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Toward better microbial safety of wheat sprouts: chlorophyllin-based photosensitization of seeds

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Sprouted seeds are gaining popularity worldwide due to their high nutritional value. At the same time, they are among the most highly contaminated fresh produce and have been recognized as the primary source of food-borne pathogens, such as *E. coli* O157 and harmful microfungi. The antifungal and antibacterial properties of chlorophyllin-based photosensitization *in vitro* together with successful application of this treatment for microbial control in wheat sprouts have been investigated. First, we examined the antimicrobial efficiency of chlorophyllin (Chl, 1.5×10^{-5}–5×10^{-3} M) activated *in vitro* by visible light (405 nm, radiant exposure: 18 J cm^{-2}) against the food-borne pathogen *Escherichia coli* and plant pathogen *Fusarium oxysporum*. Results revealed that this treatment (1.5×10^{-5} M Chl, incubation time 1 h, 405 nm, radiant exposure: 18 J cm^{-2}) can reduce the *E. coli* population by 95%. Moreover, at higher chlorophyllin concentrations (5×10^{-4}–5×10^{-3} M Chl), it is possible to delay the growth of *F. oxysporum* by 51–74%. The decontamination of wheat seeds by chlorophyllin-based photosensitization (5×10^{-4} M Chl, 405 nm, radiant exposure: 18 J cm^{-2}) remarkably reduced the viability of surface-attached mesophilic bacteria (~2.5log CFU g^{-1}), *E. coli* (~1.5log CFU g^{-1}) and yeasts/fungi (~1.5log CFU g^{-1}). Moreover, SEM images confirmed that this treatment did not damage the grain surface microstructure. Most importantly, Chl-based photosensitization did not reduce the seed germination rate or seedling growth and had no impact on the visual qualities of sprouts. In conclusion, the chlorophyllin-based photosensitization treatment, being nonthermal, environmentally friendly and cost-effective, has huge potential for microbial control of highly contaminated germinated wheat sprouts and seeds used to produce sprouts, especially in organic farming.

1. Introduction

The consumption of non-thermally processed and ready-to-eat fresh produce seems a fashionable choice, nowadays. For instance, sprouted seeds are gaining popularity across the world due to their high amounts of proteins, phenols, vitamins and important microelements.[1]

Simultaneously, germinated seeds are among the most highly contaminated fresh produce and have been recognized as the primary source of food-borne pathogens such as *Escherichia coli* O157, *Bacillus cereus*, and many serotypes of *Salmonella*.[2,3] A multistate outbreak of *E. coli* O157:H7 infections occurred in the United States in 1997.[4] Later, during 1998–2010, 33 outbreaks from sprouts were documented in the United States, affecting 1330 reported people.[5] A large outbreak of hemolytic–uremic syndrome caused by Shiga-toxin-producing *E. coli* O104:H4 occurred in Germany in May 2011. This outbreak was associated with the consumption of fenugreek sprouts produced in Egypt and regrettably caused 54 deaths in several countries in Europe and North America.[6–8] Moreover, highly contaminated sprouts can be an issue of bioterrorism.[9]

Zahoranová *et al.*[10] showed that wheat (*Triticum aestivum*) grains were contaminated not just with food-borne pathogens, but also with filamentous fungi (*Aspergillus clavatus*, *A. flavus*, *A. niger*, *Fusarium nivale*, *F. culmorum*, *Trichothecium roseum*, and *Penicillium* spp.). In one of our previous studies, we identified more than 10 different microfungi families on wheat grains.[11]

After a series of outbreaks associated with sprouts in the mid-1990s, the U.S. Food and Drug Administration (FDA) published guidelines[12] that recommended the use of calcium hypochlorite-based seed decontamination technology.[5] Now, after 20 years, the current consumer gives preference to organic food, which cannot be treated with chemical agents. In this context, innovative, non-chemical, sustainable and effective antimicrobial strategies are highly requested.

To this end, photosensitization is an effective antimicrobial technique against a wide range of different microorganisms

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and can be applied in a wider arena.[13,14] It has been shown that photosensitization treatment is effective even against resistant microbial forms, such as bacterial spores and biofilms, and is not mutagenic or genotoxic.[15–17] This light-based technology is based on the interaction of photosensitizer and visible light, in the presence of oxygen induced reactive oxygen species (ROS) and singlet oxygen ($^1O_2$). Eventually, intracellularly-produced ROS and $^1O_2$ kill the target microorganism without any damage to the surrounding matrix.[18–21]

One prominent and well described group of photosensitizers are porphyrins. Amongst them, chlorophyll is probably the one with the highest abundance and therefore is the cheapest. The removal of the phytyl tail of chlorophyll transforms it to water-soluble chlorophyllin (Chl), which is approved as a natural food additive (E140). Owing to its high biocompatibility, Chl is widely applied as a food colorant and food additive in dietary supplements as well as in cosmetics.[22]

In the current study we wanted to take a closer look at the possible use of Chl-based photosensitization for microbial control on highly contaminated wheat sprouts. Thus, the *in vitro* inactivation of prevalent bacteria and fungi, decontamination efficiency on wheat sprouts, evaluation of seed surface microstructure and eventually the examination of seed germination parameters were analyzed.

2. Materials and methods

2.1. Wheat seeds

Naturally contaminated wheat (*Triticum aestivum*) grains were obtained from local farmers and used for experiments.

2.2. Microorganism cultures

Fusarium oxysporum (family *Nectriaceae*) and *Escherichia coli* B were obtained from Vilnius University Life Sciences Centre culture collection.

2.3. Measurements of chlorophyllin absorption and fluorescence

Non-copperized chlorophyll sodium salt (Chl) was obtained from ROTH (Karlsruhe, Germany). Absorption spectra of investigated chlorophyllin solutions were recorded by a spectrophotometer (Heλios Gamma and Delta spectrophotometers, ThermoSpectronic, Leicestershire, Great Britain), while the fluorescence intensity was measured by a fluorescence spectrophotometer (PerkinElmer LS 55, Germany). Scan range parameters were as follows: excitation wavelength, 405 nm; emission, 550–750 nm; ex slit, 10 nm; em slit, 4 nm; scan speed, 200 nm min^{-1}. A 1 cm quartz cuvette (Hellma-analytics QS, Mullheim, Germany) was used for the measurement of optical properties of Chl.

2.4. LED-based light source for the inactivation of microorganisms and decontamination of sprouts

Since the Chl absorption maximum is 405 nm, for the construction of a light source, InGaN light emitting diodes (LED) with an emission maximum at 405 nm and bandwidth of 13 nm at full-width half maximum (LED Engine, San Jose, USA; Inc. LZ1-00UA00) were used. The system consisted of an illumination chamber and supply unit. A cooling system was integrated into the light prototype to dissipate heat from the chamber and minimize any heat transfer to the sample. Two rectangular 6 × 10 arrays (top and bottom) consisting of 60 LEDs powered by a 20 V DC power supply were integrated into the chamber. The light irradiance at the surface of samples reached 9.6 mW cm^{-2} (6 cm from the light source). Light irradiance was measured by a 3 Sigma "Coherent" power and energy meter (California, USA) equipped with pyroelectrical detector J25LP04 (199 nm–700 nm; measurement accuracy ±2%). Light radiant exposure was calculated as light irradiance multiplied by illumination time. The sample exposure time was adjusted according to the equation:

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

where E is the energy density (radiant exposure) in J cm^{-2}, P is the irradiance (light intensity) in W cm^{-2}, and t is the time in seconds.

2.5. *E. coli* growth conditions

E. coli was maintained at 37 °C for 24 h on Luria–Bertani agar (LBA; Liofilchem, Roseto degli Abruzzi, Italy). Before experiments, the bacterial culture was grown overnight (about 16 h) at 37 °C in 20 mL of Luria–Bertani medium (LB; Liofilchem), with agitation at 120 rpm (Environmental Shaker – Incubator ES – 20; Biosan, Riga, Latvia). Afterwards, the bacterial culture was diluted 20 times with fresh LB medium (OD_{540} = 0.164) and grown at 37 °C in a shaker (120 rpm) to ~1 × 10^8 CFU mL^{-1} (OD_{540} = 0.9). Prior to the experiments, the cell concentration was determined optically in a 10.01 mm cuvette at 540 nm before cells were diluted in LB as required. The optical density of growing bacteria was determined using a Helios Gamma spectrophotometer. Cells were then harvested by centrifugation (10 min, 3420*g*) (Mikro 200, Hettich zentrifugen, Germany), resuspended in 0.9% NaCl to ~10^7 CFU mL^{-1} and used for further experiments on the inoculation of wheat seeds and inactivation *in vitro*.

2.6. *E. coli* inactivation by chlorophyllin-based photosensitization *in vitro*

A 20 mL aliquot of bacterial suspension (~1 × 10^7 CFU ml^{-1} in 0.9% NaCl) with 1.5 × 10^{-5} M Chl was incubated in the dark at 37 °C for 1 h (120 rpm). Double negative control (no photosensitizer, no light) and light only samples were incubated with 0.9% NaCl under the same conditions. Afterwards, 150 μL aliquots of bacterial suspension were withdrawn, placed into sterile flat bottom wells and illuminated with 405 nm wavelength light for 10–50 min (radiant exposure: 6–30 J cm^{-2}). The double-negative control sample was not illuminated. In all cases, samples for dark toxicity were covered with aluminum foil. The antibacterial effect of Chl-based photosensitization was evaluated by the spread plate method (100 μL of appropri-

ate dilutions of bacterial suspensions were surface inoculated on LBA plates and kept at 37 °C for 24 h). The surviving cell population was enumerated and expressed in percentage in relation to the double negative control.

2.7. Fusarium oxysporum growth delay by photoactivated chlorophyllin *in vitro*

Fusarium oxysporum was maintained on potato dextrose agar (PDA; Sigma-Aldrich, St Louis, USA). Two concentrations of Chl, 5×10^{-4} M and 5×10^{-3} M, were tested. Appropriate amounts of Chl were added to the autoclaved and cooled (about 50 °C) PDA medium and this mixture was plated in a Petri dish (90 × 15 mm). Double negative and light only control samples contained PDA only. Each dish was inoculated in the center with an agar disk (diameter 5 mm) bearing mycelium growth from a 7-day-old *F. oxysporum* culture. After 24 h incubation, the tested plates containing Chl (except dark toxicity control) were exposed to 18 J cm^{-2} (30 min) 405 nm light. Afterward, both treated and control dishes were sealed with parafilm to avoid the evaporation of volatile compounds and incubated at 25 °C. Diameters of colonies were measured every day until the control plates were completely covered with mycelia, expressed as percentages. Each treatment (all concentrations for each treatment) was repeated 3 times.

2.8. Inactivation of mesophilic bacteria and *E. coli* by photoactivated chlorophyllin

Wheat seeds were divided into 4 groups (100 seeds per group). Two groups were soaked in 5×10^{-4} M Chl solution in the dark for 1 h. "Control" group and "just light" group were soaked in 0.9% NaCl solution in the same conditions. After soaking, the wheat seeds were dried and then one group was exposed to light for 30 min (radiant light exposure: 18 J cm^{-2}). In our previous study, the temperatures inside the chamber and on the surface of the treated sample were measured during the entire illumination time. It was found that the temperature never exceeded 30 °C.[23] The other two groups were not illuminated. In all cases, samples for "dark toxicity" were covered with aluminum foil.

After treatment, all samples (including control) were separately mixed with appropriate volumes of 0.9% NaCl (1 g of sample: 9 ml solution) and homogenized for 60 s in sterile BagPage bags using a BagMixer (model MiniMix 100 VP, Interscience, Saint Nom La Bretèche, France). The homogenate was then serially diluted in 0.9% NaCl and placed on Luria–Bertani (LB) agar. Plates were incubated at 37 °C for 24 h. The surviving mesophilic bacteria were enumerated and expressed as $\log_{10}$ (CFU g^{-1}).

For experiments with *E. coli* inoculated on grains, the seeds were also divided into 4 groups (100 seeds each) and soaked in prepared *E. coli* ($\sim 10^7$ CFU ml^{-1} in 0.9% NaCl) inoculum (described above in 2.5) for 30 min at room temperature for better attachment onto the surface of seeds. Afterward, the same experimental protocol as for mesophilic bacteria was performed. All experiments were repeated three times.

2.9. Inactivation of seed surface – attached fungi by Chl-based photosensitization

For the antifungal test, wheat samples were divided into 4 groups (100 seeds per group). Two groups were incubated in the dark with the 5×10^{-4} M Chl solution for 1 h and the double negative control group and "light only" group were incubated with 0.9% NaCl. After incubation with Chl, all samples were dried at room temperature for 20 min, placed in the treatment chamber and exposed to light for 30 min at λ = 405 nm. The control and "dark toxicity" samples were not illuminated. After treatment, all samples (including all control samples) were separately mixed with appropriate volumes of PBS (1 g of sample: 9 mL solution) and homogenized for 60 s in sterile BagPage bags using a BagMixer. The homogenate was then serially diluted in PBS and placed on potato dextrose agar (PDA). Plates were incubated at 25 °C for 5 days. The surviving fungi were enumerated and expressed as $\log_{10}$ (CFU g^{-1}). No growth of bacteria was observed in these experimental conditions. The experiment was repeated 3 times.

2.10. Evaluation of grain microstructure

The grain microstructure was evaluated with a TM-1000 scanning electronic microscope (SEM) (Hitachi, Tokyo, Japan). The main parameters were as follows: accelerating voltage 15 kV, emission current 57.9 mA, pixel size 122.4 nm, magnification 1500. For this investigation, all grains were divided into 4 groups: double negative control, dark toxicity (Chl), light only and Chl-based photosensitization. Grains for "double negative control" and "dark toxicity Chl" experiments were not illuminated, grains for "light only" were only illuminated (36 J cm^{-2}), and grains for "Chl-based photosensitization" treatment were both incubated with Chl and illuminated (5×10^{-4} M Chl, 36 J cm^{-2}). Grains from all groups were covered with gold before SEM examination using a scanning electron microscope (SEM) EVO 50 (LEO Electron Microscopy Ltd, Cambridge, UK) with an SE (second electron) detector.

2.11. Evaluation of wheat seed germination and viability

Wheat seeds were divided into 4 groups (100 seeds per sample). Untreated (double negative control) and "light only" seeds were soaked in PBS (ratio of 1 : 4 by mass) for 12 h. The other two groups of seeds were soaked in 5×10^{-4} M Chl for 1 h. Then, the treated sample and "light only" sample were illuminated with 405 nm light for 1 h and soaked in PBS for 11 h. The "dark toxicity" sample was not illuminated; after incubation for 1 h in Chl solution, the seeds were transferred into PBS for 11 h. After treatment, all seeds (100 seeds in each sample) were germinated on cotton in Petri plates in the dark at a temperature of 25 °C for 5 days. The rate of germination was calculated every day by counting the percentage of germinated seeds. The growth of sprouts was evaluated by measuring the lengths of seedling and root of germinated seeds for 5 days after treatment. The experiment was reproduced 3 times.

2.12. Statistical analyses

All analytical experiments were carried out in triplicate. Data were subjected to analysis of variance (ANOVA) using the statistical package SPSS for Windows. Calculated mean values were compared using Duncan's multiple range test with significance defined at $p < 0.05$.

3. Results

3.1. Absorption and fluorescence spectra of chlorophyllin

Chlorophyllin, due to three COOH groups, is a hydrophilic molecule, so its absorption spectra were recorded in NaCl solution. The left side of Fig. 1A presents the dependence of Chl absorbance on its concentration (1.5×10^{-5}–3×10^{-7} M) in the visible region of the spectrum. It is obvious that the absorption maximum of Chl is at 405 nm. Thus, the light source was constructed to emit light at a 405 nm wavelength in order to get an optimal excitation of Chl.

Afterward, the fluorescence intensity of Chl in the solution was investigated as well. The fluorescence spectrum presented in Fig. 1 is typical for chlorophyllin. It is clear that the Chl fluorescence peak is around 660 nm and differs depending on Chl concentration, which means that, with concentration increase, some aggregation took place due to π–π interactions of the tetrapyrrol structures of Chl molecules. As evidence, the addition of 20 μL Triton X-100, which is usually used to disassemble aggregated molecules of Chl, significantly changed the fluorescence intensity of the investigated molecules. Increased fluorescence intensity and a slight red shift of fluorescence maximum indicated that equilibrium "aggregated-monomeric" molecules changed and most aggregated chlorophyllin molecules turned into monomers.[24]

3.2. Antibacterial effect of Chl-based photosensitization *in vitro* against *E. coli*

Data presented in Fig. 2 show the inactivation curve of *E. coli* population treated by Chl-based photosensitization. In control

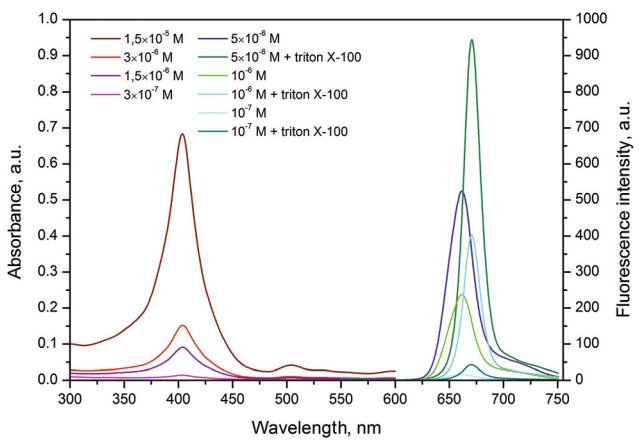

Fig. 1 Absorption and fluorescence spectra of Chl depending on concentration.

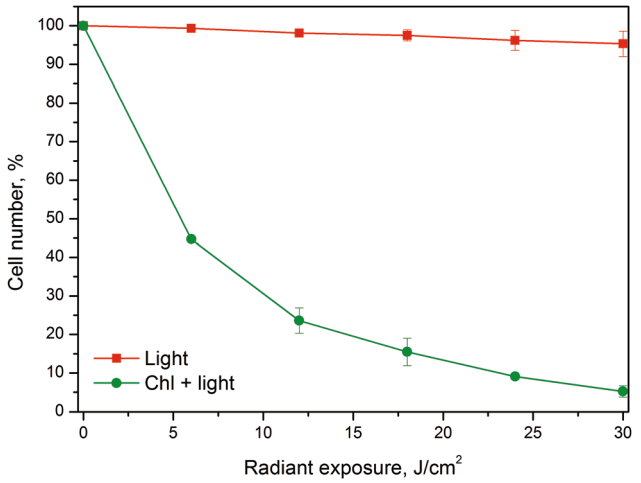

Fig. 2 Inactivation of *E. coli* by Chl-based photosensitization (1.5×10^{-5} M Chl, incubation time: 1 h, radiant exposure: 30 J cm^{-2}).

treatments (no photosensitizer, no light), the viability of *E. coli* reached 7log CFU mL^{-1} and was equated to 100%. It is clear that light up to 30 J cm^{-2} did not practically change the viability of bacteria. Chlorophyllin alone (dark toxicity) at this concentration (1.5×10^{-5} M) also had no impact on the viability of bacteria. Viability was reduced when *E. coli* was incubated with Chl and afterwards exposed to light, depending on the radiant exposure. After 10 min of illumination (radiant exposure 6 J cm^{-2}), a viability reduction of ~55% was observed, while Gram (+) bacteria in these experimental conditions were inactivated 100%.[25] It is important to note that at a long exposure time (50 min, 30 J cm^{-2}) and quite high concentration of photosensitizer (1.5×10^{-5} M), it is possible to achieve almost 95% reduction of viable Gram (–) *E. coli* population.

3.3. Delay of *Fusarium oxysporum* mycelium growth by Chl-based photosensitization *in vitro*

Fig. 3 shows that *F. oxysporum* growth can be delayed after Chl-based photosensitization treatment. No impact on the growth of fungus was found when *Fusarium* was only illuminated (18 J cm^{-2}) or only incubated with Chl (5×10^{-3} M). It was observed that the delay of growth depended on the Chl concentration used. For instance, when the fungus was incubated with 5×10^{-4} M Chl and afterwards illuminated (18 J cm^{-2}), its post-treatment growth on the 2nd day was delayed about 51%, while a 10-fold higher Chl concentration (5×10^{-3} M) and the same radiant exposure (18 J cm^{-2}) delayed the growth of *F. oxysporum* about 74%.

3.4. Decontamination of wheat seed from mesophilic bacteria, *E. coli* and fungi by Chl-based photosensitization

Data obtained in this study indicated that wheat grains were highly contaminated with mesophilic bacteria. As depicted in Fig. 4A, in control seed samples, mesophilic contamination reached almost 6log CFU g^{-1}. Chl alone (dark toxicity) (5×10^{-4} M) only slightly changed (just 0.4log CFU g^{-1} reduction)

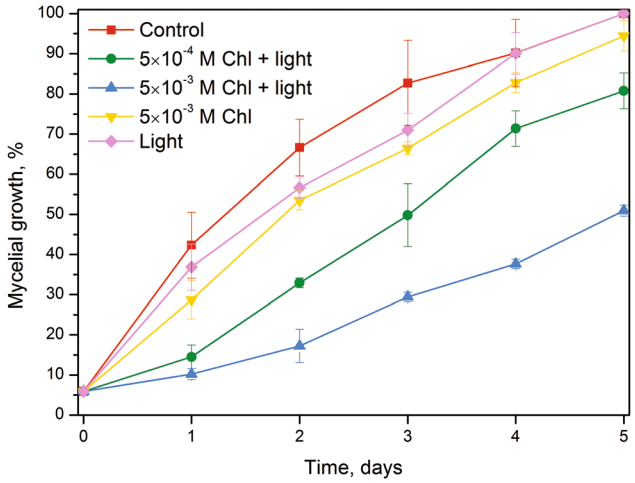

Fig. 3 *Fusarium oxysporum* mycelium growth delay after Chl-based photosensitization (radiant exposure: 18 J cm^{-2}).

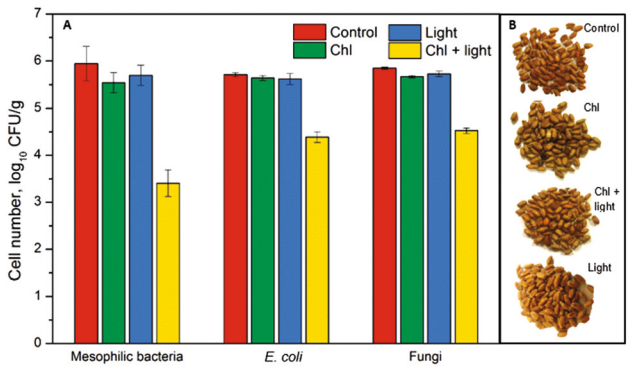

Fig. 4 (A) Inactivation of *E. coli*, mesophilic bacteria and fungi on the surface of wheat grains and (B) visual appearances of treated and control wheat seeds (5×10^{-4} M Chl, incubation time: 1 h; radiant exposure: 18 J cm^{-2}).

the microbial load on grains. Light only (18 J cm^{-2}) had no impact on bacterial counts. Only photosensitization treatment (5×10^{-4} M Chl, radiant exposure: 18 J cm^{-2}) abundantly reduced (~2.5log CFU g^{-1}) the population of mesophilic bacteria on the surface of grains.

Note that the population of *E. coli* inoculated on wheat grains was diminished by this treatment as well. In control wheat grains, *E. coli* contamination reached almost 6log CFU g^{-1}. Treatment of grains with Chl alone (1 h incubation in 5×10^{-4} M Chl, without light (dark toxicity) or with light only (18 J cm^{-2})) did not considerably reduce the *E. coli* population on grains, whereas the photosensitization treatment reduced the population of inoculated *E. coli* by ~1.5log CFU g^{-1}.

3.5. Inactivation of microfungi of wheat grains by Chl-based photosensitization

Data presented in Fig. 4 clearly indicate that after treatment with 5×10^{-4} M Chl-based photosensitization, it is possible to achieve significant reduction of fungal population on wheat grains. In double negative control or light only grains (untreated), the fungal population reached about 6log CFU g^{-1}. Treatment with Chl without light had no impact on the inactivation of fungi; however, after incubation with Chl and illumination with 405 nm light, the number of colony forming units reduced by almost 1.5log.

In order to evaluate possible treatment effects on wheat seeds, SEM images of wheat grain surfaces were analysed. As presented in Fig. 5A, the surface of control wheat seeds is rather contaminated with microfungi. Chl-based photosensitization reduced the microfungal grain surface contamination without destructive effects on the surface microstructure (Fig. 5D), while Chl without light (dark toxicity) and light only treatments had no antifungal effect (Fig. 5B and C).

3.6. Evaluation of wheat seed germination

The rate of germination of wheat seeds was observed 5 days after Chl-based photosensitization treatment. Data presented in Fig. 6 indicate that the germination process in control seeds reached 78% on the 2nd day and 90% on the 3rd day. Treatment of seeds with Chl alone (5×10^{-4} M) and only light lightly delayed the germination rate, which reached 88–93% on the 4–5th days. Much faster germination was observed in seeds that were treated by photosensitization, since on the 2nd day, their rate of germination reached 95%. Thus, it seems that this treatment slightly activated the germination process.

In the next step, the growth of sprouts was examined by measuring the lengths of root and shoot. As depicted in Fig. 7, the treatment of seeds with Chl-based photosensitization did not change the growth of root or shoot in comparison with control. For instance, the growth of roots (~13.7–13.8 cm) and

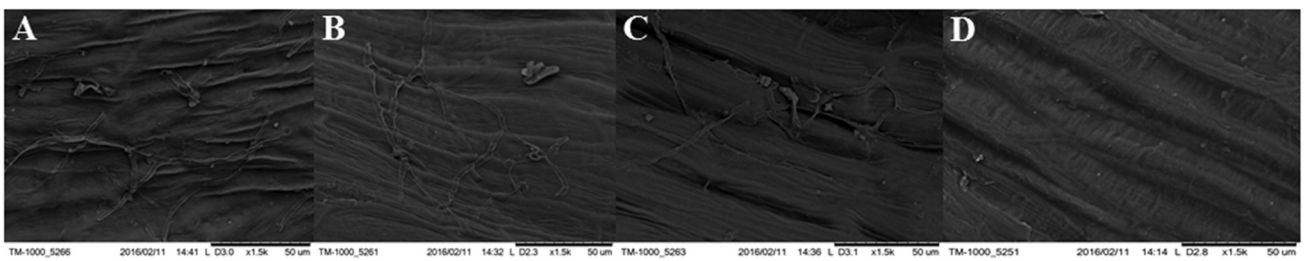

Fig. 5 SEM images of wheat grain surface: (A) double negative control sample, (B) dark toxicity, (C) light only control, and (D) treated with Chl-based photosensitization.

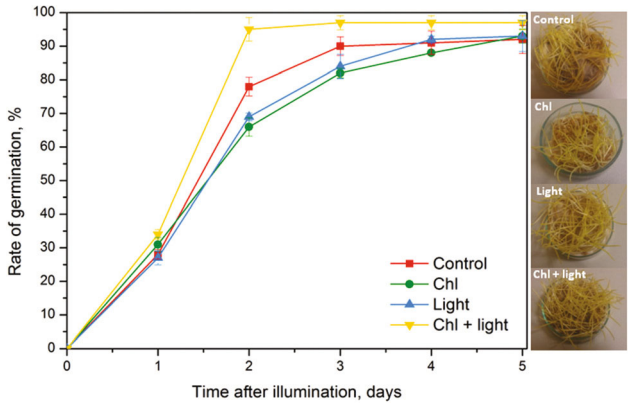

Fig. 6 Wheat seed germination after 5 × 10^{-4} M Chl-based photosensitization (incubation time: 1 h; radiant exposure: 18 J cm^{-2}).

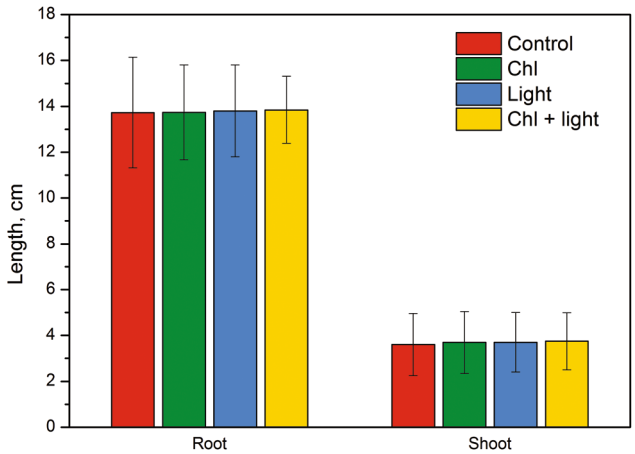

Fig. 7 Growth of root and shoot after treatment of wheat seeds by 5 × 10^{-4} M Chl-based photosensitization (incubation time: 1 h; radiant exposure: 18 J cm^{-2}).

shoots (~3.6–3.7 cm) remained the same as in all control seed groups, which is very important.

Despite the fact that after Chl-based photosensitization a higher percentage of seeds germinated and the germination process was faster, this treatment does not affect the growth of sprout shoot or root.

4. Discussion

One of the conventional sprout decontamination tools is U.S. FDA-approved ionizing irradiation. Besides being a high-cost antimicrobial treatment, it can cause the mutations of pathogens and could create new resistant strains.[26] Previously, Fan *et al.*[27] used ionizing radiation for the decontamination of different seeds. Obtained data indicate that 7 kGy irradiation reduced *E. coli* on mung bean, clover and fenugreek seeds by 5log CFU g^{-1}, but significantly inhibited seed germination rates.

The usage of chemical antimicrobial technologies is always accompanied by increased health risks due to the carcinogenic and teratogenic effects they can induce.[28,29] For instance, when hypochlorous acid, a substance present in NaOCl solution, comes into contact with an organic tissue, it acts as a solvent and releases chlorine, which interacts with protein amino groups and forms chloramines that interfere in the cell metabolism.[30] Hypochlorous acid ($HOCl^-$) and hypochlorite ions (OCl^-) lead to amino acid degradation and hydrolysis.[31] Additionally, chlorine used at high concentrations can react with organic materials, resulting in the formation of trihalomethanes, haloketones, chloropicrins, and haloacetic acids.[32] Some authors still investigate the antimicrobial efficiency of different chemical agents. For instance, Chiu & Sung[33] found that the decontamination of peas (*Pisum sativum*) by NaOCl (1 g L^{-1}), vinegar (350 mL L^{-1}), ethanol (350 mL L^{-1}) and NaCl (10 g L^{-1}) reduced the populations of aerobic bacteria by 0.34–1.57 log CFU g^{-1} and *E. coli* by 0.15–1.04 log CFU g^{-1}. However, recently, chemical chlorine-based treatment is less popular, due to concerns about its huge impact on public health and environment. Due to the risks posed by the use of chlorine in the food industry, the use of these compounds is forbidden in European countries such as the Netherlands, Sweden, Germany, and Belgium.[34] Moreover, chemical technologies are not consistent with the production of organic sprouts.

An emerging non-thermal and non-chemical food preservation technology that proposes the decontamination of surfaces by intense and short duration (microseconds) pulses of broad spectrum light (200–1000 nm) and was approved for food surface decontamination by the Food and Drug Administration in 1999 is high power pulsed light (HPPL).[35] It offers effective inactivation of pathogens, limited energy costs, and short exposure time. This treatment was very effective against all food-borne pathogens *in vitro*,[36] but less effective in the decontamination of fruits and vegetables: it reduced naturally distributed mesophilic bacteria by 1.0–1.3 log CFU g^{-1} and inoculated *Bacillus cereus* by 1.3–2.0 log CFU g^{-1} depending on the surface. The main disadvantage of this treatment is that higher treatment efficiencies induced thermal effects on the food matrix.[37,38]

Evidence on the photosensitizing activity of chlorophyllin *in vitro* has been obtained in our previous studies.[25,39–42] Later, several studies were published on the successful application of Chl-based photosensitization for the decontamination of food-related surfaces, fruits (strawberries, apricots, plums) and vegetables (cauliflower, tomatoes, basil).[23,40,41] In this recent study, we tried to apply chlorophyllin-based photosensitization for the microbial control of sprouts. Thus, the susceptibility of the prevalent food pathogen *E. coli* to Chl-based photosensitization was first evaluated. Data presented in Fig. 2 indicate that, in these experimental conditions, *E. coli* can be inactivated by 95%.

Fusarium oxysporum is a ubiquitous plant pathogen, the most widely dispersed member of the genus *Fusarium*, which causes significant problems in many crops. It was shown that fungus damages the epidermal layer of germinated wheat.[43] Data obtained in Fig. 2 showed that *F. oxysporum* growth can

be delayed by Chl-based photosensitization. It was observed that the delay of growth rate (52–75%) depended on the concentration of Chl (5×10^{-4}–5×10^{-3} M).

Data obtained in this study indicated that wheat grains were highly contaminated with mesophilic bacteria. As depicted in Fig. 4A, after the treatment of grains with Chl (dark toxicity) (5×10^{-4} M), a slight (0.4log CFU g^{-1}) reduction of microbial load was observed. Just photosensitization treatment (18 J cm^{-2}) markedly reduced (~2.5log CFU g^{-1}) the population of mesophilic bacteria. The dark toxicity of Chl alone (5×10^{-4} M, 1 h incubation) had no impact on the growth of grain surface inoculated *E. coli*, whereas photosensitization treatment reduced the population of *E. coli* by ~1.5log CFU g^{-1}. It is important to note that the Chl concentration used (5×10^{-4} M) was elaborated as a "minimal working concentration" that did not change the color or visual quality of wheat grains (Fig. 3B). Moreover, it is important to note that in our previous work it was shown that photosensitization is a more effective antimicrobial treatment than hypochlorite.[25,39] In addition, the efficiency of chlorophyllin-based photosensitization is comparable with that of the high power pulsed UV light[36,38,42] (which is FDA approved) and its cost-efficiency is more attractive.

To increase the antimicrobial efficiency, the photosensitization treatment can be combined with other antimicrobials. It is generally proven that two or more inhibition and inactivation methods at suboptimal levels are more effective than one. In our previous study, combined treatment by photosensitization and pulsed light effectively inactivates (6.7–7 log CFU g^{-1}) both the Gram-positive and the more resistant to photosensitization Gram-negative bacteria.[44] Another investigation showed that Chl combined with high power pulsed UV light reduced rather resistant Gram (–) *Salmonella* by 7.5log CFU g^{-1}. Moreover, Chl-based photosensitization can be successfully combined with other antimicrobial tools described in the literature.[45–48] These treatment combinations in the presence of light drastically reduced Gram (+) and Gram (–) pathogens to undetectable levels.

Organic cereals of good quality are widely used both in product processing and expansion of organic crop areas. In order to reduce mold on grains, different treatments have been applied. For instance, Chiu & Sung[33] used NaOCl (1 g L^{-1}), vinegar (350 mL L^{-1}), ethanol (350 mL L^{-1}) and NaCl (10 g L^{-1}) to reduce the mold on the seeds of three pea (*Pisum sativum*) varieties and found that all these antimicrobials reduced microfungi by only 0.83–1.43 log CFU g^{-1}.

Cold atmospheric plasma (CAP) treatment is an innovative and effective antimicrobial technology. Despite that, the reduction of fungi on the surface of winter wheat treated by CAP reached only about 1log CFU g^{-1}.[49] It is a pity, but the treatment conditions that resulted in the highest microbial reduction did not match the conditions required for best germination probabilities.[50]

Application of natural compounds, such as essential oils, chitosan, nisin or lysozyme, for microbial control of foods is becoming popular as an alternative to chemical agents. The potential of thyme essential oil as an antifungal preservative was tested against all of the fungal endophytes isolated from winter wheat seeds (*Alternaria alternata*, *A. infectoria*, *Aspergillus flavus*, *Epicoccum nigrum* and *Fusarium poae*). The obtained data indicate that thyme essential oil exhibited rather low fungitoxicity (15–85% depending on concentration) against all the tested fungi.[51] Moreover, biological antimicrobial control is hampered not just by low antimicrobial efficiency but also by a significant impact on organoleptic properties.[52]

In one of our previous studies, first attempts to use ALA-based photosensitization for the decontamination of wheat grains were made. The obtained data indicated that the viability of such micromycetes as *Alternaria* spp., *Penicillium* spp., and *Mucor hiemalis* on the surface of wheat grains was reduced by 75% after the ALA-based photosensitization treatment.[11]

In another study,[53] the antimicrobial activity of a new photosensitizer—cationic curcumin derivative (SACUR-3) (not yet approved as a food additive)—was investigated for the decontamination of plant surfaces from inoculated *E. coli*. The obtained data look promising, since the antimicrobial efficiency of SACUR-3-based photosensitization (100 μM, SACUR-3; 435 nm, 33.8 J cm^{-2}) on decontaminating fenugreek seeds and mung beans, which were rotated during treatment, reached 1–5.6 log CFU g^{-1}. Such efficiency may be achieved due to the rotation of seeds during light exposure, since the decontamination of mung bean germlings with more complex geometry was ineffective with a two-dimensional light source.[53]

Data presented in Fig. 4 indicated that in control grains (untreated), the total number of fungi reached almost 6log CFU g^{-1}. Treatment with Chl without light had no impact on the inactivation of fungi; however, after illumination with 405 nm wavelength light, the growth inactivation reached almost 1.5log CFU g^{-1}. To evaluate possible treatment effects on wheat seeds, SEM images of the wheat grain surface were analysed. As presented in Fig. 5A–D, Chl-based photosensitization reduced the grain surface microfungal contamination without destructive effects on its microstructure.

Germination of seeds is the most important physiological parameter that must not be inactivated by decontamination treatment. For instance, much work has been done on the application of ionizing radiation for microbial control of seeds. The obtained data revealed that ionizing radiation is one of the most effective antimicrobial tools, but at doses higher than 0.19 kGy it significantly reduced wheat seed germination compared with that of untreated seeds.[54] In addition, Fan *et al.*[27] confirmed the results of the above author and demonstrated that gamma irradiation doses higher than 4 kGy negatively impacted seed (mung bean, clover, fenugreek) germination and yield of sprouts.

In comparison, treatment of different seeds (alfalfa, broccoli, kohlrabi, kyona, mustard, red kohlrabi, red radish, red young radish, tatsoi and violet radish) with aqueous chlorine dioxide (200 mg mL^{-1}, 5 min) reduced their germination rate by 1–22%.[55]

Some authors indicated that cold plasma increased the germination of seeds by almost 10%.[56] Despite this, Zahoranová *et al.*[10] showed that only a short exposure time of plasma posi-

tively affects germination, since higher exposures drastically inhibited all measured growth parameters in comparison with untreated seeds: germination rate decreased by 14–24%, fresh and dry weights by 18–26%, and vigor index by 29–44%. These tendencies were confirmed by Los *et al.*[57] In addition, longer plasma treatment can slightly reduce the seeds' water content (from 10.48% to 9.35%).[50]

As previously described, thyme essential oil works as an antifungal agent, but the germination rates of the treated grains exhibited differential susceptibility to this treatment and can be reduced from 1% to 98%.[51]

The rate of wheat seed germination for 5 days after Chl-based photosensitization treatment is presented in Fig. 6. It is clear that the germination process in control seeds reached about 80% on the 2nd day and reached 90% on the 3rd day. The treatment of seeds with Chl alone (5×10^{-4} M) lightly delayed the germination rate, but on the 4–5th days, it reached about 90% as well. Much faster germination was observed in seeds treated by photosensitization, as on the 2nd day their vigor of germination reached 95%. Thus, it seems that this treatment slightly activated the germination process.

In the next step, the growth of sprout root and shoot was examined. As is depicted in Fig. 7, the treatment of seeds with Chl-based photosensitization did not change the growth of root or shoot in comparison with control. For instance, the growth of roots (~13.7 cm) and shoots (~3.7 cm) remained the same as in control seed group, which is important.

5. Conclusions

The germination process of cereal grains can enhance the level of many bioactive compounds and has a huge growth potential for the food industry, since they may be explored not just for ready-to-eat meals or fresh produce, but also for the development of new ingredients and products. Meanwhile, there is no technique that can decontaminate grains in an effective, sustainable and cost-effective way. In this context, Chl-based photosensitization might be a promising antimicrobial strategy for microbial control on wheat sprouts, especially organic ones. Obtained results highlighted that Chl-based photosensitization delayed the growth of *E. coli*, mesophilic bacteria and harmful microfungi on the surface of grains without damage to the surface microstructure or suppression of the seed germination rate or seedling growth.

Conflicts of interest

There are no conflicts to declare.

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