveikio 0,001% Chl–0,1% CHS konjugatu. Buvo gautas nedidelis Chl–CHS konjugato efektas nepašvitintuose pavyzdžiuose. *B. cinerea* kontrolinio varianto monokolonijos diametras buvo vertinamas kaip 100%. Po 2 dienų augimo ant terpės su Chl–CHS (nešvitintas) buvo gauta 39% slopinimas. Fotoaktyvuotas Chl–CHS konjugatas, pritaikius 76 J/cm^2 šviesos dozę, buvo dar efektyvesnis. 2 dienos po jo poveikio buvo stebima 59% monokolonijos augimo slopinimas, 3 dienos–60% (3.25 paveikslas). Taigi, remiantis gautais rezultatais galima teigti, kad pelėsinio grybo *Botrytis* augimą galima efektyviai slopinti fotoaktyvuoto Chl–CHS konjugato pagalba.



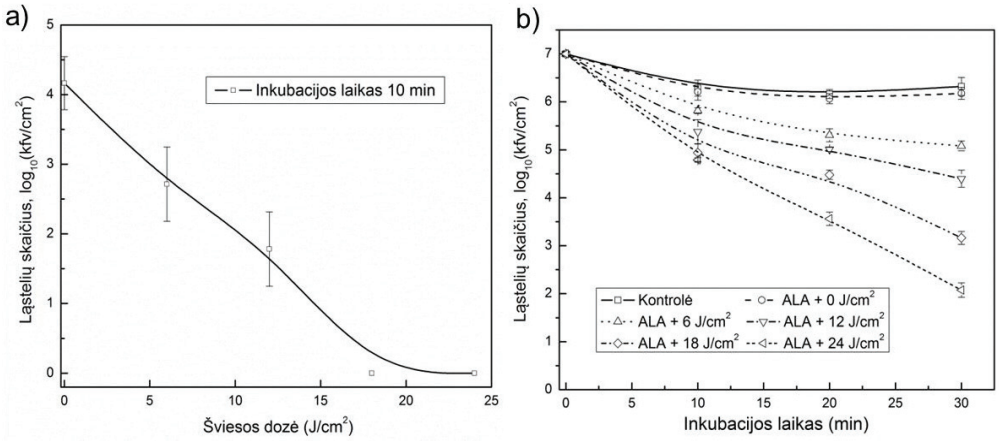
3.26 paveikslas Fotoaktyvuoto 0,001% Chl–0,1% CHS konjugato poveikio įtaka *S. enterica* ląstelių membranos integralumui: išlaisvintų viduląstelinių komponentų absorbcijos ties λ=260 nm ir λ=280 nm (inkubacijos laikas 60 min) esant skirtingai šviesos dozei a) ir paveiktos *S. enterica* SEM nuotrauka (inkubacijos laikas 30 min; švitinimo dozė 12,7 J/cm^2) b).

3.26a paveiksle pateikti rezultatai rodo, kad kontrolinėse *S. enterica* ląstelių mėginiuose nebuvo aptikta viduląstelinių komponentų išlaisvinimo. Kontrolinių mėginių filtratų absorbcija ties λ=260 nm ir λ=280 nm buvo labai nežymi. Priešingai, viduląstelinių komponentų išsiskyrimas iš fotoaktyvuotų (60 min inkubacija su 0,001% Chl–0,1% CHS, šviesos dozės 25 J/cm^2 ir 38 J/cm^2) bakterijų į aplinkinę terpę absorbciją padidino. Absorbcijos padidėjimas priklausė nuo šviesos dozės ir išaugo nuo 0,01 iki 0,138 (λ=280 nm) ir 0,01 iki 0,101 (λ=260 nm) šviesos dozę padidinus nuo 25 J/cm^2 iki 38 J/cm^2. Šie

rezultatai rodo, kad *Salmonella* ląstelėse po fotoaktyvuoto Chl–CHS konjugato poveikio įvyksta tam tikros membraninės pažaidos (3.26a paveikslas). Tokių pažeidimų atsiradimą patvirtina ir SEM nuotraukos. 3.26b paveiksle yra pateiktos fotoaktyvuoto Chl–CHS konjugato poveiktų Gram-neigiamos *S. enterica* ląstelių SEM nuotraukos iš karto po poveikio. Galima matyti, kad Chl–CHS konjugatas padengia visą bakterijos paviršių ir po sąveikos su ląstele bei fotoaktyvacijos (inkubacijos laikas 30 min; šviesos dozė 12,7 J/cm^2) įvyksta ląstelės susitraukimas ir viduląstelinių medžiagų išėjimas.

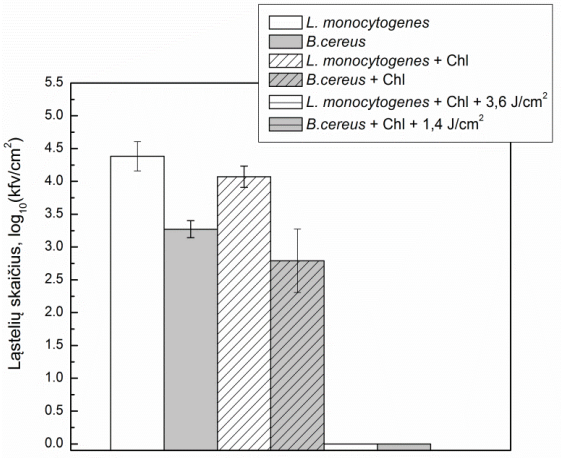
3.6. Su maistu susijusių pakuočių nukenksminimas nuo prisitvirtinusių patogenų ALA ir Chl indukuotos fotosensibilizacijos pagalba

Siekiant įvertinti ALA ir Chl indukuotos fotosensibilizacijos antibakterinį efektyvumą nukenksminant paviršius, *B. cereus, L. monocytogenes* ir *S. enterica* ląstelės buvo paskleistos ant maistui laikyti naudojamų pakuočių paviršiaus. 3.29a paveiksle pavaizduoti duomenys parodo Gram-teigiamos bakterijos *B. cereus* ALA indukuotos fotoinaktyvacijos priklausomybę nuo šviesos dozės. Akivaizdu, jog 18 J/cm^2 šviesos dozės pakanka tam, kad po 10 min inkubacijos ALA tirpale būtų pasiektas visiškas *Bacillus* ląstelių nukenksminimas ant pakuočių paviršiaus (4 log inaktyvacija).



3.29 paveikslas *B. cereus* inaktyvacija ant maistui laikyti naudojamų pakuočių paviršiaus $3\cdot10^{-3}$ M ALA indukuota fotosensibilizacija esant skirtingoms šviesos dozems a) ir *S. enterica* inaktyvacija $7{,}5\cdot10^{-3}$ M ALA indukuota fotosensibilizacija esant skirtingam inkubacijos laikui b). Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Gram-neigiamos bakterijos *S. enterica* ląstelės daug geriau prisitvirtino prie pakuočių paviršiaus ir sunkiau pasidavė ALA indukuotos fotosensibilizacijos poveikiui nei Gram-teigiamos bakterijos *B. cereus*. Pradinis *Salmonella* ląstelių skaičius ant pakuočių siekė 6,3 log. Gauti rezultatai rodo, kad šio patogeno ALA-indukuota fotoinaktyvacija ant pakuočių paviršiaus labai priklauso nuo inkubacijos laiko ir švitinimo dozės. Panaudojus 10 min inkubaciją ir 24 J/cm^2 švitinimo dozę, buvo gauta tik 1,5 log inaktyvacija (3.29b paveikslas). Padidinus inkubacijos laiką iki 30 min, išgyvenusių ląstelių skaičius sumažėjo 4,2 log, bet nepasiekė visiškos inaktyvacijos. Taigi, 3.29 paveiksle pateikti duomenys rodo, kad Gram-neigiama *S. enterica* prisitvirtinusi prie pakuočių paviršiaus yra atsparesnė ALA indukuotai fotoinaktyvacijai nei Gram-teigiama *B. cereus*.



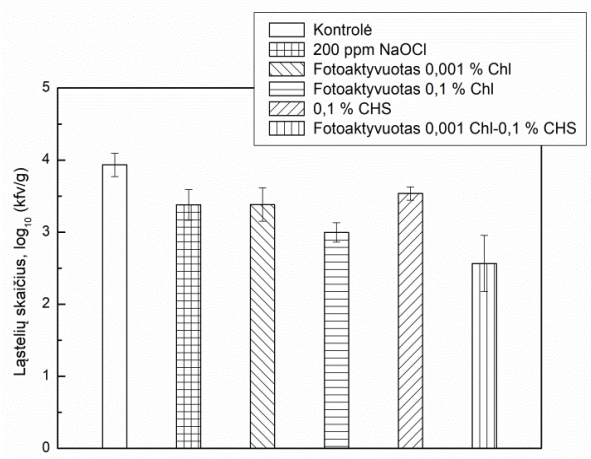
3.30 paveikslas *B. cereus* ir *L. monocytogenes* inaktyvacija ant maistui laikyti skirtų pakuočių paviršiaus 1,5·10^{-7} M Chl indukuotos fotosensibilizacijos pagalba, esant inkubacijos laikui 2 min. Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

Duomenys, pateikti 3.30 paveiksle, apibūdina Gram-teigiamų *B. cereus* ir *L. monocytogenes* Chl indukuotą fotoinaktyvaciją. Bakterijos prisitvirtinusios prie maisto pakuočių paviršiaus buvo 2 min inkubuojamos 1,5·10^{-7} M Chl tirpale ir vėliau švitinamos (šviesos dozė 1,4÷3,6 J/cm^2). Tyrimų metu buvo nustatyta, kad *Bacillus* ir *Listeria* ląstelių populiacijos ant maisto pakuočių po pritaikyto švitinimo buvo visiškai inaktyvuojamos (3,3 ir 4,4 log atitinkamai).

3.7. Braškių nukenksminimas su Chl–CHS konjugatu: skirtingų antimikrobinių poveikių palyginamoji analizė

3.7.1. Braškių mikrobiologinio užterštumo sumažinimas įvairių poveikių pagalba

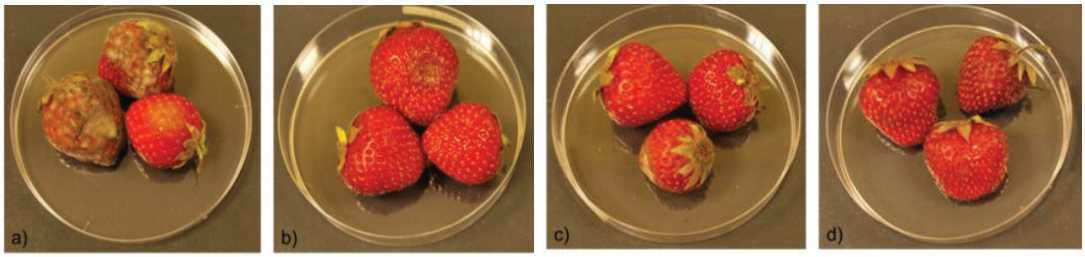
Kaip jau buvo minėta įvade, braškių uogos yra labai užterštos įvairiais pelėsiais ir mielėmis, o tai yra pagrindinė jų greito puvimo priežastis. Duomenys, pateikti 3.31 paveiksle, leidžia palyginti skirtingų paviršius nukenksminančių metodų efektyvumą. Fotosensibilizacijos, CHS ir fotoaktyvuoto Chl–CHS indukuoti poveikiai buvo palyginti su plovimu NaOCl. Buvo gauta, kad 1 min plovimas 200 ppm NaOCl sumažino pelėsių ir mielių populiacijas uogų paviršiuje 0,55 log. 0,001% Chl indukuota fotosensibilizacija pasižymėjo tokiu pat inaktyvacijos efektu, tačiau padidinus Chl koncentraciją iki 0,1% , buvo gauta šiek tiek didesnė nei 0,9 log inaktyvacija. Mažiausiu efektyvumu pasižymėjo CHS (0,4 log), o didžiausiu– fotoaktyvuotas 0,001% Chl–0,1% CHS konjugatas (1,4 log) (3.31 paveikslas).



3.31 paveikslas Palyginamoji antimikrobinių priemonių analizė: mielių ir pelėsinių grybų inaktyvacija braškių paviršiuje (200 ppm NaOCl – 1 min; 0,001% ir 0,1% Chl, 0,001% Chl–0,1% CHS konjugatas ir 30 min inkubacija, šviesos dozė 76 J/cm^2; 0,1% CTS ir 30 min inkubacija). Kiekvienas taškas yra 3÷6 eksperimentų vidurkis.

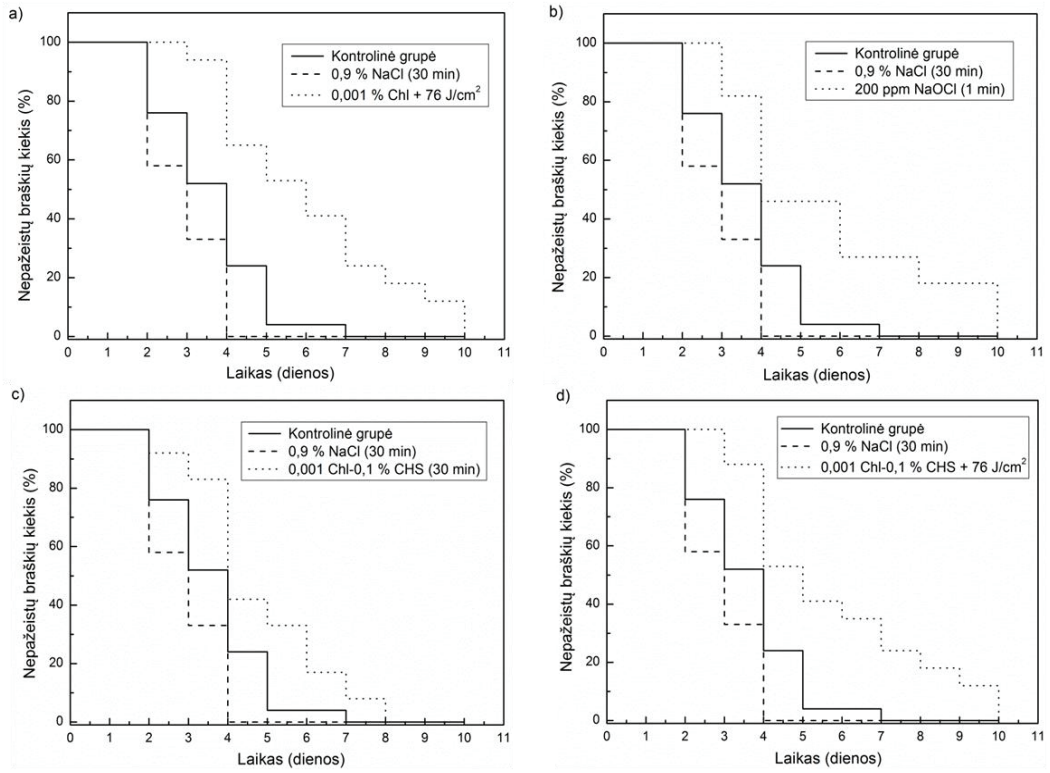
3.8.2. Apdorotų braškių vizualinės kokybės ir galiojimo trukmės įvertinimas

Pritaikant braškėms naują antimikrobinę technologiją, yra labai svarbu įvertinti jos įtaką apdorotų uogų vizualinei kokybei, nuo kurios priklauso ir jų galiojimo trukmė. Atliktų tyrimų metu buvo palyginti Chl indukuotos fotosensibilizacijos, fotoaktyvuoto Chl–CHS konjugato ir NaOCl poveikiai. Braškių galiojimo trukmė buvo vertinama vizualiai, stebint uogų paviršiaus spalvos pokyčius (matomi grybo pažeidimai) (3.32 paveikslas). Bendra apdorotų braškių išvaizda buvo nagrinėjama 10 dienų laikant jas 22 ± 2 ºC kambario temperatūroje. Laikui bėgant, buvo pastebimas braškių uogų vizualinės kokybės suprastėjimas.



3.32 paveikslas Vizualiai įvertinta apdorotų braškių kokybė (4 dienos saugojimo 22 ± 2 ºC): a) kontrolė b) 0,001% Chl–0,1% CHS konjugatas be šviesos; c) fotoaktyvuotas 0,001% Chl (šviesos dozė 76 J/cm^2); d) fotoaktyvuotas 0,001% Chl–0,1% CHS konjugatas (šviesos dozė 76 J/cm^2).

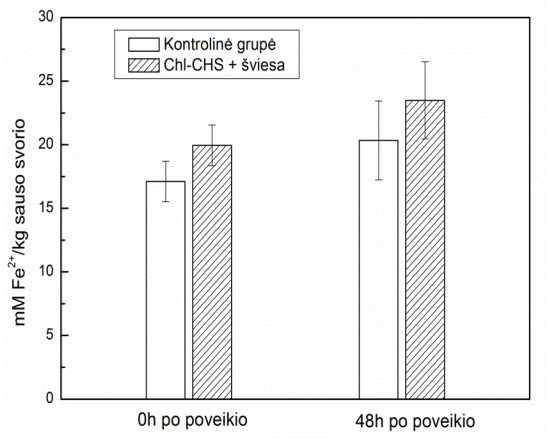
Vizualinės kokybės tyrimų metu gauti duomenys parodė, kad yra įmanoma šiek tiek pristabdyti braškių gedimą, kai jos padengtos Chl–CHS konjugatu ir apšvitintos. Septintą saugojimo dieną visos kontrolinės braškės buvo sugedusios, tuo tarpu uogų padengtų konjugatu galiojimo trukmė pailgėjo 1 dieną (3.33c paveikslas). Dar didesnį teigiamą efektą braškių galiojimo trukmei turėjo Chl indukuotos fotosensibilizacijos (3.33a paveikslas) ir fotoaktyvuoto Chl–CHS konjugato (3.33d paveikslas) poveikiai: braškių galiojimo trukmė pailgėjo 3 dienomis. Toks pats teigiamas efektas buvo gautas panaudojus 200 ppm NaOCl (3.33b paveikslas). Visgi Chl ir Chl–CHS konjugatas yra daug patrauklesnės braškių apsaugos priemonės, kadangi jos yra priskiriamos prie natūralių medžiagų.



3.33 paveikslas Nepažeistų braškių kiekio kitimas (laikymas 22 ± 2ºC) po $1{,}5 \cdot 10^{-5}$ M Chl indukuotos fotosensibilizacijos (30 min inkubacija, šviesos dozė 76 J/cm^2) a), 200 ppm NaOCl (1 min inkubacija) b), 0,001% Chl–0,1% CHS konjugato (30 min inkubacija) (c), fotoaktyvuoto 0,001% Chl–0,1% CHS konjugato (30 min inkubacija, šviesos dozė 76 J/cm^2) d) poveikių.

3.8.4. Bendras antioksidacinis aktyvumas

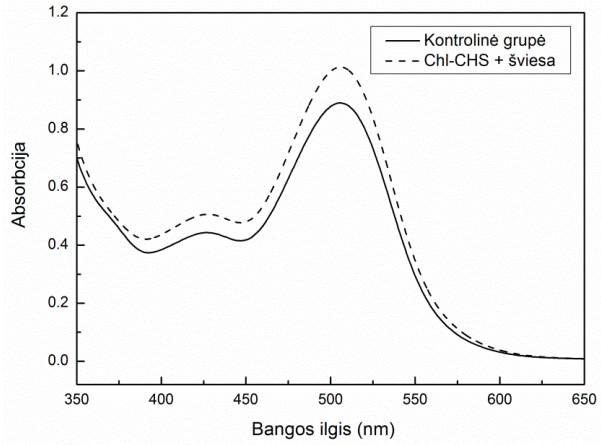
Tyrimų metu, panaudojus FRAP metodiką, buvo išanalizuotas braškių uogų antioksidacinis aktyvumas iš karto (0 val.) ir praėjus 48 val. po fotoaktyvuoto Chl–CHS konjugato poveikio. Iš duomenų, pateiktų 3.34 paveiksle matyti, kad kontrolinių braškių antioksidacinis aktyvumas lygus 17,11 mmol Fe^{2+}/kg, o apdorotų fotoaktyvuotu Chl–CHS konjugatu – 20,33 mM Fe^{2+}/kg. Gautas skirtumas nėra statistiškai reikšmingas. Tai reiškia, kad fotoaktyvuotas Chl–CHS konjugatas nepasižymėjo didesne geležies jonų antioksidacine galia ir nepakeitė antioksidacinių braškių savybių.



3.34 paveikslas Antioksidacinis braškių aktyvumas iškarto ir po 48 val. po fotoaktyvuoto 0,001% Chl–0,1% CHS konjugato poveikio (5 ± 0,5 ºC).

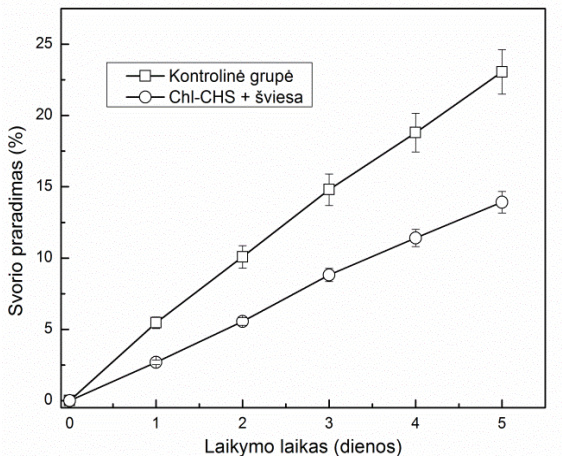
3.8.5. Braškių spalvos ir svorio kitimo matavimai

Kita svarbi braškių savybė, kuri gali pasikeisti po poveikio, tai uogų išvaizda. Yra svarbu, kad technologija apsauganti nuo kenkėjų nepakeistų braškių vaisių spalvos. Siekiant nustatyti, ar braškių vaisių apdorojimas fotoaktyvuotu Chl–CHS konjugatu (šviesos dozė 76 J/cm^2) neturėjo jokio neigiamo efekto jų spalvai, iš karto po poveikio buvo išanalizuoti uogų ekstrakto absorbcijos spektrai (350÷650) nm šviesos bangos ilgių srityje. 3.35 paveikslėlyje matyti, kad reikšmingų spalvų pokyčių neįvyko, o tai reiškia, kad fotosensibilizacijos-indukuota inaktyvacija neturi reikšmingos įtakos braškių uogų spalvai.



3.35 paveikslas Fotoaktyvuoto 0,001% Chl–0,1% CHS konjugato (šviesos dozė 76 J/cm^2) įtaka braškių spalvai.

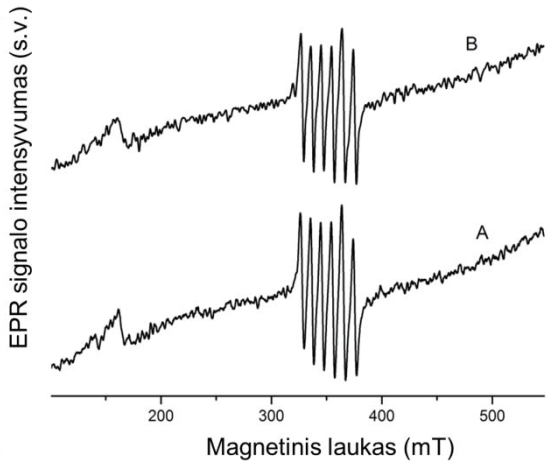
Taip pat labai svarbu įvertinti braškių uogų svorio kitimus po fotoaktyvuoto Chl–CHS konjugato poveikio. 3.36 paveiksle parodytas kontrolinių ir apdorotų uogų svorio sumažėjimas 5 dienų bėgyje. Visų mėginių (po 20 uogų) svoris saugojimo metu sumažėjo. Po 5 dienų saugojimo, kontrolinės grupės braškės prarado 22% svorio, o padengtos Chl–CHS konjugatu tik 12%.



3.36 paveikslas Neapdorotų ir paveiktų fotoaktyvuotu Chl–CHS konjugatu braškių svorio kitimas 5 dienų laikotarpyje.

3.8.6. Ilgai gyvuojančių laisvųjų radikalų nustatymas braškėse po fotoaktyvuoto Chl–CHS konjugato poveikio: EPR spektroskopija

3.37 paveiksle pateikti braškių paviršiaus elektronų paramagnetinio rezonanso (EPR) spektrai. Paveiktų fotoaktyvuotu Chl–CHS konjugatu braškių ekstrakto spektras sunormuotas į kontrolinio mėginio spektrą. Paveikto fotoaktyvuotu Chl–CHS konjugatu mėginio spektre matomos tos pačios juostos ties 320–380 mT, kurios priklauso įprastai braškėse sutinkamam Mn^{2+}. Kita, nedidelė signalo juosta ties 170 mT priklauso Fe^{3+}. Palyginamoji kontrolinių ir apdorotų mėginių analizė leidžia daryti prielaidą, kad fotoaktyvuotas Chl–CHS konjugatas nesukėlė apdorotose braškėse naujų laisvųjų radikalų atsiradimo.



3.37 paveikslas Neapdorotų a) ir paveiktų fotoaktyvuotu Chl–CHS konjugatu b) braškių mėginių EPR spektrai. Mn^{2+} linijos (g = 1,87608; 1,92801; 1,98124; 2,03521; 2,09085; 2,14451).

IŠVADOS

1. Gauti rezultatai rodo, kad ALA indukuota fotosensibilizacija prie parinktų eksperimentinių sąlygų ženkliai inaktyvavo Gram-neigiamą *Salmonella enterica* serovar Typhimurium (5,9 log), Gram-teigiamas *Listeria monocytogenes* (4 log) ir *Bacillus cereus* (6,4 log) skystoje terpėje ir ant maisto pakuočių paviršiaus (3,7÷4 log).
2. Darbo rezultatai parodė, kad Gram-neigiama bakterija *S. enterica* ir pelėsinis grybas *Botrytis cinerea* yra atsparesni Chl indukuotos fotosensibilizacijos poveikiui lyginant su Gram-teigiamos bakterijomis *L. monocytogenes* ir *B. cereus in vitro* ir ant maisto pakuočių paviršiaus. Chl indukuota fotosensibilizacija visiškai inaktyvavo *Listeria* ir *Bacillus* ($1{,}5\cdot10^{-7}$ M koncentracija, 2 min inkubacija, 2,4 ir 6 J/cm^2 šviesos dozės) skystoje terpėje bei ant maisto pakuočių paviršiaus, *Salmonella* skystoje terpėje–tik 2,5 log ($1{,}5\cdot10^{-5}$ M koncentracija, 60 min inkubacija, 51 J/cm^2 šviesos dozė) ir sustabdė *B. cinerea* micelio radialinį augimą 28% ($1{,}5\cdot10^{-4}$ M koncentracija, 24 val. inkubacija, 35 J/cm^2 šviesos dozė).
3. Tyrimų metu nustatyta, kad fotoaktyvuotas Chl–CHS konjugatas inaktyvavo Gram-neigiamą *S. enterica* ir Gram-teigiamą *L. monocytogenes in vitro* per 7 log, o *B. cinerea* augimą slopino 60%.
4. Gauti rezultatai parodė, kad po Chl indukuotos fotosensibilizacijos ir fotoaktyvuoto Chl–CHS konjugato poveikio įvyksta bakterijų ląstelės sienelės dezintegravimas, ko pasėkoje yra padidinamas jos pralaidumas ir sukeliamas DNR ir baltymų nutekėjimas.
5. Nustatyta, kad fotoaktyvuotas Chl–CHS konjugatas sumažino mielių/mikromicetų populiaciją braškių paviršiuje 1,4 log, ko pasėkoje jų galiojimo trukmė pailgėjo 3 dienom (40%), be esminio poveikio jų kokybei (spalvai, vandens kiekiui, antioksidacinėm savybėm ar radikalų koncentracijai).
6. Gauti rezultatai parodė, fotoaktyvuotas Chl–CHS konjugatas ateityje gali būti naudojamas sintetinant valgomus fotoaktyvius antimikrobinius

apvalkalus, kurie apsaugo braškes nuo greito gedimo ir prailgina jų saugojimo laiką, remiantis švarios ekologiškos technologijos (“clean green technology”).

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SUMMARY

Despite tremendous progress in technology and biomedical science the number of reported food-borne infections continues to rise. Health experts estimate that food-borne illnesses in the US cost 77.7 billion US dollars in direct medical expenses and lost productivity. CDC estimates that each year 31 major pathogens acquired in the US caused 9.4 million episodes of foodborne illness, 55961 hospitalizations, and 1351 deaths. Thus, development of innovative approaches and technologies for more effective inactivation of food pathogens seems an urgent task. In this context, photosensitization treatment seems promising despite the fact that it was never evaluated as tool to combat food pathogens. Thus, the main task of this study was to evaluate prospects of photosensitization as antimicrobial treatment and its possible applications in food industry.

Data obtained indicate that main food pathogens (*S. enterica*, *L. monocytogenes*, *B. cereus*) can be effectively inactivated by ALA-based photosensitization (5.9 log, 4 log, 6.4 log respectively) due to formation of endogenous porphyrins from precursor ALA ($1.5 \cdot 10^{-3}$ M) and following illumination (λ=405 nm, 24 J/cm^2 illumination dose). It is important to note that all investigated pathogens (*S. enterica*, *L. monocytogenes*, *B. cereus*) attached to the surface of food packaging (polyolefine) can be effectively inactivated as well (3.7÷4 log). Obtained data support the idea that photosensitization as non-thermal and effective antimicrobial treatment has potential to develop into environmentally safe, surface decontamination technique.

Antimicrobial efficiency of Chl-based photosensitization was tested against the same bacteria and gray mold (*B. cinerea*). Data indicate that Gram-positive *L. monocytogenes* and *B. cereus* were significantly (to an undetectable level) inactivated by Chl-based photosensitization *in vitro* and on the surface of packaging. Data presented in the work indicated that incubation of *B. cinerea* with $1.5 \cdot 10^{-4}$ M Chl and illumination with visible light (illumination dose 35 J/cm^2)

resulted in 3.35 cm diameter growth of fungus mycelium 2 days after Chl-based photosensitization treatment compared to the control (28% inhibition). Due to the fact that Chl-based photosensitization inactivated Gram-negative *Salmonella* just 1.8 log the novel approaches to enhance the inactivation of Gram-negative food pathogens using Chl–CHS conjugate have been applied. CHS alone incubated for 2 h with *Salmonella* reduced viability by 2.15 log, whereas photoactivated Chl–CHS conjugate (0.001%–0.1%, illumination dose 38 J/cm^2) diminished bacterial viability by 7 log. Moreover, it inhibited *B. cinerea* growth by 60%.

SEM images indicated that Chl–CHS conjugate at these experimental conditions covered all bacterial surfaces. Significant cell membrane disintegration was the main lethal injury induced in Gram-negative bacteria by this treatment.

Analysis of strawberry decontamination from naturally distributed yeasts/molds revealed that CHS alone reduced their population just by 0,4 log, Chl-based photosensitization 0,9 log, whereas photoactivated Chl–CHS coatings caused 1.4 log reduction of yeast/molds on the surface of strawberry.

Electron paramagnetic resonance spectroscopy confirmed that no additional photosensitization-induced long-lasting free radicals have been found in strawberry matrix. Visual quality (color, texture) of treated strawberries was not affected as well.

In conclusion, photoactive Chl–CHS conjugate exhibited strong antimicrobial action against more resistant to photosensitization Gram-negative *S. enterica* in comparison with Gram-positive bacteria *in vitro*. It reduced significantly the viability of strawberry surface–attached yeasts/molds without any negative impact on visual quality of berries. Experimental data support the idea that photoactivated Chl–CHS conjugate can be a useful tool for the future development of edible photoactive antimicrobial coatings which can preserve strawberries and prolong their shelf-life according to requirements of “clean green technology”.

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Kvalifikacijos tobulinimas

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