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Innovative Nonthermal Technologies: Chlorophyllin and Visible Light Significantly Reduce Microbial Load on Basil

Egle Paskeviciute, Bernadeta Zudyte and Zivile Luksiene*

Vilnius University, Institute of Photonics and Nanotechnology, Sauletekio 10, 10223 Vilnius, Lithuania

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SUMMARY

Due to the high amount of biologically active compounds, basil is one of the most popular herbs. However, several outbreaks have been reported in the world due to the consumption of basil contaminated with different food pathogens. The aim of this study is to apply nonthermal and ecologically friendly approach based on photosensitization for microbial control of basil which was naturally contaminated with mesophils and inoculated with thermoresistant food pathogen *Listeria monocytogenes* 56Ly. The obtained data indicate that soaking the basil in $1.5 \cdot 10^{-4}$ M chlorophyllin (Chl) for 15 min and illumination with light for 15 min at 405 nm significantly reduced total aerobic microorganisms on basil by 1.3 log CFU/g, and thermoresistant *L. monocytogenes* 56Ly from 6.1 log CFU/g in control to 4.5 log CFU/g in the treated samples. It is important to note that this treatment had no impact on enzymatic activity of polyphenol oxidase and pectinesterase. Results obtained in this study support the idea that photosensitization technique with its high selectivity, antimicrobial efficiency and nonthermal nature can serve in the future for the development of safe nonthermal and environmentally friendly preservation technology for different fruits and vegetables.

Key words: photosensitization, antimicrobial technology, fresh produce, *Ocimum basilicum*

INTRODUCTION

Basil is one of the most popular pot-herbs mostly due to high amount of biologically active compounds, such as volatile oils, tannins, terpenes, flavonoids, glycosides and macro- and microelements. Moreover, for many years basil has been applied as medical herb to treat inflammation (*1*).

However, despite its high antimicrobial activity, due to phenolic compounds (carvacrol, eugenol, linalool, and thymol) (*2,3*), several outbreaks have been reported after consumption of minimally processed foods that included basil (*4-6*). In 2017 European Food Safety Authority (*7*) confirmed that *Listeria monocytogenes* is one of the most often detected pathogens in ready-to-eat food in Europe. For instance, Estonia reported 3.6 % of ready-to--eat products contaminated with *Listeria monocytogenes*. In 2008, the consumption of basil contaminated with *Salmonella enterica* induced outbreak of salmonellosis in Israel and it spread out in four countries (*8*). Moreover, in the United Kingdom, Berger *et al.* (*9*) reported a large outbreak of salmonellosis (55 people) due to consumption of basil. Thus, it is evident that conventional antimicrobial technologies for decontamination of fresh produce are not efficient enough.

FAO and WHO convened an Expert Meeting in 2007 and concluded that from a global perspective, leafy green vegetables currently presented the greatest concern in terms of microbiological hazards (*10*). Moreover, fresh produce contaminated with different pathogens can pose a bioterrorism threat (*11*).

Most recent methods applied for microbial control of foods are thermal or chemical. Both of them are not desirable nowadays, because they induce plenty of uncontrolled chemical

*Corresponding author:
E-mail: Zivile.Luksiene@tmi.vu.lt

ORCID IDs: 0000-0002-4147-1437 (Zudyte), 0000-0003-3544-0252 (Paskeviciute), 0000-0001-6807-2770 (Luksiene)

reactions and sometimes significantly reduce nutritional value of fresh produce (*12*). Inevitably, a new strategy to inactivate pathogenic and harmful microorganisms in cost-effective and environmentally friendly way is highly needed. To this end, modern biophotonic technology based on photosensitization might serve as a promising tool to decontaminate food or food-related surfaces from different foodborne pathogens and harmful microfungi. This inevitably poses questions: What is it? and How does it work? In general, photosensitization is a treatment involving the administration of a photosensitizer (photoactive compound) that selectively accumulates in the target cells. The interaction of two non-toxic elements, photoactive compound and visible light in the presence of oxygen results in a plethora of reactive oxygen species-based cytotoxic reactions and consequently induces selective destruction of target microorganism (*13*). Data obtained in our previous studies clearly indicate that photosensitization might be useful nonthermal and non-chemical tool for inactivation of Gram(+) food pathogens *Listeria monocytogenes*, *Bacillus cereus*, yeasts, moulds and mesophils. It is important to note that microbial spores and biofilms are also susceptible to chlorophyllin (Chl)-based photosensitization (*14-17*). Moreover, Chl-based photosensitization was successfully applied for preservation of strawberries, significantly reducing food pathogens, mesophils and moulds distributed on the surface. Most important is the fact that this treatment can extend the shelf life of strawberries by two days without negative impact on nutritional quality or organoleptic properties (*18*).

The firmness of fruit/vegetable tissues depends on pectic material, which is a matrix component in the plant cell and fibrillar component of the cell wall. Degradation of the pectic material of fruits and vegetables, raw or processed, has been implicated in changes in texture and viscosity of the products. Several processes which have been used in the industry for preservation of fruits and vegetables (blanching, freezing, dehydration, pasteurization) cause irreversible physical damage on cellular tissues (*19*).

One of the biggest challenges in fresh food industry is enzymatic browning of the surface of freshly cut produce (*20*). It is generally considered as detrimental to food quality from both sensory and nutritional points of view. This process depends on the presence of the enzyme polyphenoloxidase (PPO) in freshly cut fruits and vegetables. Enzymatic browning and inhibition of PPO during storage and processing could be controlled with acidic solutions (*20*). Ascorbic acid, L-cysteine and sulfites have been shown to be very good browning inhibitors against PPO (*21*). However, ascorbic acid is a very reactive compound and is rapidly oxidized to dehydroascorbic acid, which can react with other compounds and cause changes in fruit quality. Sulfites, despite their wide effectiveness, have restrictions as a food additive because of their adverse effects on human health. L-cysteine can negatively affect the taste of the products (*20*).

The aim of this work is to apply Chl-based photosensitization for microbial control of fresh produce, for instance basil, which was naturally contaminated with mesophils and inoculated with thermoresistant *Listeria monocytogenes* 56Ly. Additionally, some quality properties associated with enzymatic activity of pectinesterase and polyphenol oxidase were evaluated.

MATERIALS AND METHODS

Plant material and inoculation with L. monocytogenes 56Ly

Sweet basil (*Ocimum basilicum*) was obtained from a local supermarket (Villnius, Lithuania) and used immediately. Thermoresistant strain *L. monocytogenes* 56Ly (kindly provided by Prof M. E. Guerzoni (University of Bologna , Bologna, Italy) was grown at 37 °C and maintained on tryptone soya agar supplemented with 0.6 % yeast extract (TSYEA; Liofilchem, Roseto degli Abruzzi, Italy). Before experiments, the bacterial culture was grown overnight (approx. 14 h) at 37 °C in 20 mL of the same medium as described above with agitation at 120 rpm on an environmental shaker-incubator (ES-20; Biosan, Riga, Latvia). This culture was then diluted 20 times with the fresh medium ($A_{540\,nm}$=0.164) and grown at 37 °C to the mid-log phase (approx. $1.16 \cdot 10^9$ CFU/mL, $A_{540\,nm}$=0.9) in a shaker (120 rpm; ES-20, Biosan). Absorbance of bacterial culture was determined in a 10.01-mm glass cuvette at λ=540 nm (Helios Gamma & Delta spectrophotometers; Thermo Spectronic, Cambridge, UK). Afterwards, the bacterial cells were harvested by centrifugation (20 min, 6 °C, 5000×*g*; MPW-260R; MPW Med. Instruments, Warsaw, Poland) and resuspended to approx. $5.8 \cdot 10^9$ CFU/mL in 0.1 M phosphate-buffered saline (PBS, pH=7.2; Oxoid™, Thermo Fisher Scientific, Waltham, MA, USA). This stock suspension was diluted to approx. 10^7 CFU/mL, poured over basil and left for 30 min at room temperature in the dark for cell attachment. Basil was then dried in a laminar (Ezermester ISZ, Kecskemét, Hungary) at room temperature (21 °C) and prepared for photosensitization.

Light sources for decontamination of basil

LED-based light source for decontamination of basil was constructed in our laboratory. The maximum emitting light of the prototype was 405 nm. The light intensity on the surface of experimental sample (6 cm from the light source) reached 10 mW/cm^2. The light dose delivered to the surface of basil was calculated as light intensity multiplied by time. Light power density measurements were carried out using a light energy 3sigma meter (Coherent, Santa Clara, CA, USA) equipped with pyroelectric detector J25LP04.

Photosensitization treatment

Naturally contaminated or pathogen-inoculated basil was soaked in $1.5 \cdot 10^{-4}$ M chlorophyllin (Chl; Roth, Karlsruhe, Germany) solution for 5 min. The dried basil was placed in the treatment chamber in a sterile uncovered Petri dish and exposed to light intensity of 10 mW/cm^2 at λ=405 nm for 15 min. Control samples were soaked in Chl solution but not illuminated in the chamber.

Evaluation of antimicrobial efficiency of the treatment

All samples (including control) were mixed separately with an appropriate volume of 0.1 M PBS (1 g of sample in 10 mL buffer) and homogenized for 60 s in a sterile BagPage bags using a BagMixer (model MiniMix 100 VP; Interscience, Saint-Nom-La-Bretèche, France) immediately after treatment. The homogenate was then serially diluted in 0.9 % NaCl (Liofilchem) and placed on agar. Total aerobic bacterial and *L. monocytogenes* counts were determined by plating appropriately diluted samples onto TSYEA plates (Liofilchem) and incubated at 30 °C for 48 h. Plates were incubated at 37 °C for 24 h. The surviving cell populations were enumerated in every Petri dish and mean values were expressed as log CFU/g.

Determination of pectinesterase activity

Pectinesterase was determined by titration method based on estimation of free carboxyl groups, which are formed by pectinesterase action in pectin (*22*). A volume of 100 mL of pectin solution (1 %) was added to 500-mL beaker, and then 30 mL of 1 M NaCl and 60 mL water were added. Solution was placed in a water bath (30 °C) and pH was kept at pH=7.5. A volume of 10 mL of enzyme solution was added and alkali consumption was measured. A unit of activity was defined as the amount of enzyme that releases 1 μmol of carboxyl groups per min under the aforementioned conditions. Pectinesterase activity (in U/mL) was calculated using the following formula:

$$\text{Activity}=V(\text{NaOH})\cdot c(\text{NaOH})\cdot 1000/(t\cdot V(\text{titrand})) \quad /1/$$

where c(NaOH)=1 M, V(titrand)=700 mL and t is time.

Determination of polyphenoloxidase activity

Basil extract was obtained by blending basil leaves with equivalent mass of water, which was then centrifuged in MPW-260R centrifuge (MPW Med. Instruments) to remove solids. Then, 2 mL of buffer (pH=7) and 2 mL of 0.1 % catechol were added to 0.1 mL of basil extract. The increase in the absorbance, measured with spectrophotometer (Helios Gamma & Delta spectrophotometers; Thermo Spectronic) indicated the formation of reaction product benzoquinone. Polyphenol oxidase activity of the basil extract was determined from the graph of the change in the absorbance against time.

Statistics

Every experiment was done in triplicate. Standard error was estimated for every experimental point and marked in a figure as an error bar. The data were analyzed with Origin® 8 software (*23*). The significance of the results was assessed by the analysis of variance (ANOVA) model. A value of $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Decontamination of basil by chlorophyllin-based photosensitization

It is important to note that basil is among the most microbiologically contaminated spices and herbs. For instance, Garbowska *et al.* (*24*) found that even total aerobic mesophils on the basil reached more than 10^5-10^6 CFU/g. Another study confirmed that 1.2 % herbs sold in retail was contaminated with *Salmonella* spp. and *E. coli* ($3.8\cdot10^3$ CFU/g) (*25*). Moreover, basil from different markets (Belgium, Israel and Cyprus) had different contamination levels. For instance, 45 % of samples from Cyprus were contaminated with >1 log CFU/g *E. coli* and even 14 % of samples were contaminated with >3 log CFU/g *E. coli*. In comparison, just 1-2 % of basil from Belgium or Israel was contaminated with *E. coli* (*26*).

In order to achieve better microbial control, U.S. Food and Drug Administration (FDA) has approved several sanitizers (*27*). Chlorine is the most frequently used disinfectant for fresh fruits and vegetables, but its efficiency is not high enough. For instance, Francis *et al.* (*28*) concluded that decontamination of lettuce with chlorine (200 ppm) reduced *L. monocytogenes* count by 1.7 log CFU/g, whereas decontamination of cabbage reduced microbial load just by 1.2 log CFU/g.

The FDA also approved acidified sodium chlorite as a spray/dip in the range of 500-1200 mg/L. This treatment reduced bacterial population by 1-2.5 log CFU/g (at 100-500 mg/L) (*13*). The main disadvantage of this treatment is high concentration of the agent and long treatment time. Most important is the fact that degradation of chlorine may lead to the accumulation of chlorinated trihalomethanes, haloacetic acids and chloramines in food and environment (*28*).

Another study investigated the antimicrobial efficiency of 0.2 % lactic acid against *E. coli* O157:H7 and *Yarrowia enterocolitica* inoculated on lettuce (*29*). The obtained data indicate that lactic acid reduced *E. coli* and *Y. enterocolitica* population by 0.4 and 2.4 log CFU/g, respectively. However, the treatment of cantaloupes with 1 % hydrogen peroxide at 20 °C for 15 min was ineffective against *E. coli* and *Salmonella* (*27*).

High power pulsed light is an emerging nonthermal antimicrobial technology suitable for preservation of fresh produce (*30*). However, the main disadvantage of this treatment is increasing temperature on the surface of a product at longer treatment time, which eventually reduces the visual and nutritional quality of fresh produce (*31*).

The main advantage of chlorophyllin-based photosensitization is its high efficiency against a wide range of microorganisms, Gram-positive and Gram-negative bacteria, including their more resistant spores, as well as biofilms (*32*). Moreover, fungi and yeasts are susceptible to this treatment as well. No volatile compounds are usually produced during the treatment (*30*). In our previous study, it was confirmed that temperature on the surface of the produce never exceed

26 °C, thus it does not affect the fruit texture and visual quality (*31*). These experimental results prompted us to apply this treatment for microbial control of fresh produce.

Data in **Fig. 1** show the absorption spectra of chlorophyllin (Chl) in the concentration range 10^{-7}-10^{-5} M. Thus, Chl absorption maximum is at λ=405 nm, and much lower absorption peak exists at 660 nm. Therefore, LED-based light source used for decontamination of basil emitted light at λ=405 nm for the optimal excitation of Chl.

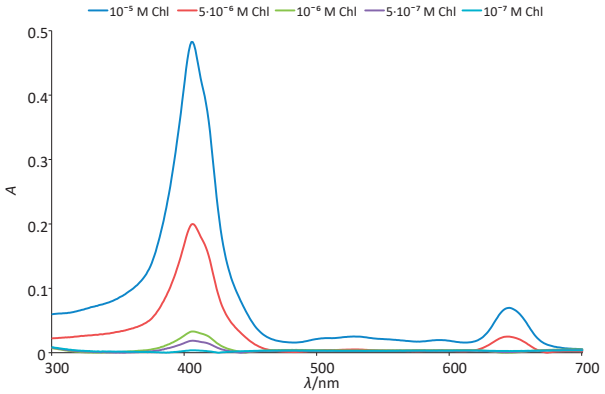

Fig. 1. Chlorophyllin (Chl) absorption spectra

The data in **Fig. 2** show that the population of total aerobic microorganisms in untreated control basil reached 4.4 log CFU/g. Soaking the basil in $1.5 \cdot 10^{-4}$ M Chl for 15 min and illumination with light for 15 min at 405 nm reduced the total aerobic microorganism count by 1.3 log CFU/g. In the next experiment, thermoresistant *L. monocytogenes* was inoculated on the basil. In the control group of inoculated basil, *Listeria* population reached 6.1 log CFU/g, while after soaking of basil in Chl solution ($1.5 \cdot 10^{-4}$ M) and following illumination (15 min at 405 nm) the population of inoculated *Listeria monocytogenes* reduced significantly ($p<0.05$) by 1.6 log CFU/g.

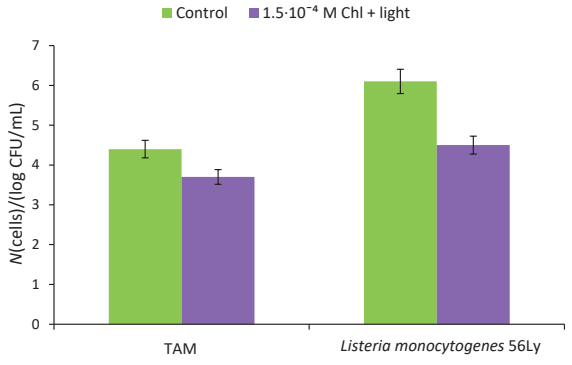

Fig. 2. Basil decontamination by chlorophyllin (Chl) -based photosensitization (total aerobic microorganisms (TAM) and *Listeria monocytogenes* 56Ly)

Since photosensitization treatment depends on light intensity and exposure time, there is always a possibility to increase treatment efficiency. Moreover, in order to increase antimicrobial efficiency, it is possible to apply hurdle technology approach (*33*). Our previous study confirmed that combination of photosensitization with the emerging, FDA approved high-power pulsed light technology is very effective (*34*). Aponiene and Luksiene (*35*) propose a useful experimental protocol for effective antimicrobial treatment combining photosensitization with photocatalysis.

Activity of pectinesterase and polyphenol oxidase in treated basil

In the plant cell wall pectinesterase catalyses hydrolysis of the methyl ester groups of pectic material, thus affecting plant cell wall structure and fruit firmness (*36*). It has been the subject of frequent technological study because of its importance in the alteration of the texture of fruits and vegetables. Sometimes pectinesterase is used for firming fruits and vegetables by infusion under vacuum (*37*). To estimate specific changes of product deterioration, the activity of pectinesterase in the treated and control basil was evaluated. The activity of pectinesterase (**Fig. 3**) immediately after photosensitization ($1.5 \cdot 10^{-4}$ M Chl, 15 min, 405 nm) did not differ from control (15.1 U/mL). It is interesting to note that storage of basil for 24 h significantly ($p<0.05$) enhanced pectinesterase activity from 15.1 to 20.1 U/mL.

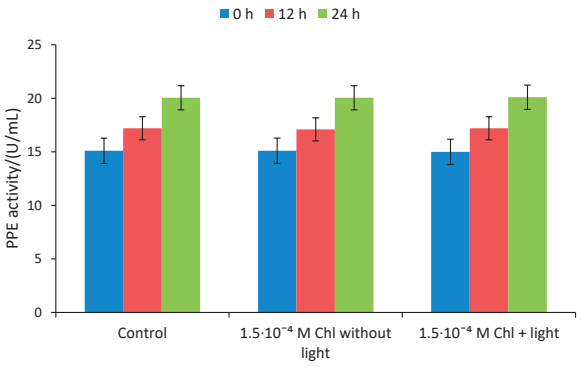

Fig. 3. Pectinesterase (PPE) activity after photosensitization ($1.5 \cdot 10^{-4}$ M chlorophyllin (Chl), 15 min illumination) in comparison with control at the beginning and after 12 and 24 h of treatment

One of the biggest challenges in fresh food industry is enzymatic browning of freshly cut produce (*20*). Browning of the cut surface of some fruits and vegetables is due to the presence of enzymes called polyphenol oxidases. These enzymes are released by the broken cells and they catalyse the reaction between the colourless molecules called polyphenols and molecular oxygen. This reaction creates coloured compounds and these new compounds can spontaneously cross-react with one another to form black-brown complexes called melanins (*38*).

Various technologies have been described to show inhibitory effects on enzymatic browning of freshly cut produce. Many chemical additives (ascorbic, phytic or citric acid, calcium chloride, hydrogen peroxide, chitosan, potassium sorbate, *etc.*) have been used for inhibition of browning and extension

of shelf life of fruits and vegetables (*39*). Moreover, the efficiency of temperature changes, modified atmosphere, enzyme inhibitors, or the removal/substitution of substrates was analyzed (*28*). Also, several types of chemicals have been tested for possible control of browning. Some of them act directly as inhibitors of oxidative enzymes (for instance chelating agents), while others act by rendering the medium inadequate for the development of the browning reaction (acidulants) (*28*).

In the study by Chen *et al.* (*39*) fresh-cut asparagus lettuce was treated with ClO_2 at different concentrations (10, 40 and 100 mg/L) for different times (5, 10 and 20 min). Following treatments, lettuce slices were stored at 4 °C for 14 days. The activities of polyphenol oxidase and peroxidase in the lettuce were reduced by ClO_2, and degradation of colour was also delayed. Thus, ClO_2 concentration and treatment time were two significant factors affecting the reduction of enzymatic browning of fresh-cut asparagus lettuce (*39*). In this study, the activity of polyphenol oxidase in basil after photosensitization-based treatment was assessed. Data in **Fig. 4** indicate that Chl-based photosensitization had no impact on the activity of polyphenol oxidase and it remained the same as in control herbs. However, the enzyme activity of basil enhanced from 100 to 203 U/mL during storage for 24 h in both, control and treated group. Despite the fact that differences between differently treated groups are insignificant ($p>0.05$), results obtained at different times (0, 12 and 24 h) after treatment are significantly different ($p<0.05$).

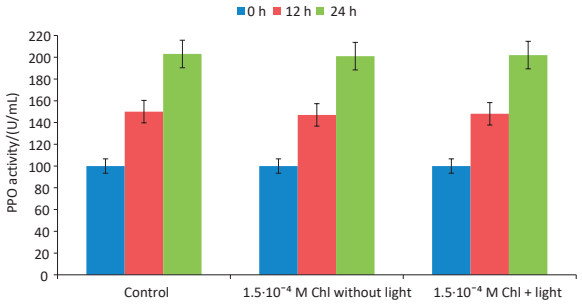

Fig. 4. Polyphenol oxidase (PPO) activity after photosensitization ($1.5 \cdot 10^{-4}$ M Chl, 15 min illumination) in comparison with control at the beginning and after 12 and 24 h of treatment

CONCLUSIONS

Chlorophyllin-based photosensitization treatment is effective antimicrobial tool and can be applied for microbial control of basil. This treatment significantly reduced surface-attached mesophilic bacteria and thermoresistant *Listeria monocytogenes*. It is important to note that chlorophyllin-based photosensitization had no impact on the activity of pectinesterase, which is responsible for basil firmness, or polyphenol oxidase, which is responsible for basil browning. Results obtained in this study support the idea that chlorophyllin-based photosensitization with its high antimicrobial efficiency, non-chemical and nonthermal nature can serve in the future for the development of completely safe and environmentally friendly preservation technology for different fruits and vegetables.

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