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Complete List of Authors:	Luksiene, Zivile; Vilnius university, Inst Applied Research Buchovec, Irina; vilnius university, Inst. Applied Research Lukseviciute, Viktorija; vilnius university, Inst. Applied Research Marsalka, Arunas; vilnius university, Dep. of General physics and spectroscopy Reklaitis, Ignas; vilnius university, Inst. Applied Research

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

Irina Buchovec[1], Viktorija Lukseviciute[1], Arunas Marsalka[2], Ignas Reklaitis[1], Zivile Luksiene[1]

[1]Vilnius University, Institute of Applied Research, Sauletekio 10, 10223, Vilnius, Lithuania
[2]Vilnius University, Department of General Physics and Spectroscopy, Sauletekio 9, 10223, Vilnius, Lithuania

Corresponding author: Zivile Luksiene, Vilnius University, Institute of Applied Research, Sauletekio 10, 10223, Vilnius, Lithuania
e-mail: Zivile.Luksiene@tmi.vu.lt; Tel.: +370 5 2366085

This study is focused on the novel approaches to enhance the inactivation of Gram (-) food pathogen *Salmonella enterica* and harmful molds *in vitro* and on the surface of strawberries using 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 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 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 Chl-CHS complex at these experimental conditions covered all 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}) coating diminished pathogen population on the surface of strawberry by 2.2 log. Decontamination of strawberries from naturally distributed yeasts/molds revealed that chitosan alone reduced population of yeasts/molds just by 0.4 log, Chl-based photosensitization just 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 strawberry matrix. Visual quality (color, texture) of treated strawberries was not affected as well. 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 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 the concerns about the microbial food safety 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 became 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 millions in tons of

51 production worldwide.[3] According to U.S. Food and Drug Administration (FDA) survey 1 out of 143
52 imported strawberry samples tested positive for *Salmonella*.[4]
53 The other challenge is extremely short postharvest life of strawberries. Due to high susceptibility
54 to mechanical injury and spoilage induced by plant pathogenic fungi[5] losses of the harvest reach 30–
55 40% if no chemical control is applied.[6]
56 Therefore, to find innovative and more effective techniques to decontaminate strawberries from
57 foodborne pathogens and molds seems important. Data obtained in our previous study clearly
58 indicate that photosensitization might be useful non-thermal and not-chemical tool for
59 decontamination of strawberries from Gram (+) food pathogen *Listeria*, yeasts, molds and mesophils
60 distributed on the surface[7]. Most important is the fact that this treatment can expand the shelf-life of
61 strawberries by 2 days[7], and it is comparable with the antimicrobial effects of high power pulsed
62 light.[8] Meanwhile, lower susceptibility of Gram (-) pathogens to photosensitization is well
63 documented, and remains the main disadvantage of this treatment.[9]
64 Chitosan is a biodegradable, nontoxic polymer produced by chitin deacetylation. It demonstrates
65 antimicrobial activity against wide variety of bacteria, filamentous fungi and yeasts. Antimicrobial
66 activity of chitosan is a complex superposition of many chemical, physical and environmental factors
67 and by no means depends on specificity of microorganism.[10] Most interesting is the fact that chitosan
68 is nutritional supplement which possesses excellent film-forming properties. Chitosan-based edible
69 coatings reduced microbial contamination of strawberries and slightly extended their shelf-life,
70 maintaining nutritional quality.[11]
71 The aim of this study is to increase susceptibility of Gram (-) food pathogen *S. enterica* to
72 chlorophyllin-based photosensitization combining it with antimicrobial properties of positively-
73 charged chitosan by immobilization of chlorophyllin into chitosan polymer. Impact of photoactivated
74 Chl–CHS coating on microbial contamination and visual quality of strawberries will be evaluated as
75 well.
76
77 ## 2. Materials and methods
78
79 ### 2.1. Chemicals
80 Not copperized chlorophyll sodium salt (Chl) was obtained from Roth (Karlsruhe, Germany). Low
81 molecular weight chitosan (CHS, Brookfield viscosity of 1% (all concentrations in percents refers to
82 w/v) solution in 1% acetic acid at 20 ºC 140 cP) was obtained from Aldrich (Saint Louis, USA).
83 Triton X-100 was purchased from MERCK (Darmstadt, Germany). Deionized water used in all
84 experiments had specific conductivity less than 1×10^{-6} S cm^{-1}.
85 Aqueous stock solution of CHS (pH = 2.4 at 20 ºC) containing 1% of CHS and 0.18% of HCl was
86 prepared dissolving in water appropriate amounts of HCl and then CHS. Aqueous stock solution of
87 1.5×10^{-5} M Chl was prepared by dissolution of Chl in water. Aqueous stock solution of
88 chlorophyllin–chitosan complex (Chl–CHS) (pH = 2.4 at 20 ºC) containing 1% of CHS, 1.5×10^{-5} M
89 Chl and 0.18% of HCl was prepared by drop wise addition of aqueous 0.05% Chl solution into
90 rapidly spinning aqueous solution containing 1.25% of CHS and 0.23% of HCl. After addition of
91 Chl-CHS complex to bacterial suspension in NaCl, the pH of final bacterial suspension changed to
92 3.95.
93
94 ### 2.2. Absorption and fluorescence measurements of Chl–CHS complex
95 Absorption spectrum of Chl–CHS solution was recorded by spectrophotometer Heλios Gamma &
96 Delta spectrophotometers, ThermoSpectronic (Leicestershire, Great Britain), fluorescence spectrum
97 was recorded by Perkin Elmer fluorescence spectrophotometer LS-55 (Rodgau, Germany). Scan
98 range parameters were as follows: excitation wavelength – 405 nm; emission – 550–750 nm; ex Slit –
99 10 nm; em Slit – 4 nm; scan speed (nm min^{-1}) – 200. 3 mL quartz cuvette (Hellma-analytics QS,
100 Mullheim, Germany) was used for measurements. 1.5×10^{-5} M Chl–0.1% CHS complex diluted by
101 0.9% NaCl was used for absorption and fluorescence measurements. To observe monomeric Chl
102 forms of this complex 20 µL of Triton X-100 to 20 mL of suspension was added.

103
104 **2.3. Cultivation of the microorganism**
105 The target bacteria, *Salmonella enterica* serovar Typhimurium strain DS88 [SL5676 SmR
106 (pLM32)] resistant to tetracycline, were kindly provided by Prof. D. H. Bamford (University of
107 Helsinki, Finland).
108 *S. enterica* was grown in Luria-Bertani medium (LB) (Liofilchem, Roseto Degli Abruzzi, Italy;
109 pH = 7.1) incubated overnight at 37 ºC. The overnight culture was 20-times diluted with fresh LB
110 medium (optical density at 540 nm (OD_{540} was 0.164) and grown at 37 ºC to the mid-log phase (5 ×
111 10^8 CFU mL^{-1}, OD_{540} = 1.3). Cells were then harvested by centrifugation (10 min, 6 ºC, 3574 × g)
112 (MPW-260R; MPW Med. instruments, Warsaw, Poland) and resuspended in a buffer 1 × 10^{-1} M PBS
113 (pH = 7.4) and normal saline 0.9% NaCl (pH 7.3), depending on treatment requirements, to give ~2.5
114 × 10^9 CFU mL^{-1}. These stock suspensions were diluted to approximately 1 × 10^7 CFU mL^{-1} and
115 immediately used for the experiments.
116
117 118 **2.4. Scanning electron microscopy (SEM)**
119 The effect of Chl–CHS complex on the morphology of *Salmonella* was examined by SEM.
120 Bacterial suspension (approximately 1 × 10^7 CFU mL^{-1}) containing 1.5 × 10^{-5} M Chl–0.1% CHS
121 complex was incubated at 37 ºC in the dark. In the next step, the samples consisting 20 µL of
122 bacterial suspension were withdrawn, transferred to aluminum stubs, air-dried and sputter coated
123 with 15 nm gold layer using Q150T ES sputter coater (Quorum Technologies, Lewes, England). The
124 scanning was performed with an Apollo 300 (CamScan, Bingham, UK) scanning electron
microscope at an accelerating voltage of 20 kV.
125
126 **2.5. Light sources for inactivation of bacteria**
127 An InGaN light emitting diodes (LED) array (LED Engine, San Jose, USA; Inc. LZ1-00UA00)
128 was used for construction of light source for the photoinactivation of bacteria. It consisted of
129 illumination chamber and supply unit (Fig. 1a). A cooling system was integrated in the light
130 prototype to dissipate heat from the source and minimize any heat transfer to the sample. LED
131 emission maximum was at 405 nm with a band width of 13 nm at full-width half maximum. Two
132 rectangular 6 × 10 arrays (top and bottom), consisted of 60 LEDs, powered by a 20 V DC power
133 supply were integrated in the chamber. The light intensity at the surface of samples from top and
134 bottom LED reached approximately 10 mW cm^{-2} (6 cm from the light source) and 11 mW cm^{-2} (3.5
135 cm from the light source), respectively. Light intensity was measured by *3 Sigma* power and energy
136 meter "Coherent" (California, USA) equipped with a piro-electrical detector J25LP04. Light dose
137 was calculated as light intensity multiplied by irradiation time. The sample exposure time was
138 adjusted according to the equation:
139 $E=P{\cdot}t$, (1)
140 where E is the energy density (dose) in J cm^{-2}, P is the irradiance (light intensity) in W cm^{-2}, and t is
141 the time in seconds. Three-dimensional model of distribution of power density of the emitted light
142 from the top and bottom in the prototype is presented in Fig. 1b. Almost the same power density
143 distribution was registered from LEDs in the bottom of prototype however distributions from the top
144 and from the bottom cannot be placed in one picture since it would overlap. The variation of light
145 intensities on the illumination square was insignificant, since we use just central part of it (± 0.5 mW
146 cm^{-2}).

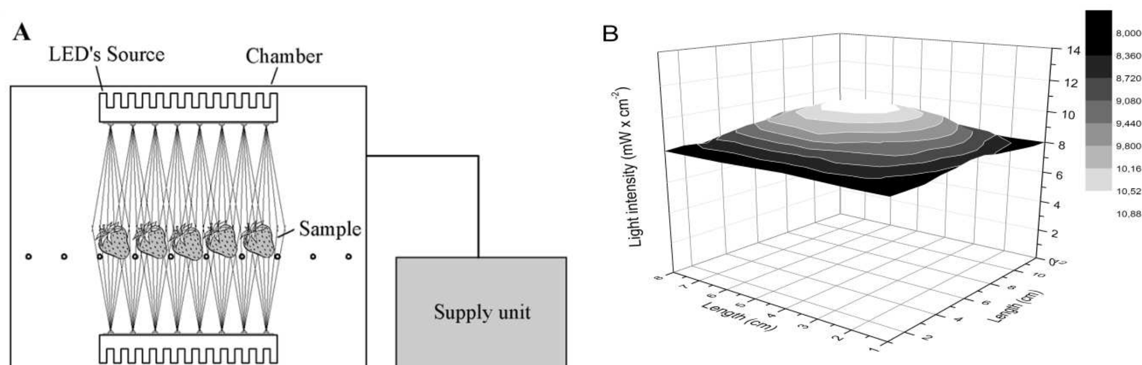


Fig. 1

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 the shaker (130 rev min^{-1}) at 37 ºC. The samples were removed after 1 min, 15 min, 30 min, 60 min and 120 min. 150 µL of the samples were placed into sterile flat bottom wells and then samples (4-5) were exposed to light (light dose 38 J cm^{-2}). Antibacterial efficiency of treatments was evaluated by the spread plate method, comparing viability of treated and not treated bacteria. 100 µL of a diluted bacterial suspension after treatment was surface inoculated on the separate LB agar (LBA) plate. Afterwards LBA plates were kept in the thermostat for 24 h at 37 ºC. Bacterial populations were recalculated from CFU mL^{-1} into log_{10} mL^{-1}.

2.7. Evaluation of membrane integrity of treated bacteria

The bacterial cell membrane integrity was examined by 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 1.5×10^{-5} M Chl–0.1% CHS complex (at 37 ºC in the dark) was irradiated (doses of 25 and 38 J cm^{-2}). Aliquots of 1.5 mL cell suspension were taken out and filtered to remove the bacteria. The UV absorbance of cell supernatant at 260 nm and 280 nm was determined using spectrophotometer (He*λ*ios Gamma *&* Delta ThermoSpectronic, Leicestershire, Great Britain).

2.8. Decontamination of strawberry from yeasts/molds by coating with Chl-CHS and treating with light

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, chitosan coating), in 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), other were just illuminated (5) or not treated (6, control). The samples 3, 4 and 5 were placed in the treatment chamber in a sterile Petri dishes, dried and exposed to 405 nm light for 60 min (light dose 38 J cm^{-2}). CHS (1), dark toxicity (2) and control (6) samples were not illuminated. The 1 g of each strawberry and 9 ml of 0.9% NaCl solution placed to sterile BagPage (Interscience, Saint-Nom-la-Bretèche, France) and homogenized using BagMixer (Interscience, Saint-Nom-la-Bretèche, France) (in detail[7-8]). Antifungal activity of 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 the separate dichloran glycerol (DG18) agar (Liofilchem, Italy). Afterwards plates were kept in the thermostat for 144 h at 30 ºC. Fungal populations were recalculated from CFU g^{-1} into log_{10} g^{-1}. Every sample consisted of 1 berry, and experiments were repeated 3-6 times.

189 Visual quality of strawberries was evaluated checking spots, induced by growth of spoilage
190 microorganisms according to methods described in[7].
191
192 **2.9 Inactivation of inoculated *S. enterica* on the surface of strawberries by coating with Chl-**
193 **CHS complex and light**
194 The target bacteria, *Salmonella enterica* was cultivated and prepared as mentioned above in 2.3.
195 Berries were soaked in bacterial suspension for 30 min and incubated in the dark at 37 °C. After
196 inoculation, strawberries were soaked in 0.1% CHS (1, chitosan coating), in 1.5×10^{-5} M Chl–0.1%
197 CHS (2, dark toxicity, Chl-CHS coating), in 1.5×10^{-5} M Chl–0.1% CHS (3, photoactivated, Chl-
198 CHS coating), in 1.5×10^{-5} M Chl (4, photoactivated using 19 J cm^{-2}), in 1.5×10^{-5} M Chl (5,
199 photoactivated using 38 J cm^{-2}), in 0.9% NaCl solution (6, only illuminated) or in 0.9% NaCl
200 solution (7, control) for another 30 min. Then samples 3-6 were placed in the treatment chamber on a
201 sterile quartz glass plate, dried and exposed to 405 nm light for 60 min (light dose 38 J cm^{-2}) or 30
202 min (light dose 19 J cm^{-2}). Control (neither treated with the complex a nor with light) samples and
203 treated with 0.1% CHS samples were not illuminated. The following preparation of samples was the
204 same as described in 2.8, except for the growth medium for *Salmonella* which was selective Briliant
205 Green Lactose Sucrose Agar (Roth, Karlsruhe, Germany), growth conditions were 24 h at 37 ºC.
206 Every sample consisted of 1 berry. Experiment was repeated 3-4 times.
207
208 **2.10 Electron spin resonance, EPR**
209 EPR spectra were registered with Bruker Elexsys E580 FT-EPR spectrometer (Billerica, USA)
210 working in X-band.
211 Before recording spectra the surface of treated and not treated strawberries was peeled, and the
212 peelings were homogenized. The capillaries (BLAUBRAND micropipettes, intraMark, Hinckley,
213 Great Britain) were filled with the mass of strawberry. After that capillaries were put into the
214 standard EPR tube.
215
216 **2.11. Statistical analysis**
217 The experiments were triplicated for each set of exposure. A standard error was calculated for
218 every experimental point and marked in a figure as an error bar. Sometimes the bars were too small
219 to be visible. The data were analyzed using Origin 7.5 software (*OriginLab Corporation*,
220 Northampton, MA 01060, USA). The significance of the results was assessed by the analysis of
221 variance (ANOVA). A value of $p < 0.005$ was considered as significant.
222
223
224 **3. Results**
225
226 **3.1. Absorption and fluorescence spectra of Chl–CHS complex**
227 In order to confirm the structure of chlorophyllin-chitosan complex the absorption and
228 fluorescence spectra were analyzed. Fig. 2 indicated that the absorption spectrum of Chl–CHS
229 complex in solution had peaks at $\lambda = 405$ nm and at $\lambda = 652$ nm. Fluorescence spectra presented in
230 the same picture indicated very low (100 a. u.) fluorescence intensity (peak at 648 nm) of complex.
231 Just adding of 0.001% triton to the complex solution monomerized chlorophyllin and increased the
232 fluorescence intensity to 660 a.u. (peak at 674 nm) Taking into account the structure of both
233 compounds, the interaction between positively charged chitosan NH_3^+ group and negatively charged
234 chlorophyllin COO^- group is most probable (Fig. 2).

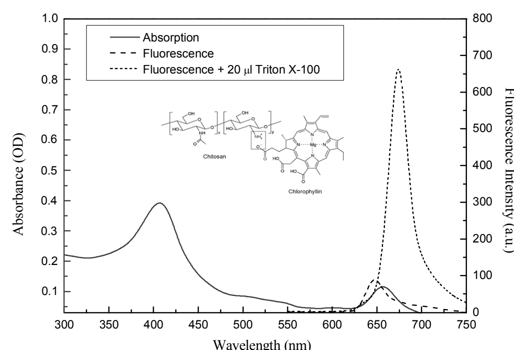


Fig. 2

3.2. Inactivation of *Salmonella enterica*

Multiple experimental data confirmed that the light alone at dose 38 J cm^{-2} did not diminish the viability of bacteria (Luksiene et al., 2010, Buchovec et al,2009). 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 cells with Chl (0–120 min) and subsequent illumination with visible light (405 nm, light dose 38 J cm^{-2}) decreased the viability of cells more considerably: in this case the photosensitization treatment led to 1.8 log reduction (Fig. 3).

The antimicrobial properties of Chl–CHS complex were assessed comparing its antimicrobial efficiency with that of CHS alone.

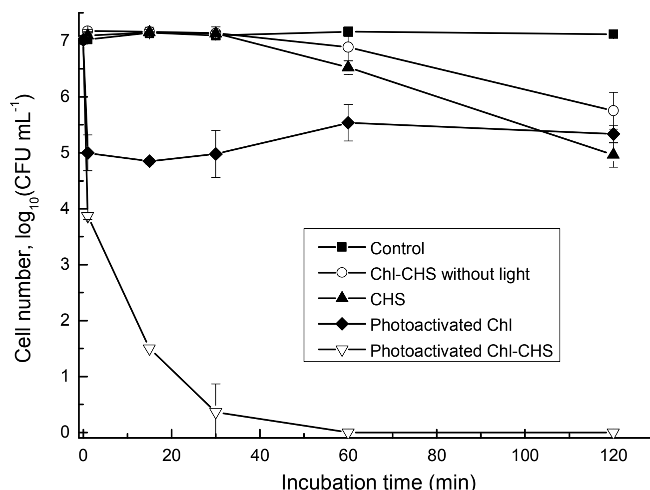


Fig. 3

Thus, dark toxicity of Chl–CHS complex slightly depends on incubation time. Viability of *Salmonella* incubated with CHS alone (0–120 min) was diminished by 2.15 log. It indicated that Chl–CHS complex exhibited some antibacterial action which was close to that of CHS alone (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 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 10^{-1} M PBS shifted from 7.4 to 6.8 when the cell suspension was mixed with Chl or Chl-CHS complex. However, when PBS was replaced by 0.9% NaCl, pH value after mixing with Chl or Chl-CHS complex decreased from 7.3 to 3.95.

In the next step it was important to assess the interaction Chl-CHS –*Salmonella*. SEM images, presented in Fig. 4 indicated that Chl–CHS biopolymer covered all surface of this Gram (-) bacterium.

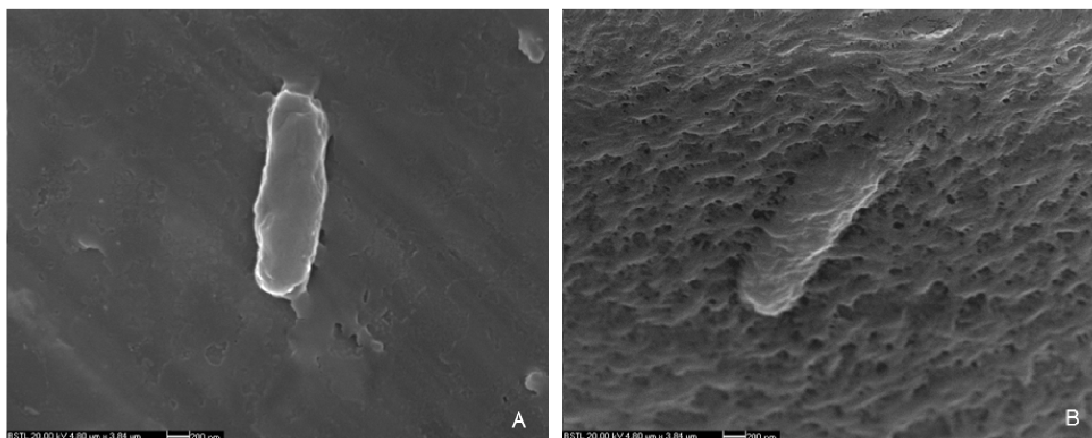


Fig. 4

3.3. Evaluation of cell membrane integrity in *S. enterica* after treatment with photoactive Chl–CHS complex

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 control and treated samples (Fig. 5). The results indicated that the release of intracellular material absorbing at λ_{260nm} and λ_{280nm} in control supernatant was insignificant and did not depend on light dose (light dose 0–46.8 J cm^{-2}). On the contrary, the release of intracellular components (both absorbing at λ_{260nm} and λ_{280nm}) increased while increasing light dose. For instance, absorption at λ_{260nm} increased from 0.01 OD to 0.14 OD and absorption at λ_{280nm} increased from 0.01 OD to 0.1 OD, when *S. enterica* was treated by photoactivated Chl–CHS complex (60 min incubated with Chl and afterwards illuminated, light dose 38 J cm^{-2}).

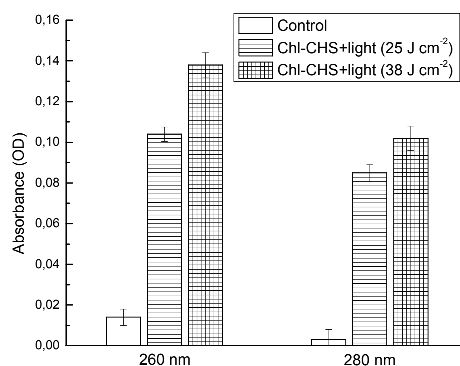
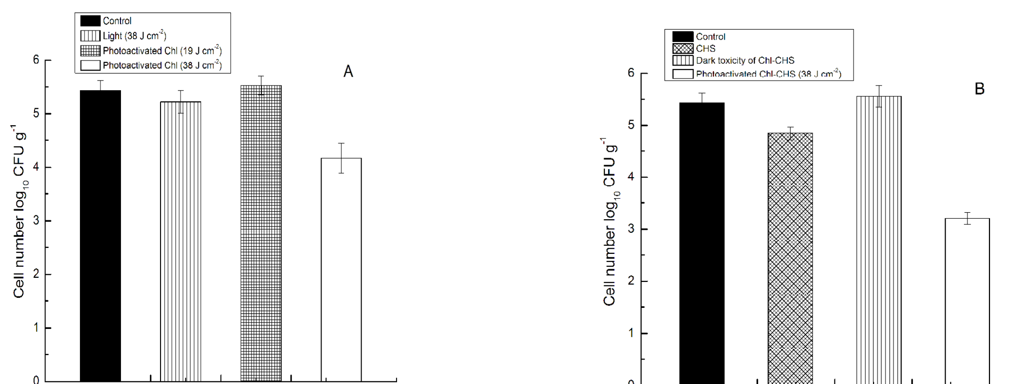


Fig. 5

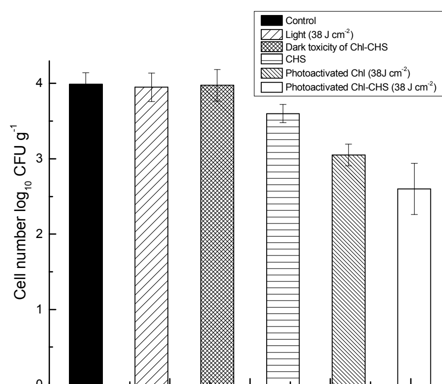
3.4. Microbial decontamination of strawberries by different treatments

It is clear from Fig. 3 that *S. enterica* has low susceptibility to Chl-based photosensitization, and just photoactivated Chl-CHS reduced 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 g^{-1} *Salmonella* counts have been found. Exposure to light (405 nm) alone even at 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 higher light dose (38 J cm^{-2}) reduced *S. enterica* viability by 1.3 log. Afterwards effects of chitosan

289 and photoactivated Chl-CHS complex on decontamination of strawberries have been evaluated (Fig.
290 6b). It is clear that antimicrobial activity of 0.1% CHS alone (incubation time 30 min) against *S.*
291 *enterica* was rather low, since it reduced microbial load from 5.4 log to 4.8 log. Dark toxicity of Chl-
292 CHS at 30 min. incubation was insignificant and did not differ from the control (5.5 log). But the
293 remarkable decrease of viable pathogens was observed (from 5.4 log to 3.2 log) after the illumination
294 of strawberries coated with Chl-CHS (1.5×10^{-5} M Chl–0.1% CHS, 30 min, λ = 405 nm, dose - 38 J
295 cm^{-2}).
296



297 **Fig. 6.**



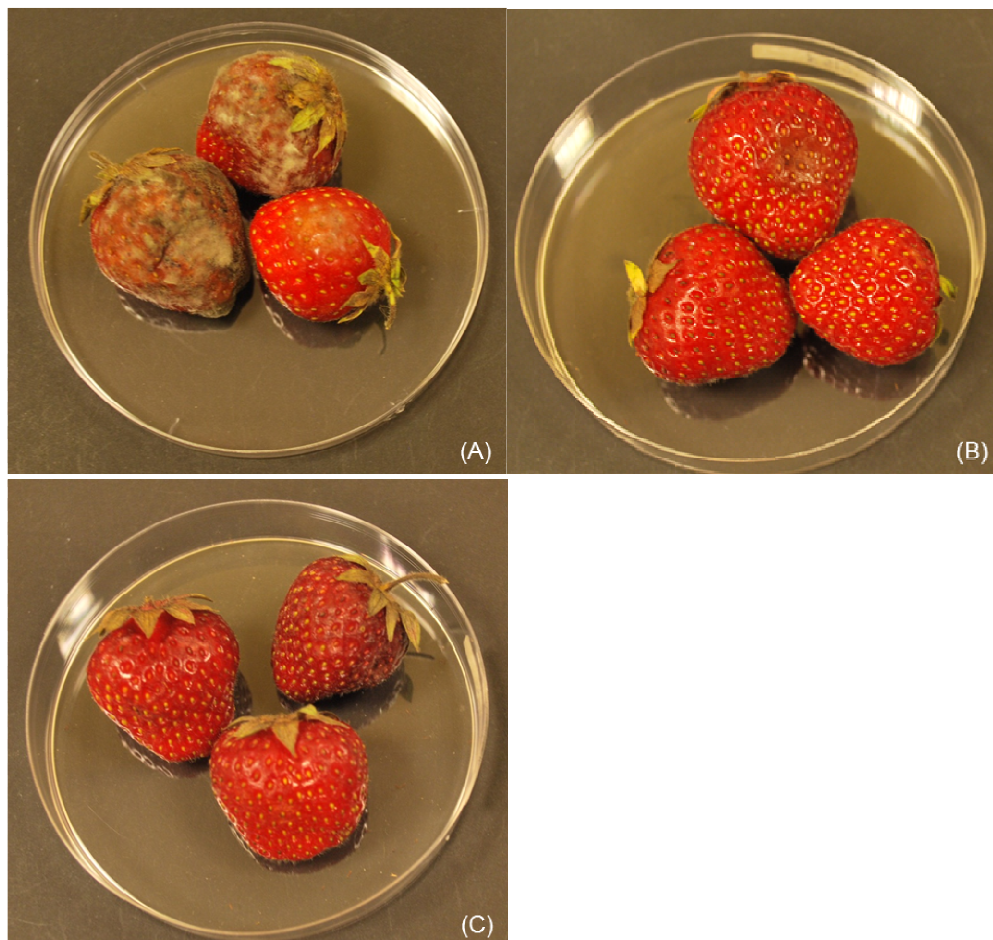
298
299
300 **Fig. 7**
301 As it was mentioned above strawberries are highly contaminated with molds and yeasts what is the
302 main reason for their fast spoilage. Thus, naturally contaminated berries (4 log) were coated with
303 Chl-CHS for 30 min and afterwards illuminated with higher dose of visible light (38 J cm^{-2}) since
304 molds exhibited lower susceptibility to photosensitization than bacteria. Data presented in Fig. 7
305 allowed us to compare antimicrobial efficiencies of different treatments. Obtained data indicated that
306 the light alone or Chl-CHS without light had no effect on natural contamination of strawberries.
307 Chitosan alone diminished contamination of strawberries by 0.4 log, whereas Chl-based
308 photosensitization reduced yeasts and molds up to 0.9 log. But the highest inactivation of yeasts and
309 molds was found when strawberries were treated by photoactivated Chl–CHS coating (1.4 log).
310

311 **3.5 Visual quality of treated strawberries**

312 The overall appearance of treated strawberries during storage at 22 ± 2 ºC for 4 days was
313 examinated. Generally, the visual overall quality of strawberries gradually decreased over storage
314 time. Our data on visual quality of control, strawberries coated with Chl-CHS without illumination,
315 and strawberries treated by photoactivated Chl-CHS (in every case 60 strawberries have been used)

316 indicate that it is possible to achieve some delay of spoilage when berries are coated with Chl-CHS
317 ant illuminated. For instance, in (Fig. 8A) control strawberries 4 days after treatment were totally
318 infected (visually detected spots of infection), whereas coating of strawberries with chlorophyllin-
319 chitosan (Fig. 8B, dark toxicity) reduced the natural spoilage. But, it is obvious that photoactivated
320 Chl–CHS complex (Fig. 8C) was most effective tool in delaying strawberry spoilage.
321

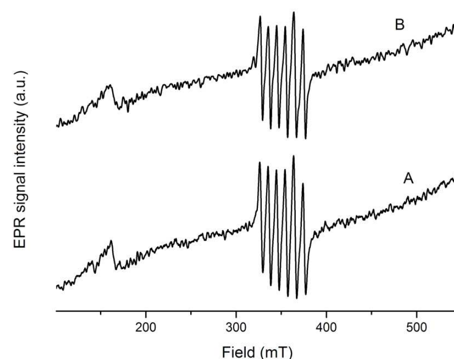
322



323
324 **Fig. 8**
325

326 **3.6 Detection of free radicals in treated strawberries by electron paramagnetic resonance**
327 **(EPR)**

328 Data presented in Fig. 9 showed the EPR spectra of control and treated by photoactivated Chl–
329 CHS strawberries in a wide field range (from 10 mT to 600 mT). The spectrum consisted of typical 6
330 signals which were separated from each other by ~ 9 mT. According to Raffi and Stocker[14] these
331 signals from 320 mT to 380 mT belong to Mn^{2+} which is normaly in strawberries in measurable
332 amounts. The number of lines from the hyperfine interaction was determined by the formula:
333 $n = 2NI + 1$,
334 where n is a number of spectral lines, N is the number of equivalent nuclei, I is the spin.
335 In our case, $N = 1$, $I = 5/2$ from the manganese nucleus, thus amount of spectral lines was 6.
336 According to P. Leveque et al.[15] the signal in lower field (170 mT) belongs to the Fe^{3+}.
337 Comparison of spectra of control and treated strawberries indicated that this treatment did not induce
338 additional free radicals in the matrix of strawberry.



339
340 **Fig. 9**
341
342
343
4. Discussion

344 In order to increase the susceptibility of Gram (-) pathogens to Chl-based photosensitization the
345 complexation of Chl with CHS was performed. Chitosan (polyβ-(1, 4)-acetyl-D-glucosamine) is
346 cationic linear polysaccharide, obtained from deacetylated derivative of chitin - most abundant
347 polysaccharide in nature after cellulose.[16] It is tasteless fiber, non-toxic and biodegradable. Moreover,
348 chitosan is nutritional supplement which exhibits film-forming properties. These features enable us to
349 apply it as an edible coating for different types of food.[17]
350 Results presented in this study clearly indicated, that inactivation of Gram (-) pathogen *S. enterica*
351 can be enhanced combining antimicrobial properties of Chl-based photosensitization with that of
352 chitosan (Fig. 3). It is clear that antimicrobial properties of CHS alone are insignificant at short
353 incubation time (0-30 min)(0 log). Just at longer incubation time (120 min) it reduced viability of *S.*
354 *enterica* by 2 log. Chl-based photosensitization inactivated *Salmonella* by 2 log but not more, and
355 inactivation efficiency did not depend on incubation time (Fig. 3). It enables to presume that Chl
356 interacts with the bacterium just superficially. Remarkable and very fast decrease of *Salmonella*
357 viability (7 log) was observed when bacteria were treated by photoactivated Chl–CHS complex (light
358 dose 38 J cm^{-2}). By no means, question arises, whether low pH (in final Chl-CHS cell suspension in
359 0.9% NaCl, pH = 3.95) or light alone (405 nm) can diminish *Salmonella* population. Data, published
360 in our previous [18,19] indicated that *Salmonella* cells preserved their viability 100% when being
361 suspended and incubated for rather long time (120 min) in 0.9% NaCl acidified by HCl to pH 4.6.
362 Thus, just minor impact of pH on viability of Chl-CHS treated *Salmonella* can be anticipated. In
363 2012 Murdoch et al.[20] published data about possibility to inactivate *Escherichia*, *Salmonella*,
364 *Shigella*, *Listeria*, and *Mycobacterium* in suspension by LED-based light (405 nm). Meanwhile,
365 statistically significant inactivation of *Salmonella* was achieved at light dose 150 J cm^{-2}, whereas in
366 our experiments just 38 J cm^{-2} light dose has been used.
367 In order to understand whether Chl–CHS interacts with the bacterial surface the analysis of SEM
368 images was performed. Data clearly indicate that *Salmonella*, incubated with 1.5×10^{-5} M Chl–0.1%
369 CHS is fully covered by it (Fig. 4). Thus, the main target of photoactivated Chl-CHS complex might
370 be cell membrane. As evidence, the intensive release of intracellular components absorbing at λ_{260nm}
371 and λ_{280nm} (DNA and proteins) was detected after this treatment. Moreover, the release of
372 intracellular components to some extend depended on light dose. It might be addressed to the
373 intensive membrane disintegration induced by the photoactivated Chl–CHS complex (Fig. 5). Chl
374 being negatively-charged has weak interaction with negatively charged *Salmonella*. But
375 immobilization of Chl into positively-charged CHS polymer enhanced the electrostatic interaction of
376 Chl with bacterium. After the illumination of cells incubated with complex the multiple cell
377 membrane injuries and effective killing were triggered.
378 The conventional treatment to reduce microbial load on the surface of fruits is based on preharvest

379 disease control by fungicides. As a result, the multiple fungicide residues were found in more than
380 60% of strawberries.[21] Moreover, so aggressive compounds are harmful to human and environment.[22]
381 Most important is the fact that all harmful microbes developed high resistance to the fungicides. To
382 combat microbes conventional water-based sanitizers are not enough effective.[23] Widely accepted
383 hypochlorite (NaOCl) (200 µg mL^{-1}, 2 min incubation) reduced the microbial contamination of
384 strawberries just by 0.45 log.[24] Moreover, hypochlorous acid interacts with organic matter and
385 releases chlorine, which eventually causes the formation of highly mutagenic compounds,
386 trihalomethanes.[25,26]. Ultrasound takes short time (5-10 min), meanwhile, power higher than 60 W
387 diminished significantly the quality of berries.[27] An emerging approach to control strawberry
388 microbial contamination is atmospheric pressure cold plasma (ACP). Misra et al.[28] observed that the
389 total mesophiles and yeasts/ molds of strawberries treated for 5 min with ACP were reduced by 2 log
390 within 24 h after treatment. Meanwhile it is difficult to control this process. According to Alexandre
391 et al.[24] UV treatment reduced the spoilage by 1 log when strawberries were treated at 4 °C.
392 International Consultative Group on Food Irradiation[29] allows irradiation of strawberries with
393 maximum dose of 3 kGy. Yu et al.[30] approved that this dose extended the shel-life of berries by a
394 factor of 2, but induced significant changes in texture and color. Moreover, the irradiated fruits are
395 not popular among consumers.[31, 32]
396 Photosensitization seems to offer a promising alternative as effective non-thermal antimicrobial
397 treatment which is environmental friendly, saves water and energy at very reasonable costs.[33] After
398 spraying of the photosensitizer on the surface of fruit most surface-distributed pathogens, harmful
399 bacteria, viruses and molds bind to the photosensitizer.[34-37] The following illumination of fruits with
400 light induced photocytotoxic reactions and death in surface-attached microorganisms without any
401 harmful effects on the environment.[38-40]
402 It is obvious that not every photosensitizer which is of high chemical purity and exhibits high
403 killing efficiency can be used for food safety purposes. In this case photosensitizer must fulfil
404 additional mandatory requirements, such as low cost, status of food additive or food component,
405 which works at very low concentration with any effects on nutritional as well as organoleptic
406 properties of the foods.[33,36] Hence, Chl is copper-free water-soluble food additive (E140) used as food
407 colorant in dietary supplements and in cosmetics.[41] According to our data Chl interacted with the
408 bacterial wall/outer membrane and just after the necessary light dose destroyed its integrity.[33,36] Thus,
409 the most important is the fact that this treatment has low mutagenicity, and microbes did not develop
410 resistance to it.[42] The main disadvantage of photosensitization is lower susceptibility of Gram (-)
411 pathogens to neutral or negatively-charged photosensitizer-based photoinactivation.[43-44]
412 It must be mentioned, that over the last decade interest has been rapidly growing in the
413 development of bio-based packaging of fruits. It can enhance the safety and preserve nutritional/
414 sensory attributes of fruits. Moreover, it reduces environmental pollution by non-biodegradable
415 packaging. Such edible coating can control water migration both in and out of fruit to maintain
416 desired moisture content. In addition, it protects fruits from the contamination, inhibits microbial
417 proliferation and extends the shelf-life of products.[11,16,45] For instance, the chitosan coating prevented
418 mechanical injury of perishable berries, reduced moisture losses, controled gas (CO_2, O^{-2}) and
419 extended the shelf-life of strawberries.[46] Thus, in the next step it was important to evaluate whether
420 *Salmonella* inoculated on the surface of strawberry can be controlled by photoactivated Chl-CHS
421 coating. Data presented in Fig. 6 revealed, that this treatment diminished *Salmonella* on strawberries
422 by 2.2 log, whereas CHS alone just 0.6 log. It is obvious that photoactivated Chl-CHS coating is
423 really effective tool against Gram (-) *S. enterica* distributed on the surface of strawberry. Moreover,
424 the antimicrobial activity of coating at longer incubation time must be stronger, since the
425 antimicrobial effects of chitosan depended on the time (Fig.3).
426 In the next step the efficiency of photoactivated Chl-CHS coating against naturally distributed
427 yeasts/molds on the surface of strawberry was evaluated. Data presented in Fig. 7 indicated that Chl-
428 based photosensitization (1.5×10^{-5} M) reduced naturally surface-attached microbes by 0.9 log.
429 Antimicrobial effect of chitosan coating at short incubation time is very mild (0.4 log). The highest
430 inactivation of yeasts/molds was found when strawberries were treated by photoactivated Chl–CHS

431 coating (1.4 log). By no means, the irregularity and the different light reflecting properties of the
432 strawberry surface can possibly account for the lower antimicrobial efficiency of Chl-CHS coating in
433 comparison with data *in vitro*.[8] It must be emphasized that antimicrobial efficiency of this treatment
434 can be enhanced by more powerful LED's.
435 The visual quality of treated strawberries is key-parameter for consumers. It is important to note
436 that at these experimental set up no effects on color of strawberries have been found. Data presented
437 in Fig. 8 indicated that visual berry texture was not damaged after coating with Chl-CHS and
438 following illumination. Just significant delay of spoilage of treated berries in comparison with control
439 was observed during storage (Fig. 8A- C).
440 Leveque et al.[15] found out that EPR imaging could be applied for the monitoring of free radicals in
441 various food samples. Other authors used EPR spectroscopy to evaluate antioxidant activity of spices
442 and herbs.[47-50] Moreover, EPR method was succesfully applied to distinguish irradiated and not
443 irradiated fruits and vegetables. Raffi and Stocker claimed that it is possible to detect irradiated
444 berries (due to free radicals) as long as 25 days (stored at 4-5 °C).[14] As photosensitization treatment
445 involves radical reactions it was important to check whether photoactivated Chl-CHS coating
446 induced additional long lasting reactive oxygen species in strawberries. Data indicated that both
447 registered spectra (control and treated strawberries) (Fig. 9) exhibited strong signal due to the 6 lines
448 of Mn^{2+}(which is a transition metal ion linked to enzymes in the strawberry).[14] Comparison of EPR
449 spectra in control and treated strawberries revealed that no radical-based fundamental changes
450 occured 1 hour after treatment. It means, that despite the high antioxidant activity of these berries this
451 treatment does not induce long-lasting free radicals in the strawberries, as for instance do 2 kGy
452 ionizing radiation.[14] Hence, the obtained data indicate that photoactivated Chl-CHS coating has
453 potential to combat harmful and pathogenic microorganisms distributed on the surface of
454 strawberries and can serve in the future for the development of photoactive biodegradable edible
455 coating with more pronounced antimicrobial properties.
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458
459 **5. Conclusions**
460
461 In conclusion, photoactive chlorophyllin-chitosan complex exhibits high antimicrobial capacity
462 against Gram (-) food pathogen *S. enterica* and *in vitro*. It is able to cover *Salmonella* surface and
463 after the photoactivation induced intensive membrane disintegration and total destruction of
464 pathogens.
465 Moreover, our data indicated that the application of edible and active in visible light Chl–CHS
466 coating preserved strawberries much better in comparison with chitosan coating, since it reduced fruit
467 contamination by *S. enterica* and yeasts/molds to desirable levels. Experimental data support the idea
468 that Chl–CHS coating photoactivated with visible light can be a useful tool for the preservation of
469 strawberries according to requirements of "clean green technology concept".
470
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474 microscopy.
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616 # Figure captions
617
618
619 **Fig. 1** Schematic presentation of LED-based light source prototype (A) and three-dimensional
620 distribution of average light intensity from the top and bottom (B).
621
622 **Fig. 2** Chemical formula, absorption and fluorescence spectra of 1.5 × 10^{-5} M Chl–0.1% CHS
623 solution in 1 × 10^{-1} M PBS (pH 6.9) and fluorescence spectra of 0.001% Triton X-100 solution in 1.5
624 × 10^{-5} M Chl–0.1% CHS.
625
626 **Fig. 3** Inactivation of *Salmonella enterica* as a function of incubation time when bacteria were treated
627 by: photoactivated 1.5 × 10^{-5} M Chl (light dose 38 J cm^{-2}), 0.1% CHS, and photoactivated 1.5 × 10^{-5}
628 M Chl–0.1% CHS complex (light dose 38 J cm^{-2}) in saline. Every point is the average of 3–6
629 experiments, and error bars sometimes are too small to be more visible.
630
631 **Fig. 4** Scanning electron microscopy image of *Salmonella enterica* Serovar Typhimurium strain
632 DS88 (SL 5676 Smr pLM2) cells after treatment by 1.5 × 10^{-5} M Chl–0.1% CHS complex: not
633 treated control (A), treated bacteria (B).
634
635 **Fig. 5** Effects of photoactivated 1.5 × 10^{-5} M Chl–0.1% CHS complex on the leakage of UV-
636 absorbing materials at 260 and 280 nm of *S. enterica* (20 min and 30 min illumination, light doses 25
637 J cm^{-2} and 38 J cm^{-2} respectively). Every point is the average of 3 experiments.
638
639 **Fig. 6 A:**Inactivation of *Salmonella enterica* Serovar Typhimurium strain DS88 inoculated on the
640 surface of strawberry by Chl-based photosensitization: 405 nm light (light dose 38 J cm^{-2}) and
641 photoactivated 1.5 × 10^{-5} M Chl (light doses 19 and 38 J cm^{-2}); (B): Inactivation of *Salmonella*
642 *enterica* Serovar Typhimurium strain DS88 inoculated on the surface of strawberry by 1.5 × 10^{-5} M
643 Chl–0.1% CHS complex: 0.1% CHS (incubation time 30 min), Chl-CHS dark toxicity (incubation
644 time 30 min), and photoactivated 1.5 × 10^{-5} M Chl–0.1% CHS complex (light dose 38 J cm^{-2},
645 incubation time 30 min). Every point is the average of 3 experiments, dark toxicity and 405 nm light
646 show no significant difference from control ($p>0.005$).
647
648 **Fig. 7** Comparative analysis of different antimicrobial tools: efficiencies of inactivation of yeasts/
649 moldss on the surface of strawberries (405 nm light (light dose 38 J cm^{-2}), dark toxicity of 1.5 × 10^{-5}
650 M Chl–0.1% CHS complex (incubation time 30 min), 0.1% CHS (incubation time 30 min),
651 photoactivated 1.5 × 10^{-5} M Chl (light dose 38 J cm^{-2}) and photoactivated 1.5 × 10^{-5} M Chl–0.1%
652 CHS complex (light dose 38 J cm^{-2}, incubation time 30 min). Every point is the average of 3–6
653 experiments, dark toxicity and 405 nm light show no significant difference from control, ($p>0.005$).
654
655 **Fig. 8** Visual quality of strawberries 4 days after treatment: control berries (A); berries coated with
656 chlorophyllin-chitosan (dark toxicity) (B), and berries treated by photoactivated chlorophyllin-
657 chitosan (C).
658
659 **Fig. 9** EPR spectra of strawberries: control (A) and (B) treated by photoactivated Chl–CHS
660 strawberries. Mn^{2+} lines (g = 1.87608, 1.92801, 1.98124, 2.03521, 2.09085 and 2.14451).
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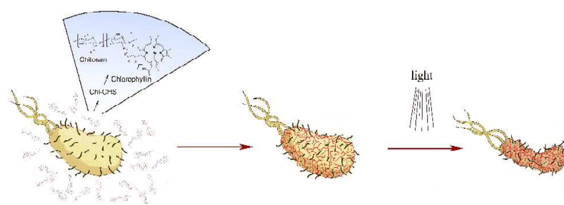

664
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666 **Illustrated abstract**
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