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# Effects of pulsed light and sanitizer wash combination on inactivation of *Escherichia coli* O157:H7, microbial loads and apparent quality of spinach leaves☆

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ABSTRACT

The purpose of this study was to investigate the efficacy of pulsed light (PL), a new formula of sanitizer (HEN) consisting of hydrogen peroxide, EDTA and Nisin, as well as synergy of PL and HEN sanitizer (PL-HEN) wash in inactivating *E. coli* O157:H7 on spinach. The treatment effect on microbial loads and apparent quality during 13 days storage at 4 °C was also determined. A bacterial cocktail containing three strains of *E. coli* O157:H7 was used as inoculum based on their association with produce-related outbreaks. Spinach leaves were spot inoculated on surface before treating with PL (1–63J/$cm^2$), HEN sanitizer wash (2 min) or their combinations. PL inactivation was influenced significantly at low doses. Treatment dose of 15.75 J/$cm^2$, equivalent to 15 s intense PL treatment, was found optimal above which adverse quality effect was evident. The optimal PL dose resulted 2.7 log CFU/g reduction of *E. coli* O157:H7 while a rapid 2 min wash in sanitizer formulation HEN, provided comparatively low, 1.8 log CFU/g, reduction of the pathogen. Two different sequences of PL and HEN treatment combinations were tested. In PL-HEN treatment, inoculated leaves were first treated at optimal PL dose (15.75 J/$cm^2$) followed by 2 min immersion in HEN whereas in HEN-PL treatment, leaves were first washed in HEN before PL exposure. HEN-PL treatment indicated a compound inactivation activity (4.6 logs reduction) while PL-HEN treatment indicated a strong synergistic inactivation as *E. coli* cells were not detectable after treatment indicating > 5 log reduction. The PL-HEN treatment not only significantly reduced spoilage microbial populations on spinach but also slowed their growth during storage. Furthermore, the visual and firmness quality of spinach were not significantly affected by the PL-HEN treatment. Overall, our results demonstrate that integrated PL-HEN technology can be used to enhance microbial safety of spinach.

## 1. Introduction

Consumption of fresh fruits and vegetables is highly recommended due to its numerous health benefits (Ignarro et al., 2007; Liu et al., 2000) and hence the recent surge in global production, distribution and consumption of minimally processed fresh produce (Abadias et al., 2008; Olaimat and Holley, 2012). This increase in consumption triggered an upsurge in number of produce related illness outbreaks in the US and worldwide in recent years. Fresh produce was responsible for 19% of total outbreaks from 2004 to 2013 in the US (CSPI, 2015). Spinach is a popular, nutritious vegetable. The consumption of spinach in the US has increased since 2009 and is predicted to rise (PBHF, 2015). Spinach provides a good ecological niche for the proliferation of numerous microorganisms including enteric pathogens linked to its consumption (Beuchat, 2002; Aruscavage et al., 2006). The 2006 North American *E. coli* outbreak was linked to the contamination of bagged baby spinach by *Escherichia coli* O157:H7 (Cooley et al., 2007). The outbreak caused approximately 205 confirmed illnesses, 31 cases of

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hemolytic uremic syndrome and three deaths (Barrera et al., 2012; Grant et al., 2008) and the outbreak was spread over 22 U.S. States (CDC, 2006). Contamination of spinach may occur via a number of routes, including composts and manures, irrigation water, wild and domesticated animal feces, and pathogen-carrying employees during growing or harvesting or processing. Therefore, the exact time of contamination is usually not known and by the time produce reach the packinghouse, it may retain a population of 4–6 log (Parnell et al., 2005). Therefore, postharvest decontamination is extremely important for controlling foodborne pathogens in spinach to safeguard against outbreaks of foodborne illness.

Sanitizer washing is a critical step in the process of preparation of spinach and other produce for the ready-to-eat market. Among various sanitizers studied, chlorine in the form of hypochlorite (HOCl) is widely used in the produce industry (Weng et al., 2016; Luo et al., 2012) where about two-thirds of manufacturers follow chlorine based wash as the prime step to prevent cross contamination (Gunduz et al., 2010). However, the effectiveness of chlorine is limited to about 1–2 log reductions of pathogens (Brackett, 1999; Herdt and Feng, 2009; Davidson et al., 2013) partly due to limited ability of the sanitizer to reach pathogens internalized in the protected sites of plant tissues either natural pores (stomata or lenticels) or damaged tissue (wounds or cut surfaces) due to the low diffusion penetration capability of sanitizer molecules (Shynkaryk et al., 2015). Also, it is difficult to maintain effective concentration of chlorine in the wash tank as chlorine reacts readily with organic matters escaped from the produce in wash solution, causing a rapid depletion of chlorine (Shen et al., 2012). Therefore, chlorine is added frequently to maintain an effective free chlorine level in the washing tank. This recurring addition of chlorine to washing liquid with high organic loads creates formation of organochlorine byproducts, suspected carcinogens (Richardson et al., 1998) which may impose new regulatory restrictions (Allende et al., 2004; Artes et al., 2009). In view of these concerns, researchers are interested in developing a safe and effective decontamination strategy, alternative to chlorine based sanitizers.

During the past decade, several nonthermal intervention methods and sanitizer treatments have been proposed and investigated. These include sanitizers as organic acids (Almasoud et al., 2015; Mukhopadhyay et al., 2015; Neal et al., 2012), electrolyzed water (Park et al., 2009; Al-Holy and Rasco, 2015), ozone (Olmez, 2012; Karaca and Velioglu, 2014), antimicrobial coating (Mukhopadhyay et al., 2018a; Park and Zhao, 2004) or physical interventions such as ultraviolet (UV-C) light (Mukhopadhyay et al., 2014; Sommers et al., 2010), cold plasma (Min et al., 2017) and high hydrostatic pressure (Kiera et al., 2008; Huang et al., 2013; Mukhopadhyay et al., 2016, 2017). These nonthermal treatments and methods have the ability to inactivate microorganisms to varying degrees depending on the type of pathogen, produce and the nature of contaminated surface.

Recently, pulsed light (PL) has come under attention as an effective non-thermal processing method for microbial decontamination of fresh fruits and vegetables. PL is an U.S. FDA approved technology (21CFR179.41) for food processing and handling (FDA, 2015). PL utilizes short, intense pulses of broad spectrum light ranging from UV to near infrared (200–1100 nm) to inactivate microorganisms on food surfaces (Mukhopadhyay et al., 2018b). The germicidal effect of PL light is mainly due to photochemical damage to deoxyribonucleic acid (DNA) of microorganisms interrupting cell replication from the exposure of UV component of the PL. In addition to photochemical, a photothermal and a photo physical effect may also be involved in surface decontamination by PL (Gomez-Lopez et al., 2007). Several studies have been published on high potential of PL technology for the inactivation of pathogenic and spoilage microorganisms and its effect on shelf life and quality (Oms-Oliu et al., 2010; Bialka and Demirci, 2008; Ramos-Villarroel et al., 2012; Lagunas-Solar et al., 2006). However, prolonged exposure to PL to achieve a pasteurizing intensity can cause deleterious effects on the sensory quality of produce due to generation of heat during PL treatment (Bialka and Demirci, 2008; Huang, Y. and Chen, H., 2014). Therefore, a short exposure or low dose PL treatment in combination with active antimicrobial sanitizer wash may prove appropriate to control target microorganisms on produce. To the best of our knowledge, no investigation has been conducted on inactivation of *E. coli* O157:H7 on spinach using PL and sanitizer wash combinations. Thus, the aim of this study was to evaluate the inactivation efficacy of combined non-thermal treatment based on PL light and active sanitizer wash against *E. coli* O157:H7 on spinach as an effective strategy alternative to current chlorine wash. The other objective was to examine the efficacy of the combined treatments to control the growth of native microflora responsible for spoilage during storage and treatment effects on sensory quality.

## 2. Materials and methods

### 2.1. Bacterial strains and preparation of inoculum

A bacterial cocktail composed of three strains of *E. coli* O157:H7 (C9490, E02128 and F00475) were used for this work. Selection of these strains was based on their association, mainly with produce related outbreaks. *E. coli* O157:H7 (E02128) was associated with a lettuce outbreak and *E. coli* O157:H7 (F00475) was isolated from a spinach outbreak in 2006 (Uhlich et al., 2008), while *E. coli* O157:H7 (C9490) was isolated from an uncooked hamburger outbreak that occurred in the 1990s (CDC, 1997). These isolates were obtained from in-house (USDA-ARS-ERRC) culture collection. The bacterial strains were grown by two successive loop transfers of individual strains incubated at 37 °C for 24 h in 5 ml Tryptic Soy Broth (TSB, BBL, BD Difco, Sparks, MD). A final transfer of 1 ml was made into 9 ml TSB with incubation at 37 °C for 18 h. The bacterial cells were harvested by centrifugation (4000 × g, 10 min) at 4 °C. Cell pellets were washed twice in 0.1% (w/v) peptone water (PW, BBL, BD Difco) and was finally suspended in PW to achieve a population level of about $10^8$ CFU/ml. To enumerate the population densities in each cell suspension, appropriate dilutions (in 0.1% PW) were spread plated, in duplicate, on to tryptic soy agar (TSA; BD Difco) plates. Equal volumes of each culture were combined in a separate sterile test tube to obtain a cocktail of three strains of *E. coli* O157:H7 (about 8 log CFU/ml) prior to inoculation of spinach.

### 2.2. Sample preparation and inoculation

Fresh and unblemished baby spinach (*Spinacia oleracea* L. variety Catalina) were purchased from local markets (Philadelphia, PA, USA) one day prior to performing the experiments and stored overnight at 4 °C. On the day of experiment, spinach leaves were taken out of the refrigerator and placed inside a hood for 2 h to acclimatize to room temperature. Leaves were washed for 2 min in 200 ppm chlorine solutions followed by a rinse with sterile deionized water and arranged in a single layer and air-dried for 1 h in a laminar hood at ambient temperature (22 °C) before being inoculated. The chlorine pre-wash was used to reduce natural microbiota and minimize antagonistic effect of natural microbiota to inoculated pathogens (https://www.sciencedirect.com/science/article/pii/S0168160517301046?via%3Dihub, Niemira and Cooke, 2010). However, for a set of spinach, destine for determination of background microbial loads, prewash was not performed. Three spinach leaves were used for each treatment per replicate. Three replicates of experiments were conducted. Experiments were independently replicated at different times (weeks). Freshly grown inoculum, and fresh new batch of spinach were used for each replicate. Spinach leaves were inoculated with 50 µl of *E. coli* O157:H7 cocktail suspensions by depositing droplets with a micropipette at ambient temperature. Samples were dried in the bio-hood for 2 h at 22 °C before being treated with PL and/or antimicrobials. All treatments were conducted in triplicate in separate experiments.

### 2.3. Inactivation of E. coli O157:H7 using PL treatment

PL experiments were performed with a laboratory scale PL system (RC-847 SteriPulse- XL system, Xenon Corp., Wilmington, MA, U.S.A.) consisting of a controller module, a treatment chamber and an air cooling module. The quartz lamp (LH 840) of the system is capable of generating PL in the wavelength of 180–1100 nm, with 40% of the energy being in the UV region. Pulses were delivered at a rate of 3 pulses/s with pulse width of approx. 500μs. Per manufacturer specification, each pulse delivers 1.27 J/cm$^2$ for an input of 3.8 kV at 19.3 mm from the quartz window of the lamp. The broadband energy in J/cm$^2$ of each pulse was quantified using a Vega laser power meter (P/N: 7Z01560, OPHIR Photonics, North Logan, UT, USA) containing a pyroelectric energy sensor (P/N: 7Z02936, OPHIR Photonics, North Logan, UT, USA). The distance between the lamp and the quartz window was 5.8 cm. For fluence measurement, the meter wavelength setting was 300 nm. To measure the PL energy, the pyroelectric sensor was placed at the center of the bottom of PL chamber, which was about 14 cm from the quartz window. For the PL experiment, spinach leaves (3 leaves, ca. 2.3 g) were placed in a sterile petri dish without cover with the inoculation spot facing the PL lamp. The distance between the quartz window to the top of the spinach leaves was about 14 cm. At this distance, the fluence delivered to the sample by single pulse was 0.35 J/cm$^2$. Samples were treated with PL for 1, 5, 10, 15, 30 and 60 s, corresponding to a total fluence dose of 1.05, 5.25, 10.5, 15.75, 31.5 and 63 J/cm$^2$, respectively. Untreated samples were used as control. All experiments and analyses were carried out independently in triplicate.

### 2.4. Antimicrobial treatment of spinach leaves

In addition to PL treatment spinach leaves were subjected to a new antimicrobial wash to evaluate its decontamination efficacy. The antimicrobial solution was prepared combining several GRAS (generally regarded as safe) compounds as 3% hydrogen peroxide (HP), 0.02 mM, ethylenediaminetetraacetic acid (EDTA) and 20 mg/ml Nisin (Sigma, St Louis, MO, USA), pH 6.6. This sanitizer formulation was referred to as HEN for easier description throughout the manuscript. Three percent (3%) HP was prepared by diluting stock solution of 30% HP (Sigma Aldrich, St Louis, MO, USA) in sterile DI water. The pH value of the antimicrobial solution was measured using a digital TS 625 pH meter (Thomas Scientific, Swedesboro, NJ, USA). The antimicrobial solution was prepared fresh for each experiment. Two different inactivation strategies were explored, namely, PL treatment followed by HEN wash (PL-HEN) and HEN wash followed by PL treatment (HEN-PL). Briefly, about 250 mL of antimicrobial solution (HEN) were prepared fresh in a clean, sterile 500 mL beaker and PL treated or untreated spinach leaves were immersed in the antimicrobial solution and washed under mild agitation (~250 rpm) for 2 min at room temperature (22 ± 2 °C). Agitation was sufficient to ensure complete coverage of the leaves during the entire treatment time. For PL treatment after sanitizer wash, leaves were carefully removed and placed in sterile petri dishes without covers and treated as mentioned in section 2.3. Trials were conducted independently in triplicate.

### 2.5. Microbial enumeration

For determination of the number of survivors, treated spinach leaves (~2.3 ± 0.4 g) were placed into stomacher bags containing 10 ml of neutralizing buffer (BD, Difco) and pummeled in filtered stomacher bags with a Stomacher 400 laboratory blender (Seward, Worthington, UK) for 2 min at 230 rpm to obtain a homogenate. Decimal serial dilutions of the homogenate were then prepared as needed and the surviving populations were evaluated by plating 0.1 mL (in duplicate) on nonselective tryptic soy agar medium (TSA, BD, Difco). Plates were held at room temperature (22 °C) for 2 h inside a biosafety cabinet (NuareTM, Plymouth, MN, USA) prior to overlaying with a selective agar. The waiting period was to allow for possible recovery of injured bacteria. After 2 h, TSA plates were overlaid with the selective medium Sorbitol MacConkey agar (SMAC, BD Difco). The plates were incubated for 24 h at 37 °C, the colonies were counted and the populations were expressed as log CFU/g. The experiments were conducted three times, independently, with duplicate determination of colony forming units (CFU).

In cases when population fell below detection limit, the presence of pathogens in the sample was also confirmed by enrichment methods. For the enrichment, approximately, 10 g leaves of all treated and untreated leaves were placed in a Stomacher bag along with 40 ml Modified tryptone soya broth (mTSB, Oxoid) and incubated at 37 °C for 18 h for *E. coli* O157:H7. Aliquots (0.1 ml) of undiluted homogenate from the enriched samples were plated on Cefixume Tellurite Sorbitol MacConkey agar (CT-SMAC, BBL/Difco) with incubation at 37 °C for 24 h. Any colonies were confirmed to be *E. coli* O157:H7 as described by Hitchins et al. (1995) (*FDA-BAM*). The experiments were conducted three times with duplicate determination of colony forming units (CFU).

For native microbial population, total aerobes (TAB) were enumerated by plating on plate count agar (PCA, BD Difco) with incubation at 35 °C for 24–48 h, and mold and yeast (M&Y) were enumerated on dichloran rose bengal chlortetracycline agar (DRBC, BD Difco) with incubation at 25 °C for 5 days. The experiments were conducted three times, independently, with duplicate determination of colony forming units (CFU).

### 2.6. Effects on quality

Treated and untreated Spinach leaves were placed in a perforated plastic deli container and stored at 4 °C. Samples were removed from the refrigerator on day 1, day 5, day 9 and day 13 to measure color and texture.

#### 2.6.1. Color analysis

The color of control and treated spinach was measured at 1, 5, 9 and 13 days of storage at 4 °C. Color (CIE L*, a*, b*) was measured using Hunter Lab Ultra Scan VIS with Easy Match QC Software, Version 4.87 (Hunter Associates Laboratory, Inc., Reston, VA, USA). Hunter Lab values (L*, a* and b*) were measured using RSEX (Reflectance Specular Excluded), 0.375-inch measuring aperture, with D65/10° illuminant-viewing geometry, and standardized with black and white tiles. Two readings were taken on one side (top side) of each spinach leaf as described by Jiang et al. (2017), a total of 8 readings per container. Experiments were conducted in triplicate. Hue and chroma values were calculated from the following equations: Hue = $\tan^{-1}$ (b*/a*) and chroma = $(a^{*2} + b^{*2})^{1/2}$.

#### 2.6.2. Texture evaluation

The texture of control and treated spinach was measured at 1, 5, 9 and 13 days of storage at 4 °C. Maximum force (in grams) was measured using a Texture Analyzer (Model TA.XT Plus) with Texture Exponent Software, Version 6.1.4.0 (Texture Technology Corp., Scarsdale, NY, USA). The procedure employed was a return-to-start (RTS) method with return distance of 30 mm, measuring the force under compression in auto mode with a Kramer Shear Cell with 5 blades at a distance of 10 mm, recording the peak of maximum force (Jiang et al., 2017). Force - time curves were recorded at a speed of 2 mm/s. Readings were taken on five leaves, a total of 2 reading per container. Experiments were conducted in triplicate.

### 2.7. Statistical analyses

Three independent trials were performed on separate days with separate batches of spinach, and two samples per treatment were analyzed using SAS (version 9.2) statistical package (SAS Institute Inc.,

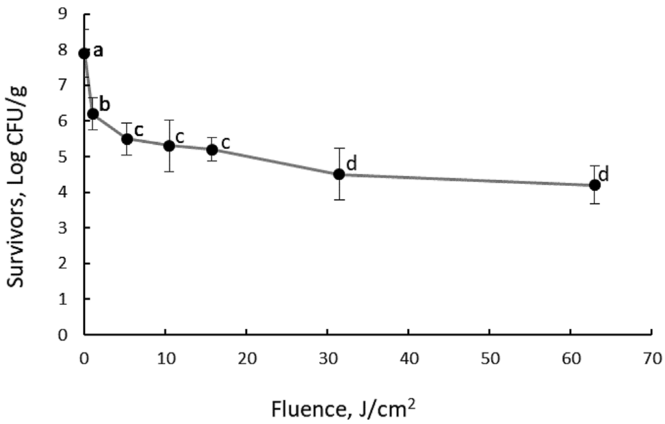


**Fig. 1.** Influence of PL treatment on survival of *E. coli* O157:H7 inoculated on spinach leaves. Initial counts of *E. coli* O157:H7 on spinach was 7.9 ± 0.67. Data represent mean population ($log_{10}$ CFU/g) of three replicate measurements ± 1 standard deviations. Error bars represent the standard deviation of the mean. Mean values with different letters are significantly different ($p < 0.05$).

Cary, NC, USA) for analysis of variance (ANOVA) and the Bonferroni LSD method (Miller, 1981) to estimate significant differences ($p < 0.05$) between mean values. Significance was defined at $p < 0.05$.

## 3. Results and discussion

### 3.1. Inactivation of E. coli O157:H7 on spinach with PL dosages and selection of optimal dose

Results of the study aimed to investigate the behavior of *E. coli* O157:H7 on spinach as influenced by different PL dose treatments, at 22 °C are presented in Fig. 1. The recovered initial population (mean value) of *E. coli* O157:H7 from spinach leaves was 7.9 ± 0.67 log CFU/g. The population of *E. coli* O157:H7 decreased with increasing PL doses and even at a lower dose, significant lethal effect against the survival of the pathogen was evident. Thus, merely 3 light pulses or 1 s exposure (equivalent to fluence 1.05 J/cm$^2$) to PL was enough to produce 1.7 log CFU/g reduction of *E. coli* O157:H7. As PL fluence increased from 0 to 31.5 J/cm$^2$ (30 s exposure), there was a rapid decrease in surviving *E. coli* O157:H7 populations (mean value) from the initial level of 7.9 to 4.5 log CFU/g indicating 3.4 log reduction of *E. coli* O157:H7 populations. This represents a 2-fold (or 100%) increase in pathogen reduction as the exposure time increase from 1 s to 30 s (Fig. 1). It is evident from the figure (Fig. 1), although, the population of pathogens continued to decrease with further increase in PL dose, the increase in PL dose or treatment time did not provide a proportional response in the efficacy of the treatment. In fact, the *E. coli* O157:H7 populations were reduced from 4.5 to only 4.2 log CFU/g as the treatment intensity increased by 2-fold from 31.5 to 63 J/cm$^2$. However, the log reduction was significantly influenced by low doses ranging from 1.05 to 5.25 J/cm$^2$. In fact, Fig. 1 indicates a low fluence, much lower than 1 J/cm$^2$, was enough to achieve a log reduction greater than 1 for the pathogen. Fig. 1, also shows a clear trend that higher fluence or treatment time leads to greater reductions of *E. coli* O157:H7. Although the inactivation continued to increase with treatment intensity, no significant dose differences observed at higher doses (> 5.25 J/cm$^2$). This is probably due to the fact that the UV-C component of PL, which is primarily responsible for the germicidal action, inactivates microorganisms by preventing the DNA replication. The damage at cellular level starts with the initial dose. As dose exceeds cellular injury threshold, rapid lethal destruction of cells occurs. The inactivation process continues with additional dose increment, but cellular death begins to level off (Sastry et al., 2000).

Other authors reported similar inactivation criteria for *E. coli* O157:H7 on produce surface. Bialka and Demirci (2007, 2008) applied PL on strawberries, blueberries and raspberries at varying UV doses and times. Maximum reductions of *E. coli* O157:H7 and *Salmonella* were reported to be 3.9 and 3.4 log CFU/g at 72 and 59.2 J/cm$^{-2}$ respectively on raspberries. Whereas on the surface of strawberries, maximum reductions of 2.1 and 2.8 log CFU/g were achieved at 25.7 and 34.2 J/cm$^2$ with no visible damage to the fruits. On blueberries, maximum reductions of 4.3 and 2.9 log CFU/g were obtained after 60 s of treatment for *Salmonella* and *E. coli* O157:H7, respectively. No change on sensory or visual quality was observed (Bialka and Demirci, 2007). Ramos-Villarroel et al. (2012) reported reductions of 2.66 and 3.03 log CFU/g for *L. innocua* and *E. coli* O157:H7 respectively on fresh-cut mushroom at a fluence of 12 J/cm$^2$. For spinach, Aguero et al. (2016) achieved 1.7 and 2.3 log CFU/g reductions for *E. coli* O157:H7 at dose of 10 kJ/m$^2$ and 120 kJ/m$^2$ respectively. Results presented in this work is in general agreement with published report.

During treatment, chamber temperature containing leaves was increased due to the generation of heat from PL photothermal effect. The generation of heat during PL treatment has been reported in the literature (Bialka and Demirci, 2008; Ramos-Villarroel et al., 2011). Visual observation indicated noticeable wilting of the leaves for treatments exceeding 15 s. This is probably due to the loss of water from the leaf surface as the excess heat generated by the treatment is absorbed by the exposed leaf surface. Noticeable damage to the 30 and 60 s treated leaves was apparent visually after treatment (Fig. 2). Hence, taking into consideration of this fact, 15 s treatment equivalent to a PL dose of 15.75 J/cm$^2$ was selected as optimal dose in this study.

### 3.2. Effects of antimicrobial sanitizer wash and various combinations of PL and antimicrobial wash on inactivation of E. coli O157:H7 on spinach

Efficacy of antimicrobial wash and various combinations of antimicrobial wash with PL treatment on inactivation of *E. coli* O157:H7 on spinach leave are shown in Fig. 3. The recovered mean population of *E. coli* O157:H7 from untreated (control) spinach was 6.3 ± 0.51 log CFU/g. According to Figs. 3 and 2 min wash in antimicrobial formulation HEN provided about 1.8 log CFU/g reduction of the target organism *E. coli* O157:H7 whereas 15 s (15.75 J/cm$^2$) PL treatment presented a stronger (~50% higher) inactivation compared to antimicrobial wash. None of these treatments alone could deliver a pasteurization level reduction (> 5 log) of the pathogen as per recommended microbiological criteria for safe and sanitary processing


**Fig. 2.** Visual appearance of PL treated spinach leaves. Uninoculated fresh spinach leaves were placed in Petri dish and treated with pulsed light, 1–60 s and the picture were taken after treatment.

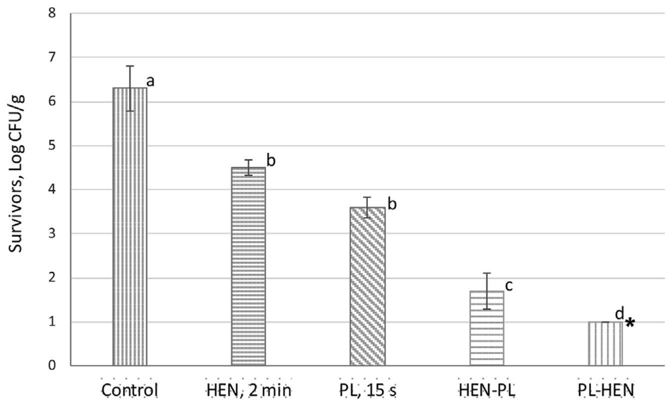


**Fig. 3.** Survival of *E. coli* O157:H7 in spinach leaves as affected by PL treatment, HEN antimicrobial wash and two combinations of PL treatment with HEN wash. PL – pulsed light; HEN- antimicrobial formulation containing hydrogen peroxide, ethylenediaminetetraacetic acid Nisin. Data represent mean population ($log_{10}$ CFU/g) of three replicates ± 1 standard deviation. Any two means with no letter in common are significantly ($p < 0.05$) different by the Bonferroni LSD method. Recovered population of *E. coli* O157:H7 from untreated leaves (control) was 6.3 ± 0.51 log CFU/g. Vertical error bars represent the standard deviation of the mean. *- population under detection limit (< 1 log CFU/g).

(FDA, 2001). However, the integration of PL treatment with antimicrobial wash indicated strong inactivation activity against the bacterial human pathogen. Antimicrobial wash was chosen in combination treatment protocol with PL since the antimicrobial components of HEN prevent bacterial growth effectively through an entirely different mechanism than PL inactivation mode. Two different sequences of combination treatments were considered. In one test, spinach leaves were first washed in HEN solution before PL treatment (HEN-PL) and in the other, the leaves were treated with PL followed by aqueous antimicrobial wash (PL-HEN). According to the figure (Fig. 3), both treatment strategies indicated strong inactivation activity of the target pathogen. The combined treatment of HEN-PL reduced *E. coli* O157:H7 population by 4.6 log CFU/g, indicating a compound inactivation effect of individual treatments. However, when the treatment sequence was reversed, i.e., PL treatment followed by aqueous antimicrobial wash (PL-HEN), no *E. coli* cells were detectable as the population fell below the detection limit. The log reduction achieved by this combination treatment of PL-HEN was, at the least, > 5 log CFU/g (Fig. 3), indicating a lethal synergistic inactivation scenario. PL targets mainly the surface microorganisms and has limited penetration depth in opaque media. Low inactivation for sanitizer washed leaves in HEN-PL treatment experiments may be due to the shielding of *E. coli* cells by the aqueous thin film of sanitizer which remains on the leaves after washing treatment.

Continuous UV light (0.6 kJ/m$^2$) treatment followed by 2 min wash in HEN sanitizer was reported to reduce *Salmonella enterica* populations by 4.7 logs on plum tomato surface (Mukhopadhyay et al., 2015). In the present work, the use of pulsed UV light with HEN (PL-HEN) provided higher reduction (> 5 logs) of *E. coli* O157:H7 on spinach, although no direct comparison can be made since both food substrate and the target pathogen are different in these two studies. Report on combined treatment of PL with sanitizer wash on inactivation of food borne pathogens on spinach is not available in the literature. Combined use of sanitizer washes with mild heat reported varied degrees of inactivation of foodborne pathogens on spinach. Neal et al. (2012) investigated effectiveness of combined use of multiple chemical sanitizers with mild heat on the reduction of foodborne pathogens on spinach and reported combined use of water wash, followed by 2% lactic acid with mild heat at 55 °C, could inactivate 2.3 log CFU/g of *Salmonella* and 2.7 log CFU/g of *E. coli* O157:H7 on spinach leaves. Huang and Chen (2011) investigated effects of organic acids and hydrogen peroxide alone and in binary combinations with or without mild heat (40 and 50 °C) on the inactivation of *Escherichia coli* O157:H7 on baby spinach and reported 2.7 log CFU/g reduction of *E. coli* O157:H7 with 1% lactic acid wash for 5 min at 40 °C. Washing with a binary mix solution of 1% each of lactic and citric acid or with a solution containing 1% lactic acid and 1% hydrogen peroxide at 40 °C for 5 min, provided similar results. These reports on combined treatments of multiple chemical sanitizers with mild heat demonstrated much lower log reductions for *E. coli* O157:H7 on spinach compared to results of synergistic inactivation of combined treatment achieved by PL and HEN sanitizer presented in this work. Current industrial practice of chlorinated water wash containing 200 ppm chlorine can provide only about 2-log reduction of *E. coli* O157:H7 on produce (Beuchat et al., 1998). In the present work, for spot inoculated spinach leaves, 15 s (15.75 J/cm$^2$) PL treatment and the combination treatments (PL-HEN and HEN-PL), all achieved significantly higher log reduction of *E. coli* O157:H7 than 200 ppm chlorine washing, indicating that both PL and the combination technology of PL and HEN, has the potential of being used in the spinach industry as an alternative to chlorine for decontamination purpose. Present study was conducted in small scale with only three spinach leaves (~2.3 g sample weight). For confirmation of the PL efficacy, experiments with large number of samples are necessary. Scale up study using large number of samples is also necessary for validation and commercial adaptation of the PL-HEN technology to improve spinach safety and quality.

### *3.3. Effects of PL and antimicrobial wash treatment on the native microflora of spinach*

Quality of fresh produce depends on their microbiota. Spinach, like many other produce, are known to harbor large bacterial populations. Control of spoilage microorganisms via physical or chemical treatments plays an important role in maintaining quality and freshness of produce such as spinach. Reports on the effect of combined PL and antimicrobial wash treatment on background microbial loads in spinach is not available in the literature. The effect of integrated treatment of PL and antimicrobial wash on the total aerobic bacteria (TAB) and M&Y population on surface of spinach leaves were evaluated during storage at 4 °C for 13 days. The change in the mean background microbial population of control and treated spinach is shown is Fig. 4. The initial TAB counts on spinach samples was 6.5 ± 0.4 CFU/g. The finding is in agreement with the published reports (Jiang et al., 2017; Aguero et al., 2016). As given in Fig. 4, during storage, the TAB population of untreated spinach leaves increased gradually from 6.5 ± 0.4 log CFU/g at day 1–8.6 ± 0.5 log CFU/g at day 13. This represents greater than 2 logs increase in TAB population during 13 days of storage. The combined treatment of PL-HEN, significantly ($p < 0.05$) reduced the native bacterial populations on spinach to 3.9 ± 0.4 log CFU/g (Fig. 4). This represents an initial log reduction of 2.5 log CFU/g of total aerobics. Although the TAB populations of PL-HEN treated spinach increased during storage in a manner like that observed in the case of control, the final TAB population of treated spinach at day 13 (6.2 ± 0.3) was significantly ($p < 0.05$) lower compared to that of untreated spinach.

Aguero et al. (2016) reported 2.2 log CFU/g initial reduction for the TAB population on spinach leaves by 40 kJ/m$^2$ of PL treatment. However, for shredded spinach, Gomez-Lopez et al. (2005) found a smaller initial reduction of mesophilic aerobic counts, ranging from 0.3 to 0.9 log CFU/g by PL at treatment intensity of 160 and 640 kJ/m$^2$ respectively. The authors (Gomez-Lopez et al., 2005) tested eight minimally processed vegetables including shredded spinach and cabbage and reported maximum initial log reductions up to 2.04 log CFU/g for total aerobic bacteria by PL. For fresh cut mushroom, initial reduction of microbial counts was 1.3 log CFU/g at a PL intensity of 28 J/cm$^2$ (Oms-Oliu et al., 2010). A continuous UV treatment (6 kJ/m$^2$) with 2 min HEN antimicrobial wash reported to reduce the initial TAB population

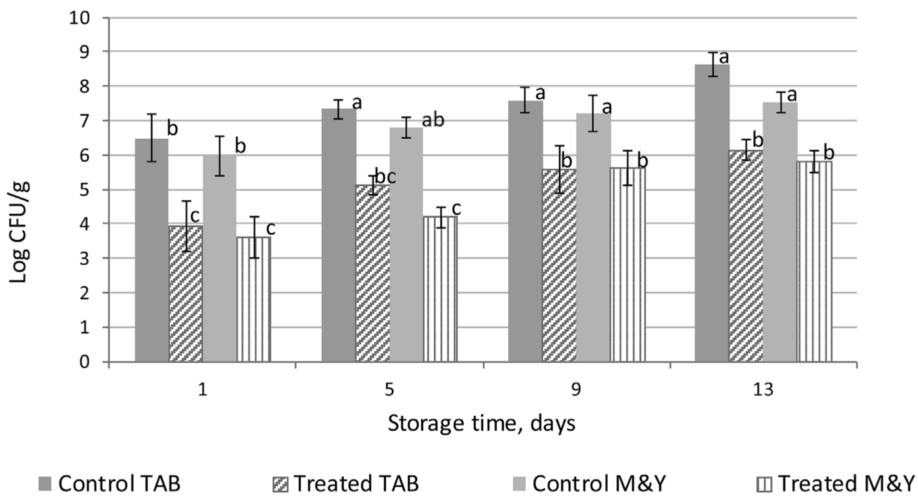


**Fig. 4.** Population of total aerobic bacteria (TAB) and Mold and Yeast (M &Y) on control and PL-HEN treated spinach during storage at 4 °C over 13 days. Spinach leaves were subjected to PL treatment for 15 s (15.75 J/cm2) followed by 2 min wash in new antimicrobial formulation HEN. Treated and untreated (control) leaves were stored at 4 °C for 13 days and analyzed for microbial loads at day 1, 5, 9 and 13.

by 1.7 log CFU/g (Mukhopadhyay et al., 2015) compared to 2.5 log reduction obtained in the present work with pulsed UV and 2 min HEN antimicrobial wash.

The initial M&Y counts on spinach samples was 5.9 ± 0.6 log CFU/g. Aguero et al. (2016) reported that the populations of M&Y on the spinach leaves were 4.1 log CFU/g while Jiang et al. (2017) found 6.4 log CFU of M&Y on spinach leaves. This variation may be due to the difference in variety and type of spinach or the pre- or post-harvest specific conditions. The combined treatment of PL and HEN sanitizer wash significantly ($p < 0.05$) reduced M&Y populations on spinach from 5.9 ± 0.6 to 3.6 ± 0.4 log CFU/g, representing 2.3 initial logs reduction ($p < 0.05$) due to the combined treatment (Fig. 4). During storage M&Y population for both control and treated spinach increased but the count for treated spinach at day 13 (5.8 ± 0.3 log CFU/g) was 1.7 log CFU/g fewer compared to the population for the control spinach. Initial reduction of M&Y population for spinach by 40 kJ/m$^2$ PL was reported 0.6 log CFU/g (Aguero et al., 2016). However, working with aerosolized $H_2O_2$, Jiang et al. (2017) found a much higher initial log reduction of 2.2 log for M&Y population on spinach leaves which is in the same order of magnitude than what found in the present study.

The variations in treatment effectiveness regarding initial log reduction of native microbiota may be due to many factors such as the type of produce, characteristics of the produce matrix, differences in resistance of the natural microbial population, location of microorganisms on and into the produce, and variations in sample preparation and microbial recovery techniques, among others.

Results indicated that combined PL-HEN technology significantly affected the initial load of microbial population in spinach and slowed their growth during refrigerated storage. This might be due to the sublethal damage to native microorganisms and a consequential decline to adapt in a refrigeration temperature environment. Hence, the PL-HEN treated spinach had lower counts than untreated spinach at the end of refrigerated storage. In contrast to high inactivation capability (log reduction > 5 log CFU/g) for the inoculated pathogen *E. coli* O157:H7, the effectiveness of the combined PL-HEN technology for native microflora was limited to about 2.4 logs. This is probably due to possible internalization of native microorganisms in the leaves and hence a higher resistance to inactivation effort.

The results presented in this work indicate an efficacious treatment strategy that may be used to ensure the microbial safety of spinach and control of spoilage organisms to promote microbial stability and extension of shelf life.

### *3.4. Effect of PL and antimicrobial treatment on quality of spinach during storage*

#### *3.4.1. Effects on spinach color*

Produce undergo a series of complex chemical and biochemical changes during post-harvest handling and storage which causes some color changes. Instrumental color changes of spinach before and after PL-HEN combination treatment and during refrigerated storage for 13 days are shown in Table 1. Color has been expressed in terms of L*, a* and b* values, where L* value indicated luminosity (level of light or darkness); a* indicated chromaticity on a green (negative number) to red (positive number), and b* value indicated chromaticity on a blue (negative number) to yellow (positive number), respectively. As presented in the table, the treatment causes no significant change in initial lightness (L*) values. However, there was continuous but nonsignificant

**Table 1**
Firmness and color parameters of untreated and PL-HEN treated spinach samples during storage under refrigerated conditions for 13 days.

| Treatment | Storage | Color parameters | | | | | Texture |
|---|---|---|---|---|---|---|---|
| (Days) | (4 °C) | L* | a* | b* | Hue | Chroma | Maximum Force, (kg) |
| Control | 1 | 35.3 ± 1.9a | −10.1 ± 0.7a | 17.5 ± 4.2a | 118.9 ± 2.1a | 20.2 ± 1.8a | 7.8 ± 1.9b |
| | 5 | 35.6 ± 2.1a | −9.5 ± 1.1a | 17.4 ± 3.8a | 118.6 ± 1.8a | 19.8 ± 2.2a | 7.1 ± 0.8b |
| | 9 | 36.2 ± 1.4a | −9.2 ± 0.7a | 18.1 ± 2.5a | 116.9 ± 2.2a | 20.3 ± 1.9a | 6.9 ± 2.2b |
| | 13 | 36.3 ± 1.7a | −9.4 ± 0.8a | 17.7 ± 3.9a | 117.9 ± 3.2a | 20.1 ± 3.2a | 7.4 ± 1.3b |
| Treated (PL-HEN) | 1 | 34.9 ± 2.7a | −8.9 ± 0.9b | 17.2 ± 2.6b | 117.2 ± 2.4a | 19.5 ± 2.6a | 7.9 ± 1.2b |
| | 5 | 35.4 ± 3.2a | −9.0 ± 1.3b | 16.5 ± 4.4b | 118.6 ± 1.9a | 18.8 ± 2.9a | 8.0 ± 2.3b |
| | 9 | 35.8 ± 1.3a | −8.8 ± 0.9b | 16.8 ± 3.7b | 117.6 ± 3.1a | 18.9 ± 2.1a | 8.3 ± 1.1a |
| | 13 | 36.1 ± 2.3a | −8.6 ± 1.2b | 17.1 ± 3.3b | 116.7 ± 1.5a | 19.1 ± 1.8a | 9.2 ± 2.9a |

Data expressed as means ± 1 standard deviations (n = 3).
Data in the same column followed by the same letter are not significantly different ($p > 0.05$).
Hue and chroma angle values were calculated from the following equations: Hue = $\tan^{-1}$ (b*/a*) and Chroma = $(a^{*2} + b^{*2})^{1/2}$.

increase in lightness values during storage. The a* values were elevated slightly but significantly due to treatment but there were no meaningful changes of a* values due to storage. The initial b* value was decreased slightly due to combined treatment with no subsequent changes during 13-day storage period. The calculated initial Hue and Chroma angles were slightly decreased after treatment but remain mostly unchanged during storage. Artes-Hernandez et al. (2009) studied effects of continuous UV-C light (4.5–11.4 kJ/m$^2$) on spinach quality and found the lightness (L*) values were decreased by as much as 10–15% due to treatment at the end of 13-day storage at 8 °C. They attributed this decrease in lightness values to the cellular damage to the leaves at high UV-C doses. This is in contrast with the findings by (Aguero et al., 2016) who observed no change in L* values of spinach due to PL treatment but subsequent increase in values during storage. In the present work of combined PL-HEN antimicrobial treatment, we found no change in L* values due to treatment but a nonsignificant increase in values during storage. For other green vegetables, such as broccoli similar findings were reported which reveals slight increase in L* values combined with nonsignificant decrease in hue angles in UV-C treated (10 kJ/m$^2$) broccoli florets during 6-day storage at 20 °C (Costa et al., 2006). In agreement with this work on broccoli (Costa et al., 2006) and the work on spinach by Artes-Hernandez et al. (2009), the present work finds a slight decrease in hue and chroma angles after treatment but no meaningful change in values during storage.

Visual appurtenance of food manifested as its color is an important quality attribute that has a strong influence on consumer perceptions and purchase behavior. The visual quality of spinach is influenced by the content of leaf pigments, ascorbic acid, carotenoids and phenol content (Spinardi et al., 2010). No significant change in the appearance was observed among control and PL-HEN antimicrobial treated spinach during storage.

### 3.4.2. Effects on texture

The firmness of control and PL-HEN treated spinach during refrigerated storage is shown in Table 1. Required maximum force for the control was 7.9 ± 1.9 kg and as shown in Table 1, the firmness quality of spinach was not affected by the treatment after 1 day of storage. There was small insignificant degradation of firmness quality during storage period for the control group. However, for the treated leaves, firmness was higher ($p > 0.05$), in excess of 20%, at day-9 and day-13 of storage. This is probably due to the relative dryness of the treated leaves from the loss of water during storage.

Mechanical properties of leafy greens vary in relation to cultivar differences and agronomic conditions (Newman et al., 2005). Younger leaves are, in general, tougher and higher forces may be required for leaf puncture. Also, location of the probe within the leaf blade may play significant role on puncture profile. To our knowledge there have been limited systematic attempts to describe the firmness profile of spinach. Fan et al. (2017) investigated effects of cold plasma activated hydrogen peroxide aerosol treatment on produce decontamination and quality and found no apparent change of spinach texture (9.52 kg) after treatment.

While there are no reports available on the effect of PL and HEN treatment on quality of leafy green spinach, the results presented in this work indicate the suitability of the current intervention method for spinach.

## 4. Conclusions

PL treatment and its combination with a sanitizer wash in the surface decontamination of fresh spinach leaves inoculated with *E. coli* O157:H7 has been investigated and its potential application in post-harvest processing, as a replacement for current chlorine based treatment, has been presented. PL treatment presented a dose dependent inactivation. The treatment enabled significant lethal reduction in contaminating microorganisms even at low doses. Only 3 light pulses or fluence equivalent to 1.05 J/cm$^2$, was enough to provide 1.7 log CFU reduction of *E. coli* O157:H7 per g of infected leaf. High doses were associated with increase of temperature due to the generation of heat which can have negative quality effects. Hence excessive exposure to PL should be avoided. A treatment dose of 15.75 J/cm$^2$ was considered optimal above which adverse quality effect to the leaves was evident. The optimal PL dose, resulted 2.7 log CFU/g reduction of *E. coli* O157:H7 while a rapid 2 min wash in a new sanitizer formulation HEN provided a comparatively lower, 1.8 log CFU/g reduction of the pathogen. Remarkably, the merger of these two technologies, PL-HEN resulted in a strong synergistic inactivation with *E. coli* O157:H7 population reduction reaching greater than 5 log cycles. The combined PL-HEN technology not only significantly affected the initial populations of native microorganisms on spinach but also slowed their growth during refrigerated storage. Furthermore, lightness, hue and chroma color parameters and the firmness of leaves were not significantly affected by the PL-HEN treatment. However, treatment resulted in some increase in leaves firmness after day-5 of storage. Overall, our results demonstrate that the integrated PL-HEN technology can be used to enhance microbial safety of spinach. Additional work is needed to address the potential of this novel efficient technique for decontamination of other fresh produce. Further, optimization of key variables and increased production cost need to be evaluated for large scale application of this technology.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.01.022.

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