A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Efficacy of Hydrogen Peroxide Vapor Treatment against Foodborne Pathogens on Fresh Produce

과산화수소 증기를 이용한 신선채소류의

병원성 미생물 저감화

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석사학위논문

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지도교수 강동현

이 논문을 석사학위 논문으로 제출함

2013년 2월

서울대학교 대학원 농생명공학부

백 경 환

백경환의 석사 학위논문을 인준함

2013년 2월

위원장 유 상 렬 (인)

부위원장 강 동 현 (인)

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ABSTRACT

In this study, the efficacy of hydrogen peroxide vapor (HPV) for reducing *Salmonella* Typhimurium, *Escherichia coli* O157: H7 and *Listeria monocytogenes* on spinach and lettuce was investigated as well as its effect on the quality used preliminary HPV treatment system and optimized HPV treatment system. The mechanism responsible for HPV treatment on bacterial cells was also studied using Transmission Electron Microscopy (TEM). Spinach and lettuce were inoculated with a cocktail containing three strains of each pathogen then treated with vaporized hydrogen peroxide for 0, 2, 4, 6, 8 and 10 min. The concentrations of hydrogen peroxide used were 0, 1, 3, 5 and 10%. With increasing treatment time and hydrogen peroxide concentration, HPV treatment showed significant ($P < 0.05$) reduction compared to the control (0%, treated with vaporized distilled water). In particular, vaporized 10% hydrogen peroxide treatment for 10 min was the most effective combination to reduce the three pathogens on spinach and lettuce. The reduction levels of *S.* Typhimurium, *E. coli* O157: H7 and *L. monocytogenes* on spinach were 4.40, 3.99 and 4.08 $\log_{10}$ CFU/g,

respectively at preliminary HPV treatment system. And the reduction levels of *S.* Typhimurium, *E. coli* O157: H7 and *L. monocytogenes* on lettuce were 3.12, 3.15 and 2.95 $\log_{10}$ CFU/g, respectively at optimized HPV treatment system. Additionally, there were no significant ($P > 0.05$) differences between the levels of cells enumerated on the appropriate selective agar when injured cells were enumerated during the most of whole treatment time. Furthermore, there were no significant ($P > 0.05$) quality changes (color, texture, and ascorbic acid contents) of spinach and lettuce among all tested samples, and hydrogen peroxide residue were not detected after 36 hr storage time in any of the treated samples. TEM showed significant damage in the cell cytoplasm and cytoplasmic membrane after treatment with HPV. Our results suggest that HPV treatment might be used as an alternative treatment to control *S.* Typhimurium, *E. coli* O157: H7 and *L. monocytogenes* on fresh produce.

Keywords: **Hydrogen peroxide vapor; Foodborne pathogens; Fresh produce; Food safety; Pasteurization**

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I. INTRODUCTION

The number of documented foodborne illnesses associated with consumption of raw vegetables and fruits has increased in recent years (1, 14, 34, 51). So, the concern about pathogens in fresh foods also has increased. Fresh produce can become contaminated with pathogenic microorganisms in the field through contact with soil amended by untreated or improperly composted manure, contaminated irrigation water, the presence of wild and domestic animals and unclean containers and tools used in harvesting (5, 7, 33, 44). In addition, contamination of produce with pathogenic microorganisms may occur during postharvest handling, processing, and distribution (7, 53). Because of this, controlling pathogenic microorganisms on fresh produce plays a paramount role in maintaining product quality and microbiological safety (21, 46).

Pathogenic microorganisms such as *Salmonella* Typhimurium, *Escherichia coli* O157: H7 and *Listeria monocytogenes* have all been

implicated in minimally processed fresh produce outbreaks of foodborne illness (5, 7, 8, 25). *S.* typhimurium is the most commonly isolated *Salmonella* serotype, and the symptoms of infection in humans are diarrhea, abdominal pain, mild fever and chills (3, 47). *E. coli* O157:H7 has come to be recognized as a major cause of diarrheal illness as well as hemolytic uremic syndrome (13, 18, 57). *L. monocytogenes* causes meningitis, encephalitis or septicaemia in immune compromised patients, pregnant women, infants and the old people (15, 44, 50).

For sanitizing fresh fruits and vegetables, water washing and chlorinated water (50 - 200 ppm chlorine) are widely used on a commercial scale (11, 45, 58). In particular, chlorine based chemicals are the most widely used sanitizers for fresh produce disinfection in the food industry. However, chlorine sanitizer occasionally produces harmful byproducts such as chloramines and trihalomethanes (2, 33, 48). Moreover, this treatment results in a microbial reduction of less than 2 $\log_{10}$ CFU/g on fresh fruits and vegetables (4, 6, 10, 11, 54, 60). Therefore, alternative treatments that are

able not only to reduce pathogenic microorganisms efficiently but also to prevent quality deterioration of fresh produce are needed to replace traditional treatment.

Currently, several studies documented that gaseous sanitizers have better penetration ability compared with aqueous sanitizers and are therefore considered more effective in reducing microorganisms on fresh produce (22, 33). Microorganisms can be attached to the surfaces of fresh produce and in additional, attached microorganisms showed increased resistance to aqueous sanitizers (16, 37, 52, 61). Consequently, microorganisms attached to produce in injured or inaccessible sites are one of the limiting factors of washing efficacy (49). One potential method to overcome this limiting factor of aqueous sanitizers is to use gaseous sanitizers. Its greater penetration ability may enable to control pathogenic microorganisms more efficiently on fresh produce. Thus, use of gaseous sanitizers may be a practical alternative to aqueous application of sanitizers by the food industry.

To date, aqueous hydrogen peroxide (H_2O_2) is a widely used biocide for

disinfection, sterilization and antisepsis (35, 36, 55, 56). The bactericidal and virucidal properties of aqueous H_2O_2 have been recognized for more than a century. H_2O_2 demonstrate wide range of organisms such as virus, bacteria, yeast and bacterial spores (9, 39). H_2O_2 acting against bacteria and viruses by forming hydroxyl free radicals (•OH) which attack essential cell components, including lipids, proteins, and DNA (20, 39, 40, 41). While aqueous H_2O_2 has a long history of use as a sterilant the concept of vapour-phage H_2O_2 sterilization was recently developed. Hydrogen Peroxide Vapor (HPV) is considered less toxic to human than other fumigants such as such as formaldehyde chlorine dioxide and ethylene oxide. These fumigants leave toxic residues after treatment (12, 29, 39). Therefore, HPV has been widely used for the decontamination of laboratory and medical equipment, health care institutions, pharmaceutical facilities, hospital rooms, ambulances, animal holding rooms and other applications (17, 26, 27, 29, 31, 39). However, to date, there has been paucity of information that has investigated the effect of HPV on controlling pathogenic microorganisms on foodstuffs.

The objectives of this study were to examine the bactericidal effectiveness of HPV on fresh produce (spinach and lettuce), and evaluate the quality changes following treatment with HPV. The mechanism responsible for HPV treatment on bacterial cells was also studied using Transmission Electron Microscopy (TEM). For these, we determined HPV concentration and exposure time required to reduce numbers of foodborne pathogens, including *S.* Typhimurium, *E. coli* O157: H7 and *L. monocytogenes* on fresh produce (spinach and lettuce) and its effect on color, texture and ascorbic acid contents changes were determined. Additionally, the levels of hydrogen peroxide residues on samples were measured after treatment.

II. MATERIALS AND METHODS

2.1. Efficacy of HPV treatment and mild heat against food borne pathogens on Spinach using preliminary system.

2.1.1. Bacterial strains

Three strains each of *Salmonella* Typhimurium (ATCC 19585, ATCC 43971, ATCC 700408), *Escherichia coli* O157: H7 (ATCC 35150, ATCC 43889, ATCC 43890) and *Listeria monocytogenes* (ATCC 15315, ATCC 19114, ATCC 19115) were used in this experiment and obtained from the Bacterial Culture Collection at Seoul National University (Seoul, Korea). All strains were stored at -80℃ in 0.7 ml of Tryptic Soy Broth (TSB; Difco Becton Dickinson, Sparks, MD, USA) and 0.3 ml of 50% glycerol (vol/vol). Working cultures were maintained on TSA (Difco) slants at 4℃ and subcultured monthly.

2.1.2. Bacterial cultures and cell suspension preparation

Each strain of *S.* Typhimurium, *E. coli* O157: H7 and *L. monocytogenes* was cultured in 5 ml TSB for 24 hr at 37℃, harvested by centrifugation at 4000 × g for 20 min at 4℃, and washed three times with Buffered Peptone Water (BPW; Difco). The final pellets were resuspended in BPW, corresponding to approximately 10^7~10^8 CFU/ml. Subsequently, suspended pellets of each strains of the three pathogens were mixed to produce culture cocktails.

2.1.3. Sample preparation and inoculation

Commercial spinach was purchased at a local grocery store (Seoul, South Korea) on the day before the experiment and kept at 4℃ prior to use. Spinach leaves (25 g) were separated and placed on sterile aluminum foil in a laminar flow hood with the fan running. Then 100 μl of previously

described culture cocktail was inoculated onto the surface of spinach leaves by depositing droplets at 15-20 locations with a micropipettor. The inoculated spinach pieces were air-dried for 2 hr in the hood with the fan running at room temperature (22 ± 2℃).

2.1.4. Preparation of treatments

Aqueous solutions containing 1%, 3%, 5% and 10% hydrogen peroxide (hydrogen peroxide, 30%, Junsei Chemical Co. Ltd., Tokyo, Japan) were prepared using sterile distilled water on a vol/vol basis. Freshly prepared solutions were used within 30 min at room temperature (22 ± 2℃). Distilled water served as a control.

2.1.5. Preliminary HPV treatment system

The experimental device (Fig. 1) consisted of a treatment chamber (80 × 50 × 50 cm), a solution tank and a volumetric pump (JWS600, Jeniewell, Seoul, South Korea), a 680-W heater (HY-HS11, HYSC, Seoul, South Korea), a data logger (34790A, Agilent Technologies, Palo Alto, CA, USA). This system was developed and constructed at Seoul National University (Seoul, South Korea). Temperatures were monitored using a K-type thermocouple, placed at the sample tray. For the HPV treatment, 1-L of solution of each previously described hydrogen peroxide concentration was placed in a 2-L solution tank. The solution was pumped to the heater by a volumetric pump, and the flow rate was maintained at 15 ml/min. When the solution dropped onto the heater, HPV generation occurred and treatment commenced. Inoculated spinach leaves were treated with HPV for 2, 4, 6, 8 and 10 min in the treatment chamber. All tests were performed at mild heating temperature (47 ± 3℃).

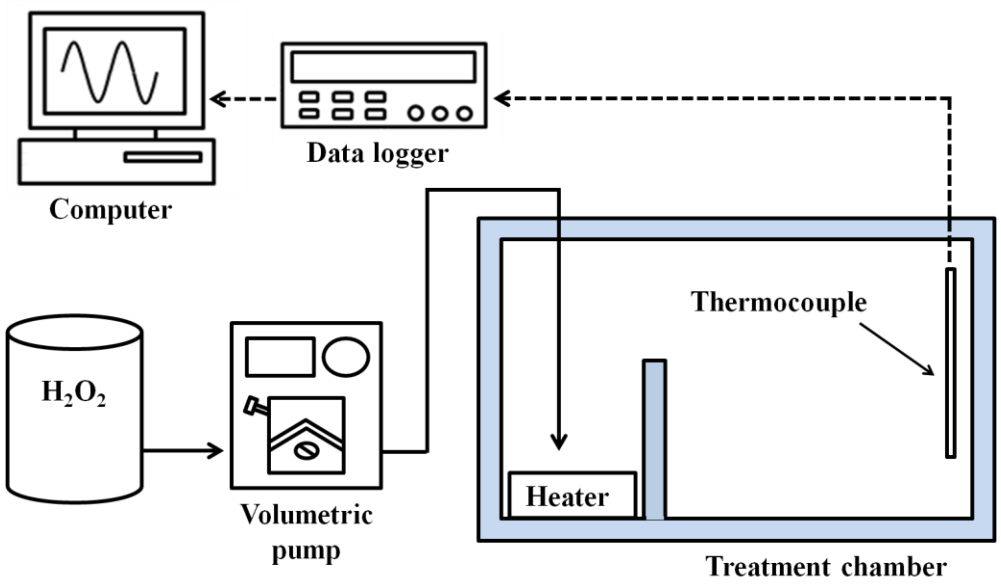


Fig. 1. Schematic diagram of hydrogen peroxide vapor treatment system used in this study.

2.1.6. Microbial enumeration

After 2, 4, 6, 8 and 10 min of treatment, each treated 25 g of spinach leaves were immediately transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of BPW and homogenized for 2 min with a stomacher (EASY MIX, AES Chemunex, Rennes, France). After homogenization, 1 ml aliquots of sample were serially 10-fold diluted with 9 ml of BPW, and 0.1 ml of sample or diluent was spread-plated onto each selective medium. Xylose Lysine Desoxycholate Agar (XLD; Difco), Sorbitol MacConkey agar (Difco) and Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Bacto Oxford Antimicrobic Supplement, Difco), were used as selective media to enumerate *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes*, respectively. Where low levels of surviving cells were anticipated, 1 ml of undiluted stomacher bag contents was equally distributed into four plates of each medium and spread-plated. All plates were incubated aerobically at 37 ℃ for 24-48 hr, then typical colonies

characteristic of *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* were enumerated.

2.1.7. Color measurement

In order to identify the color changes of spinach leaves during storage following treatments, all treated samples stored at 4℃ for 7 days. Color change of spinach leaves was measured with a Minolta colorimeter (model CR300, Minolta Co., Osaka, Japan) at 3 locations on each leaf at 0, 1, 3, 5, and 7 days after treatment. Colors were expressed as L*, a*, and b* values. The parameter L* is a measure of lightness, a* is an indicator of redness, and the parameter b* is a measure of yellowness. All experiments were performed three times.

2.1.8. Ascorbic acid determination

The concentration of ascorbic acid in spinach was determined using High-Performance Liquid Chromatography (HPLC) (Ultimate 3000, Dionex, Sunnyvale, CA, USA) equipped with an autosampler and an UV detector set at 265 nm, reported by Sapers, Douglas, Ziolowski, Miller, and Hicks (1990) with slight modification. Treated spinach was blended with a solution containing 4% metaphosphoric acid. Then, the blended mixture was filtered through Whatman No. 2 filter paper (Whatman Incorporated, Clifton, New Jersey, USA) and then through a 0.45 µm membrane filter (Micron separations Inc., Westboro, MA) with the help of a syringe. A reversed-phase C18 column (5 µm particle size, 4.6 mm diameter, 250 mm length, Dionex) was used to separate the ascorbic acid using 0.05 M potassium phosphate buffer and acetonitrile (95:5, vol/vol) as a mobile phase. The mobile phase was filtered using a 0.45 µm membrane filter (Micron separations Inc.) and degassed via vacuum before being used on the column. A flow rate of 0.5

ml/min was employed. A standard calibration curve was obtained by using L-ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA) in concentrations ranging from to 5 to 100 ppm. The standard solution was injected into the HPLC system and the corresponding peak area was obtained (Diamante et al, 2002). The ascorbic acid content of the samples was calculated and expressed in mg/100 g.

2.1.9. Transmission electron microscopy

To study the morphological changes in *L. monocytogenes* cells caused by HPV treatment Transmission Electron Microscopy (TEM) was employed in this study. The TEM specimens were prepared by the following procedures. Spinach inoculated with *L. monocytogenes* and treated with HPV was covered with 1.5% agar (Bacto agar, Difco) to prevent cell loss following treatments. The cells for TEM were fixed in modified Karnovsky's fixative containing 2% glutaraldehyde and 2% paraformaldehyde in sodium

cacodylate buffer (pH 7.2, final concentration of 0.05 M sodium cacodylate) for 2-4 hr at 4℃. After the primary fixation, each sample was rinsed three times for 10 min at 4℃ each in 0.05 M sodium cacodylate buffer (pH 7.2) to remove excess Karnovsky's fixative. For post-fixation, the specimens were treated with 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 hr at 4℃. Each specimen was rinsed twice for 10 min at room temperature with distilled water. After the washing procedure, specimens were pre-stained in 0.5% uranyl acetate overnight at 4℃. The following day, specimens were dehydrated in a graded ethanol series of 30, 50, 70, 80, 95, and three times at 100% for 10 min each. Then the specimens were placed in two changes of 100% propylene oxide (transition material) at 4℃ for 15 min each. The specimens were then infiltrated for 2 h with solution of propylene oxide and Spurr's resin. Following that, the specimens were immersed in a 50:50 propylene oxide and Spurr's resin for 2 hr and then placed in Spurr's resin overnight. Infiltrated specimens were polymerized at 70℃ for 24 hr. These specimens were sectioned (slices 70 nm in thickness) using an

ultramicrotome (MT-X; RMC, Tucson, AZ, USA). Thin sections were stained with 2% aqueous uranyl acetate for 7 min and Reynold's lead citrate for 7 min. The dried specimens were examined under a TEM (LIBRA 120; Carl Zeiss, Heidenheim, Germany) and digitally photographed.

2.1.10. Residual of hydrogen peroxide

Peroxide residues on treated spinach leaves were determined by hydogen peroxide test strips (WaterWorksTM, Industrial Test Systems, Inc., Rock Hill, SC, U.S.A.) with a minimum level of detection of 0.05 ppm. Residual of hydrogen peroxide was detected by the following procedures. Uninoculated spinach leaves were treated with various concentrations (1, 3, 5 and 10%) of HPV for 10 min as previously described. The treated spinach pieces were air-dried for 1 hr in the hood with the fan running at room temperature (22 ± 2℃). In order to identify the changes of residual of hydrogen peroxide on spinach leaves during storage following treatments, all treated samples stored

at 4℃ for 36 hr. Each treated 25 g of spinach leaves were transferred into sterile plastic bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of distilled water and homogenized at ambient laboratory temperatures. After homogenization, hydrogen peroxide test strips were used to measure the hydrogen peroxide concentration of leachate. All experiments were performed three times.

2.1.11. Statistical analysis

All experiments were repeated three times with duplicate samples and averages of duplicate plate counts from three replications were converted to $\log_{10}$ CFU/g. Data were analyzed by the ANOVA procedure of SAS (Version 8.1. SAS Institute Inc., Cary, NC, USA) for a completely randomized design. When the main effect was significant ($P < 0.05$), means were separated using the Duncan's multiple range test.

2.2. Efficacy of HPV treatment and mild heat against food borne pathogens on lettuce using optimized treatment system.

2.2.1. Sample preparation and inoculation

Commercial lettuce was purchased at a local grocery store (Seoul, South Korea) on the day before the experiment and kept at 4℃ prior to use. Lettuce leaves (25 g) were cut into 5 by 5 cm pieces and placed on sterile aluminum foil in a laminar flow hood with the fan running. Then 100 µl of previously described culture cocktail was inoculated onto the surface of leuttce leaves by depositing droplets at 15-20 locations with a micropipettor. The inoculated leuttce pieces were air-dried for 2 hr in the hood with the fan running at room temperature (22 ± 2℃).

2.2.2. Optimized HPV treatment system.

The experimental device (Fig. 2A) consisted of a treatment chamber (80 × 50 × 50 cm), a solution tank and a volumetric pump (JWS600, Jeniewell, Seoul, South Korea), a 300-W heater (JY-22300, Exso, Busan, South Korea), a data logger (34790A, Agilent Technologies, Palo Alto, CA, USA). This system was developed and constructed at Seoul National University (Seoul, South Korea). Temperatures were monitored using a K-type thermocouple, placed at the sample tray. Before chamber making, the HPV treatment system simulations (Fig. 2B) were performed with ANSYS (ANSYS Inc., Canonsburg, PA, USA), an engineering simulation software for computer-aided engineering for optimization of HPV dispersion. For the HPV treatment, 1-L of solution of each previously described hydrogen peroxide concentration was placed in a 2-L solution tank. The solution was pumped to the heater by a volumetric pump, and the flow rate was maintained at 10 ml/min. When the solution dropped onto the heater, HPV generation occurred and treatment commenced. Inoculated lettuce leaves were treated with HPV for 2, 4, 6, 8 and 10 min in the treatment chamber.

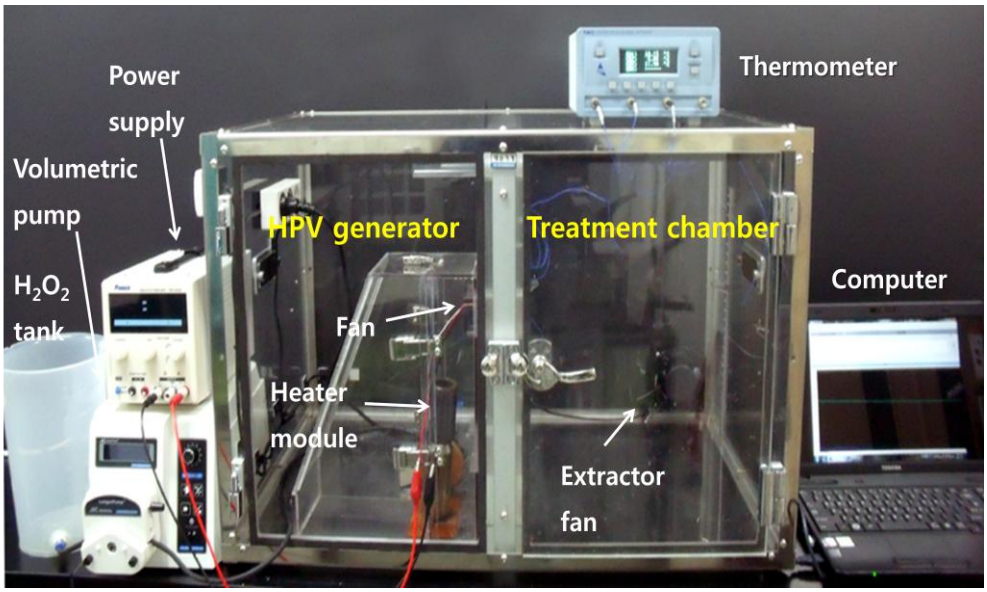


Fig. 2A. HPV treatment system at Seoul National University (Seoul, Korea).

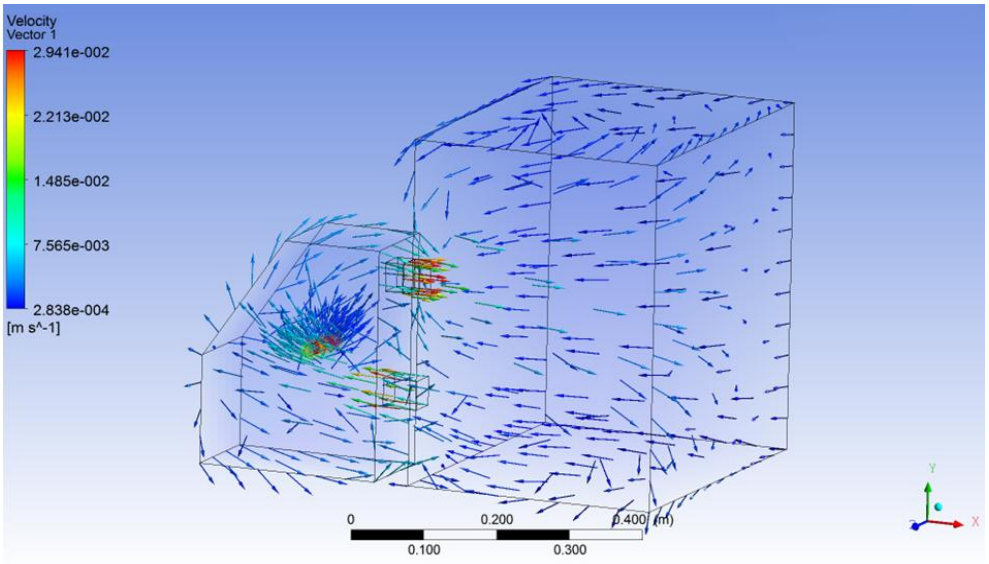


Fig. 2B. Simulated HPV distribution in this experimental system.

2.2.3. Microbial enumeration

After 2, 4, 6, 8 and 10 min of treatment, each treated 25 g of lettuce leaves were immediately transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of BPW and homogenized for 2 min with a stomacher (EASY MIX, AES Chemunex, Rennes, France). After homogenization, 1 ml aliquots of sample were serially 10-fold diluted with 9 ml of BPW, and 0.1 ml of sample or diluent was spread-plated onto each selective medium. Xylose Lysine Desoxycholate Agar (XLD; Difco), Sorbitol MacConkey agar (Difco) and Oxford Agar Base (OAB; Difco) with antimicrobic supplement (Bacto Oxford Antimicrobic Supplement, Difco), were used as selective media to enumerate *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes*, respectively. All plates were incubated aerobically at 37 ℃ for 24-48 hr, then typical colonies characteristic of *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* were enumerated.

2.2.4. Enumeration of injured cells.

Phenol red agar base with 1 % sorbitol (SPRAB; Difco) was used to enumerate injured cells of *E. coli* O157:H7. After incubation at 37 ℃ for 24 hr, typical white colonies characteristic of *E. coli* O157:H7 were enumerated. Randomly selected isolates from SPRAB plates were subjected to serological confirmation as *E. coli* O157:H7 (RIM, *E. coli* O157:H7 Latex Agglutination Test; Remel, Lenexa, KS, USA), because SPRAB is not typically used as a selective agar for enumerating *E. coli* O157:H7. The overlay (OV) method was used to enumerate injured cells of *S.* Typhimurium and *L. monocytogenes.* TSA was used as a nonselective medium to repair and resuscitate heat injured cells. Ten microliters of appropriate dilutions were spread onto TSA medium and plates were incubated at 37 ℃ for 2 hr to allow injured microorganisms to repair and resuscitate. Then the plates were overlaid with 7-8 ml of selective medium (XLD or OAB agar). After solidification, plates were further incubated for an additional 24 to 48 hr at 37 ℃. Following incubation, typical black colonies were enumerated.

2.2.5. Quality changes measurement

In order to identify the color and texture changes of lettuce leaves during storage following treatments, all treated samples stored at 4℃ for 7 days. Color change of lettuce leaves was measured with a Minolta colorimeter (model CR300, Minolta Co., Osaka, Japan) at 3 locations on each leaf at 0, 1, 3, 5, and 7 days after treatment. Colors were expressed as L*, a*, and b* values. The parameter L* is a measure of lightness, a* is an indicator of redness, and the parameter b* is a measure of yellowness. All experiments were performed three times.

Changes in lettuce leaf texture were evaluated with a Brookfield texture analyzer (model M08-373, Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) with a blade set probe. The Texture Analyzer was supplied with a load cell of 10 kg and application software (Brookfield Texture PROCT). The maximum shear force required for cutting the sample was recorded by the texture analyzer. Fifteen grams of non-treated and

treated sample was placed onto the press holder, and a blade was moved down at 2 mm/s. The peak force required to shear the samples were referred as a measure of hardness. Results were expressed in a report with values automatically calculated by the analyzer's software. All experiments were performed five times, with independently-prepared samples.

The concentration of ascorbic acid in lettuce and residual of hydrogen peroxide on lettuce were determined using previously described method.

2.2.6. Statistical analysis

All experiments were repeated three times with duplicate samples and averages of duplicate plate counts from three replications were converted to $\log_{10}$ CFU/g. Data were analyzed by the ANOVA procedure of SAS (Version 9.2. SAS Institute Inc., Cary, NC, USA) for a completely randomized design. When the main effect was significant ($P < 0.05$), means were separated using the Duncan's multiple range test.

III. RESULTS

3.1. Efficacy of HPV treatment and mild heat against food borne pathogens on Spinach using preliminary system

3.1.1. Inactivation of S. Typhimurium on spinach leaves after HPV treatment

Fig. 3A. shows the populations of *S*. Typhimurium treated by distilled water or HPV. When spinach leaves were treated with vaporized distilled water for 10 min, levels of *S*. Typhimurium were not significantly reduced ($P > 0.05$). Whereas, when inoculated spinach was treated with HPV (1-10%), levels of *S*. Typhimurium were reduced significantly ($P < 0.05$). Survival of *S*. Typhimurium decreased with increasing treatment time and hydrogen peroxide concentration. At 1% HPV, treatment for 2 min significantly reduced ($P < 0.05$) levels of *S*. Typhimurium by 0.96 $\log_{10}$ CFU/g on spinach

leaves. After 10 min of 1% HPV treatment, levels of S. Typhimurium were reduced by 2.00 $\log_{10}$ CFU/g. At 3% and 5% HPV, treatment for 2 min caused 1.05 and 1.18 $\log_{10}$ CFU/g reductions, respectively. The reductions were significantly ($P < 0.05$) different compared to non-treated samples. After 10 min of HPV treatment, levels of *S.* Typhimurium were reduced by 2.64 and 2.62 $\log_{10}$ CFU/g on spinach leaves, respectively. There were no significant differences ($P > 0.05$) in microbial levels between two hydrogen peroxide concentrations (3%, 5%) after 10 min exposure. *S.* Typhimurium was affected the most by treatment with vaporized 10% hydrogen peroxide, which reduced levels of S. Typhimurium by 2.67, 2.94, 3.37, 3.64, and 4.40 $\log_{10}$ CFU/g after 2, 4, 6, 8, and 10 min, respectively.

3.1.2. Inactivation of E. coli O157:H7 on spinach leaves after HPV treatment

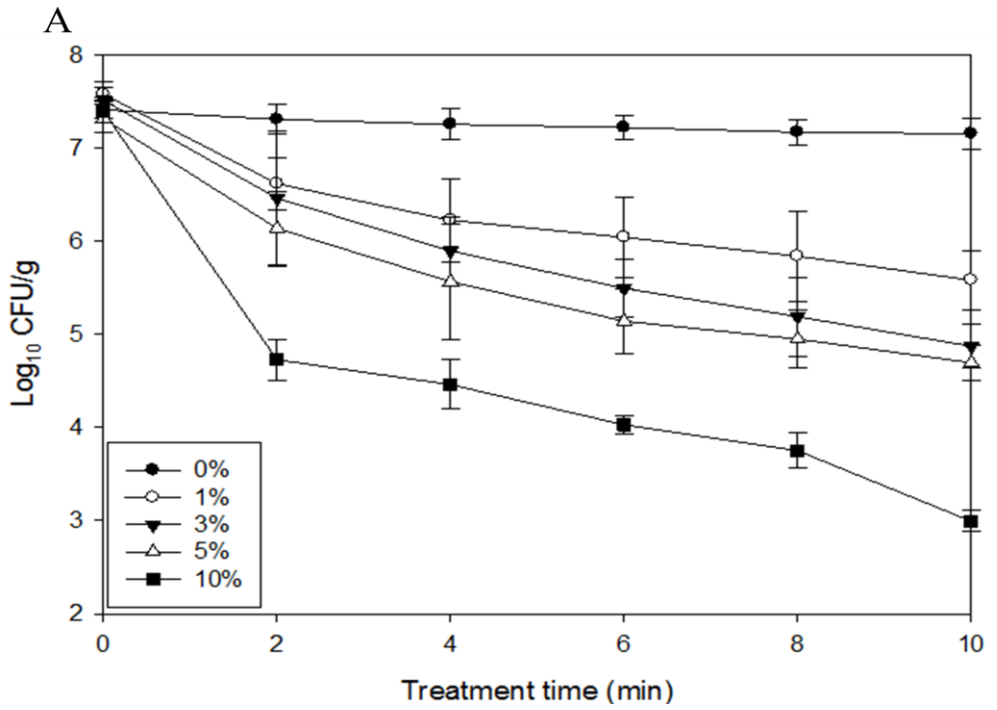
Fig. 3B. shows the populations of *E. coli* O157:H7 treated by distilled water or HPV. Spinach leaves were inoculated with approximately 10^7 CFU/g of *E. coli* O157:H7. *E. coli* O157:H7 was not significantly affected by the control treatment (distilled water) after 10 min ($P > 0.05$). When spinach leaves were treated with HPV, levels of *E. coli* O157:H7 were reduced significantly ($P < 0.05$). At 1%, 3%, and 5% HPV, treatments for 2 min caused 0.91, 1.13 and 1.59 $\log_{10}$ CFU/g reductions, respectively. The reductions were significantly ($P < 0.05$) different compared with non-treated samples. After 10 min of 1%, 3%, and 5% HPV treatment, 1.89, 2.52, and 3.23 $\log_{10}$ CFU/g reductions of *E. coli* O157:H7 occurred, respectively. There were no significant differences ($P > 0.05$) in microbial levels following treatment with two hydrogen peroxide concentrations (3%, 5%) until 10 min had elapsed. Vaporized 10% hydrogen peroxide was the most

effective concentration for reducing *E. coli* O157:H7 on spinach surfaces, resulting in reductions of 2.69, 3.30, 3.61, 3.97 and 3.99 $\log_{10}$ CFU/g after 2, 4, 6, 8, and 10 min, respectively.

3.1.3. Inactivation of L. monocytogenes on spinach leaves after HPV treatment

The survival of *L. monocytogenes* on spinach leaves after HPV treatment is shown in Fig. 3C. The initial level of *L. monocytogenes* on spinach was approximately 10^7 CFU/g. When spinach leaves were treated with vaporized distilled water for 10 min, levels of *L. monocytogenes* was not reduced significantly ($P > 0.05$). After treatment with 1% HPV, significant ($P < 0.05$) log reductions of *L. monocytogenes* were observed after 2 min, but reduction levels were less than 0.5 $\log_{10}$ CFU/g. At 1% HPV, treatment for 10 min achieved 1.10 $\log_{10}$ CFU/g reductions. When spinach leaves were treated with HPV, the inhibitory effect increased with increasing concentration of

hydrogen peroxide. Following treatment with 3% HPV, *L. monocytogenes* was decreased significantly ($P > 0.05$) after 4 min by 1.05 $\log_{10}$ CFU/g. After 10 min of HPV treatment, levels of *L. monocytogenes* were reduced by 2.30 $\log_{10}$ CFU/g. At 5% HPV, treatment for 2 min significantly reduced ($P < 0.05$) levels of *L. monocytogenes* by 1.19 $\log_{10}$ CFU/g on spinach leaves. After 10 min of 5% HPV exposure, levels of *L. monocytogenes* were reduced by 2.90 $\log_{10}$ CFU/g. *L. monocytogenes* was affected the most by treatment with 10% HPV, which reduced levels of *L. monocytogenes* by 2.07, 2.96, 3.22, 3.39, and 4.08 $\log_{10}$ CFU/g after 2, 4, 6, 8, and 10 min, respectively.


A
8
7
6
5
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3
2
Log10 CFU/g
0%
1%
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8
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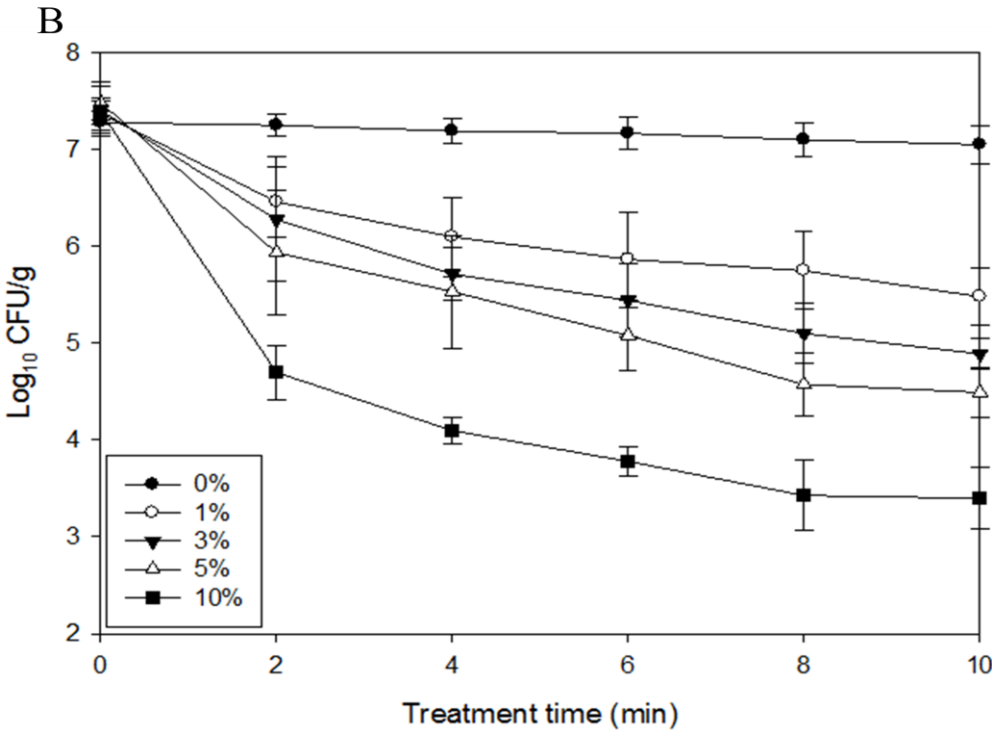
Treatment time (min)


B
8
7
6
5
4
3
2
Log10 CFU/g
0%
1%
3%
5%
10%
0
2
4
6
8
10
Treatment time (min)

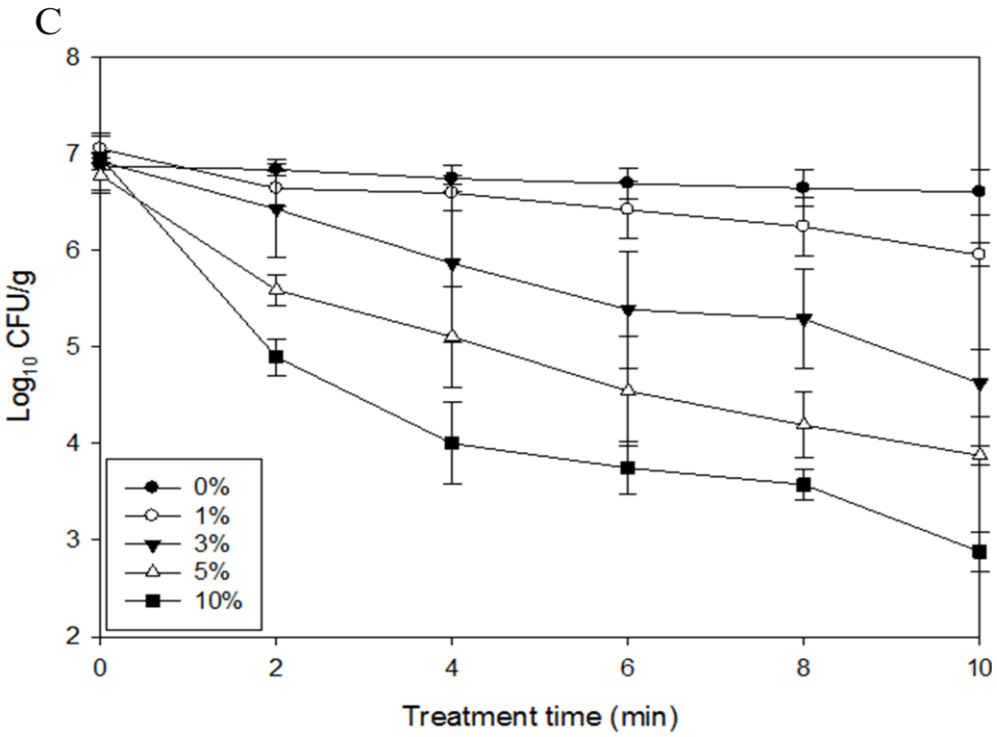


Fig. 3. Survival curves of *Salmonella* Typhimurium (A), *Escherichia coli O157:H7* (B) and *Listeria monocytogenes* (C) on spinach surfaces exposed to hydrogen peroxide vapor. The error bars indicate standard deviations calculated from triplicates.

3.1.4. Effect of HPV on spinach color

Table 1 shows Hunter color values (L*, a*, and b*) of spinach stored at 4℃ for 7 days following treatment with 0% (vaporized distilled water) or 1%, 3%, 5%, and 10% HPV for 10 min, compared with non-treated samples. There were no significant differences ($P < 0.05$) in L*, a*, and b* color measurement values among all tested samples, indicating treatment with up to 10% HPV did not significantly affect visual color quality of spinach following treatment and during 7 days storage.

Table 1. L*, a*, and b* value[a] changes for spinach stored at 4°C for 7 days following treatment with vaporized distilled water (0%) or 1%, 3%, 5% and 10% HPV for 10 min compared with non-treated samples.

Parameter[b]	Treatment concentration (%)	Storage time (day)				
		0	1	3	5	7
L*	Non-treated	36.88 ± 0.72 [NS]	37.38 ± 0.51	37.50 ± 0.25	36.89 ± 0.16	37.06 ± 0.21
	0	37.17 ± 1.12	37.44 ± 0.44	37.51 ± 1.13	37.06 ± 0.68	37.82 ± 0.04
	1	37.11 ± 1.42	37.48 ± 0.34	36.68 ± 0.50	36.86 ± 0.35	37.66 ± 0.71
	3	37.18 ± 0.40	37.16 ± 0.43	37.18 ± 0.56	37.25 ± 0.23	36.94 ± 0.39
	5	37.13 ± 1.06	36.90 ± 0.54	37.18 ± 0.61	36.83 ± 1.16	36.98 ± 0.81
	10	36.48 ± 0.14	36.76 ± 0.49	37.66 ± 0.76	37.07 ± 0.91	37.24 ± 0.86
a*	Non-treated	-11.21 ± 0.46	-11.61 ± 0.21	-11.40 ± 0.67	-10.95 ± 0.50	-10.83 ± 0.45
	0	-11.41 ± 0.38	-11.40 ± 1.57	-11.53 ± 0.54	-11.59 ± 0.75	-11.31 ± 0.28
	1	-11.70 ± 0.08	-11.68 ± 0.43	-11.15 ± 0.80	-11.51 ± 0.31	-11.51 ± 0.39
	3	-11.30 ± 0.24	-11.31 ± 0.40	-10.73 ± 0.34	-11.07 ± 0.25	-11.01 ± 0.31
	5	-11.01 ± 0.77	-10.45 ± 0.62	-10.47 ± 0.91	-10.87 ± 0.58	-10.77 ± 0.68
	10	-11.40 ± 0.74	-10.96 ± 0.66	-10.78 ± 0.22	-10.78 ± 0.28	-10.99 ± 0.37
b*	Non-treated	16.47 ± 0.36	17.13 ± 0.44	16.87 ± 0.61	16.59 ± 0.13	16.37 ± 0.28
	0	16.22 ± 0.99	16.29 ± 1.05	16.29 ± 1.22	16.86 ± 0.86	16.46 ± 0.22
	1	17.38 ± 0.79	17.94 ± 0.62	17.80 ± 1.26	17.18 ± 0.88	16.81 ± 0.63
	3	16.56 ± 1.03	17.08 ± 0.36	16.42 ± 0.31	16.77 ± 0.54	16.58 ± 0.43
	5	16.78 ± 1.79	17.19 ± 1.98	17.46 ± 2.48	17.87 ± 1.34	17.31 ± 0.60
	10	17.47 ± 1.04	17.05 ± 0.15	16.70 ± 0.08	17.10 ± 0.86	16.32 ± 1.04

[a] Mean of three replications ± standard deviation.
[b] Color parameters are L* (lightness), a* (redness), and b* (yellowness).
[NS] : no significance within L*, a*, b* value

3.1.5. Influence of HPV on ascorbic acid content

HPV effects of ascorbic acid content on spinach are presented in Table 2. The Initial ascorbic acid concentration of spinach was about 104.51 ± 13.40 mg/100g. Following treatment with 0% (vaporized distilled water) or 1%, 3%, 5%, and 10% HPV for 10 min, the ascorbic acid content of spinach was 103.74 ± 9.21, 103.73 ± 6.05, 103.51 ± 6.04, 104.00 ± 4.80, and 103.49 ± 10.05 mg/100g, respectively. There were no significant differences in ascorbic acid content in spinach among all tested samples ($P < 0.05$).

Table 2. Ascorbic acid content[a] (mg/100g) of spinach treated with 0% (vaporized distilled water) or 1%, 3%, 5% and 10% HPV for 10 min compared with non-treated samples.

Treatment concentration (%)	Ascorbic acid (mg/100g)
Control	104.51 ± 13.40 a
0	103.74 ± 9.21 a
1	103.73 ± 6.05 a
3	103.51 ± 6.04 a
5	104.00 ± 4.80 a
10	103.49 ± 10.05 a

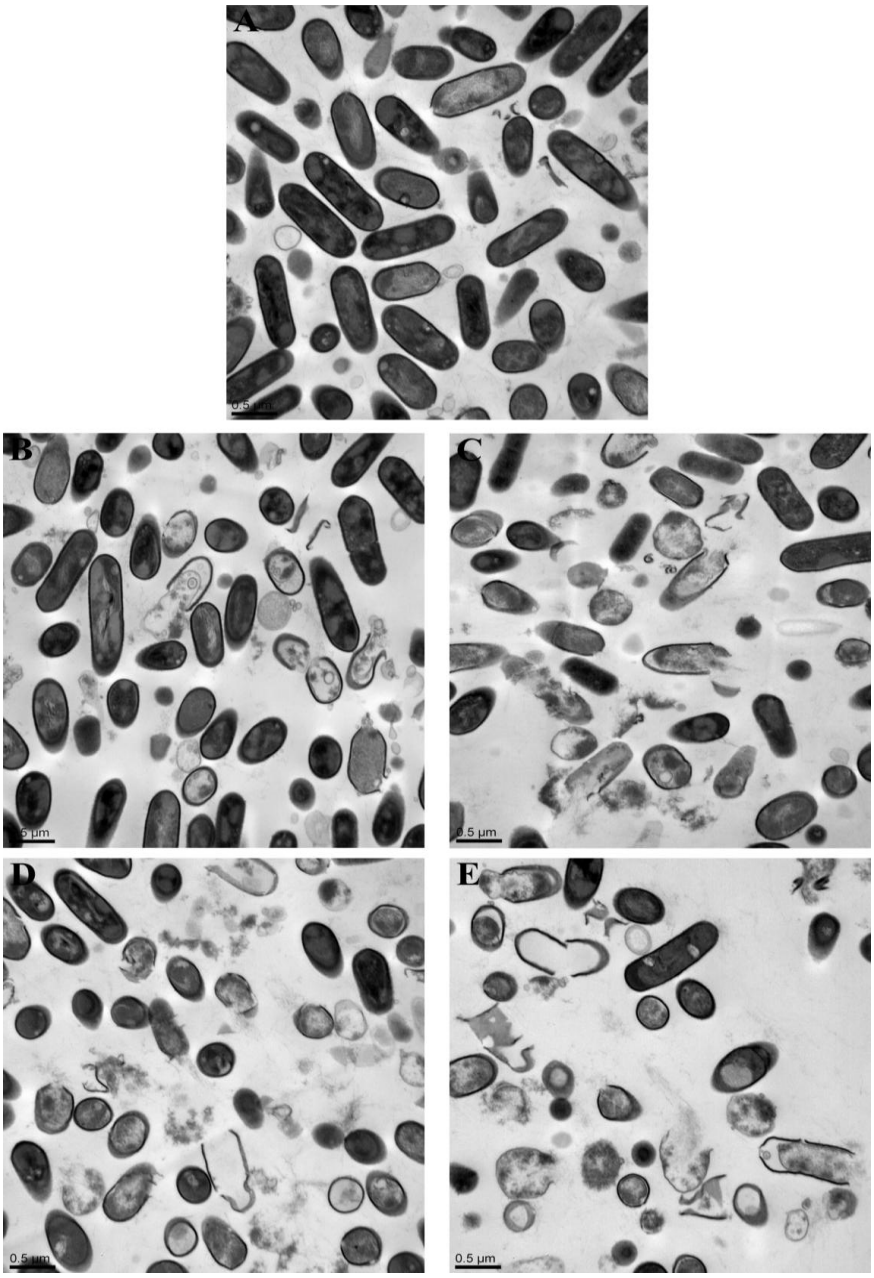
Mean of three replications ± standard deviation.
[a] Values followed by same letters within the column per parameter are not significantly different ($P > 0.05$).

3.1.6. TEM analysis of L. monocytogenes after treatment with HPV

The efficacy of HPV on the morphological changes of *L. monocytogenes* cells was evaluated by TEM. Fig. 4 shows transmission electron micrographs in bacterial cross-section projections from non-treated and HPV treated *L. monocytogenes* at HPV concentrations of 1%, 3%, 5%, and 10% for 10 min. Untreated *L. monocytogenes* cell surfaces and cytoplasm appeared uniform and homogeneous (Fig. 4A). Compared to non-treated *L. monocytogenes*, TEM results from 1%, 3%, 5%, and 10% HPV treated cells revealed that there was rupture at the cell wall with leakage of the intracellular contents or other cellular debris (Fig. 4B, Fig. 4C, Fig. 4D and Fig 4E). It is manifest that 1%, 3%, 5%, and 10% HPV treatment induced pores at several locations in the cell envelope. The morphological structure of *L. monocytogenes* was markedly damaged, destroyed, or deformed with increasing HPV concentration. Disintegration of the cell envelope led to leakage of cytoplasmic and nuclear material from the cells. Therefore, it seems clear

that HPV treatment not only kills bacteria by rupturing the cell wall, but also affects binary fission leading to loss of cytoplasmic and nuclear material, compared to intact *L. monocytogenes* cells during the process.

Fig. 4. Transmission electron microscopy photographs of *Listeria monocytogenes* on spinach surface treated with non-treated (A), 1% (B), 3% (C), 5% (D) and 10% (E) HPV for 10 min.



3.1.7. Residual of hydrogen peroxide

Hydrogen peroxide residues were detected immediately after exposure in all treated samples (Table 3). Following treatment with 1%, 3%, and 5% HPV for 10 min, the concentration of hydrogen peroxide on spinach was 40±8.33, 250±0.00, and 500±0.00 mg/liter, respectively. At 10% HPV, the concentration of hydrogen peroxide exceeded the detection limit (1000 mg/liter). The residues declined over time. After 24 hr only samples treated at 10% had detectable residues. Hydrogen peroxide residues at all exposure levels declined to undetectable levels 36 hr after treatment.

Table 3. Hydrogen peroxide residues[a] in distilled water leachate from spinach treated with 1%, 3%, 5% and 10% HPV for 10 min during different storage times.

Hydrogen peroxide concentration (%)	H_2O_2 (mg/liter) after storage (hr)			
	0	12	24	36
1	40±8.33	8.33±2.89	<0.5	-
3	250±0.00	16.67±5.77	<0.5	-
5	500±0.00	43.33±5.77	<0.5	-
10	>1000	250.00±0.00	16.67±5.77	<0.5

[a] Mean of three replications ± standard deviation.

3.2. Efficacy of HPV treatment and mild heat against food borne pathogens on lettuce using optimized treatment system.

3.2.1. Survival curves of S. Typhimurium and recovery of injured cells.

Table 4. shows the populations (log_{10} CFU/g) of *S.* Typhimurium and injured cells on the surface of lettuce following treated by distilled water or HPV. When lettuce leaves were treated with vaporized distilled water for 10 min, cell numbers of *S.* Typhimurium were not significantly reduced ($P > 0.05$). However, when inoculated lettuce was treated with HPV (1-10%), levels of *S.* Typhimurium were reduced significantly ($P < 0.05$). Survival of *S.* Typhimurium decreased with increasing treatment time and hydrogen peroxide concentration. Significant ($P < 0.05$) log reductions of *S.* Typhimurium were observed after 4 min of 1% HPV treatment. After 10 min of 1% HPV treatment, levels of S. Typhimurium were reduced by 1.48 log_{10} CFU/g. At 3% and 5% HPV, treatment for 2 min caused 0.72 and 1.23 log_{10}

CFU/g reductions, respectively. The reductions were significantly ($P < 0.05$) different compared to non-treated samples. After 10 min of HPV treatment, levels of *S.* Typhimurium were reduced by 2.09 and 2.63 $\log_{10}$ CFU/g on lettuce leaves, respectively. *S.* Typhimurium was affected the most by treatment with vaporized 10% hydrogen peroxide, which reduced levels of S. Typhimurium by 1.31, 1.72, 2.23, 2.62 and 3.12 $\log_{10}$ CFU/g after 2, 4, 6, 8, and 10 min, respectively. When surface-inoculated lettuce was treated with HPV, slightly higher numbers of the *S.* Typhimurium was detected by the agar OV method compared with XLD. However, there were no significant ($P > 0.05$) differences between the levels of cells enumerated on the appropriate selective agar (XLD) versus the agar for resuscitation (OV-XLD) during the most of whole treated samples.

TABLE 4. Populations[a] (log_{10} CFU/g) of surviving cells and cells including injured *Salmonella* Typhimurium on lettuce surfaces exposed to hydrogen peroxide vapor.

Treatment time (min)	Population (log_{10} CFU/g)									
	0%		1%		3%		5%		10%	
	XLD[d]	OV-XLD	XLD	OV-XLD	XLD	OV-XLD	XLD	OV-XLD	XLD	OV-XLD
0	7.51±0.13 A[b]a[c]	7.68±0.12 Aa	7.87±0.15 Aa	7.95±0.06 Aa	7.73±0.06 Aa	7.83±0.03 Aa	7.93±0.11 Aa	7.99±0.14 Aa	7.89±0.06 Aa	7.98±0.02 Aa
2	7.51±0.13 Aa	7.67±0.11 Aa	7.26±0.57 ABa	7.69±0.15 ABa	7.01±0.27 Ba	7.36±0.17 Ba	6.71±0.32 Ba	7.20±0.34 Ba	6.57±0.11 Ba	6.93±0.19 Ba
4	7.54±0.19 Aa	7.67±0.11 Aa	6.93±0.30 BCa	7.38±0.11 BCa	6.47±0.13 Ca	6.85±0.23 Ca	6.21±0.49 BCa	6.71±0.34 BCa	6.16±0.22 Ca	6.26±0.50 Ca
6	7.55±0.21 Aa	7.67±0.11 Aa	6.65±0.29 BCa	7.18±0.22 CDa	6.24±0.14 Ca	6.61±0.21 CDa	6.03±0.43 BCDa	6.58±0.32 CDa	5.65±0.40 Da	5.81±0.39 CDa
8	7.51±0.15 Aa	7.68±0.12 Aa	6.44±0.47 Ca	7.00±0.22 DEa	5.78±0.18 Da	6.33±0.33 DEa	5.69±0.50 CDa	6.16±0.35 DEa	5.27±0.24 Da	5.53±0.16 DEa
10	7.49±0.11 Aa	7.66±0.10 Aa	6.40±0.45 Ca	6.84±0.22 Ea	5.64±0.26 Da	5.93±0.42 Ea	5.30±0.32 Da	5.75±0.21 Ea	4.77±0.12 Eb	5.12±0.13 Ea

[a] Data represent means ± standard deviations of three replications.
[b] Means with the same capital letter in the same column are not significantly different ($P > 0.05$).
[c] Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[d] XLD, Xylose Lysine Desoxycholate; OV XLD, overlay XLD agar on TSA.

3.2.2. Survival curves of E. coli O157:H7 and recovery of injured cells.

Table 5. shows the survival curves of *E. coli* O157:H7 and injured cells on the surface of lettuce following treated by distilled water or HPV. Lettuce leaves were inoculated with approximately 10^7 CFU/g of *E. coli* O157:H7. *E. coli* O157:H7 was not significantly affected by the control treatment (distilled water) after 10 min ($P > 0.05$). When lettuce leaves were treated with HPV, levels of *E. coli* O157:H7 were reduced significantly ($P < 0.05$). At 1%, 3% and 5% HPV, the treatments for 2 min caused 0.62, 0.79 and 1.51 $\log_{10}$ CFU/g reductions, respectively. The reductions were significant ($P < 0.05$) difference compared with non-treated samples. After 10 min of 1%, 3% and 5% HPV treatment, there were 1.62, 2.14 and 2.94 $\log_{10}$ CFU/g reductions of *E. coli* O157:H7, respectively. Vaporized 10% hydrogen peroxide was the most effective concentration for reducing *E. coli* O157:H7 on lettuce surface, resulting in reductions of 1.37, 1.95, 2.43, 2.73 and 3.15 $\log_{10}$ CFU/g after 2, 4, 6, 8, and 10 min, respectively. When enumerated on

SMAC and SPRAB medium, the reductions of *E. coli* O157:H7 populations were slightly different among all tested samples. Whereas, there were not significant differences ($P > 0.05$) in levels of *E. coli* O157:H7 reduction between counts obtained with SMAC and those obtained by SPRAB during the most of whole treated samples.

TABLE 5. Populations[a] (log_{10} CFU/g) of surviving cells and cells including injured *Escherichia coli* O157:H7 on lettuce surfaces exposed to hydrogen peroxide vapor.

Treatment time (min)	Population (log_{10} CFU/g)									
	0%		1%		3%		5%		10%	
	SMAC[d]	SPRAB	SMAC	SPRAB	SMAC	SPRAB	SMAC	SPRAB	SMAC	SPRAB
0	6.88±0.18 A[b]a[c]	7.14±0.12 Aa	7.66±0.04 Aa	7.67±0.06 Aa	7.24±0.11 Aa	7.32±0.15 Aa	7.67±0.05 Aa	7.68±0.05 Aa	7.79±0.07 Aa	7.81±0.11 Aa
2	6.89±0.18 Aa	7.12±0.14 Aa	7.04±0.44 Ba	7.19±0.38 ABa	6.45±0.43 Ba	6.80±0.19 Ba	6.16±0.51 Ba	6.62±0.25 Ba	6.42±0.42 Ba	6.69±0.45 Ba
4	6.86±0.21 Aa	7.13±0.13 Aa	6.72±0.32 BCa	7.03±0.28 ABCa	6.06±0.27 BCa	6.37±0.09 Ca	5.91±0.45 BCa	6.29±0.34 BCa	5.84±0.44 BCa	6.10±0.60 BCa
6	6.86±0.20 Aa	7.17±0.12 Aa	6.51±0.22 BCDa	6.79±0.43 BCa	5.78±0.20 CDa	6.12±0.10 CDa	5.47±0.56 BCDa	6.02±0.40 CDa	5.37±0.43 CDa	5.75±0.56 Ca
8	6.90±0.17 Aa	7.17±0.12 Aa	6.30±0.29 CDa	6.63±0.52 BCa	5.47±0.26 DEa	5.80±0.21 Da	5.16±0.55 CDa	5.69±0.34 Da	5.06±0.32 DEa	5.37±0.26 CDa
10	6.86±0.20 Aa	7.09±0.17 Aa	6.04±0.47 Da	6.42±0.47 Ca	5.10±0.25 Ea	5.33±0.45 Ea	4.73±0.43 Da	5.16±0.18 Ea	4.64±0.07 Eb	4.94±0.04 Da

[a] Data represent means ± standard deviations of three replications.
[b] Means with the same capital letter in the same column are not significantly different ($P > 0.05$).
[c] Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[d] SMAC, Sorbitol MacConkey agar; SPRAB, Phenol red agar base with 1% sorbitol.

3.2.3. Survival curves of L. monocytogenes and recovery of injured cells.

Table 6. shows surviving cells or cells including those chemically injured of *L. monocytogenes* enumerated on OAB and OV-OAB, respectively, from lettuce leaves treated with distilled water or HPV. Lettuce leaves were inoculated with approximately 10^7 CFU/g of *L. monocytogenes*. *L. monocytogenes* was no significant reduction affected by the control treatment (distilled water) during 10 min ($P > 0.05$). When lettuce leaves were treated with HPV, levels of *L. monocytogenes* were reduced significantly ($P < 0.05$). At 1% HPV, the treatments for 4 min caused 0.70 $\log_{10}$ CFU/g reductions. The reduction was significant ($P < 0.05$) difference compared with non-treated samples and after 10 min of 1% HPV treatment, there was 0.99 $\log_{10}$ CFU/g reduction of *L. monocytogenes*. At 3%, 5% and 10% HPV, we observed a 0.34, 0.59 and 0.58 log reduction in CFU/g of *L. monocytogenes* after 2 min of treatment, respectively. The reductions were significant ($P < 0.05$) difference compared with non-treated samples. After

10 min of 3%, 5% and 10% HPV treatment, 1.63, 2.24 and 2.95 CFU/g of reductions were achieved, respectively. There was no significant different ($P > 0.05$) between levels of cells enumerated on OAB and OV-OAB during the most of whole treated samples.

TABLE 6. Populations[a] (log_{10} CFU/g) of surviving cells and cells including injured *Listeria monocytogenes* on lettuce surfaces exposed to hydrogen peroxide vapor.

Treatment time (min)	Population (log_{10} CFU/g)									
	0%		1%		3%		5%		10%	
	OAB[d]	OV-OAB	OAB	OV-OAB	OAB	OV-OAB	OAB	OV-OAB	OAB	OV-OAB
0	6.76±0.09 A[b]a	[c]6.86±0.06 Aa	7.37±0.21 Aa	7.49±0.24 Aa	7.12±0.07 Aa	7.22±0.03 Aa	7.32±0.12 Aa	7.38±0.10 Aa	7.10±0.10 Aa	7.19±0.10 Aa
2	6.74±0.07 Aa	6.85±0.05 Aa	6.92±0.31 ABa	7.26±0.18 ABa	6.79±0.19 Ba	7.06±0.02 Aa	6.73±0.21 Ba	7.10±0.10 Aa	6.52±0.37 Ba	6.83±0.25 Aa
4	6.73±0.06 Aa	6.88±0.08 Aa	6.67±0.32 Ba	7.14±0.18 ABCa	6.40±0.08 Cb	6.78±0.05 Ba	6.49±0.13 Ba	6.75±0.13 Ba	5.79±0.18 Ca	6.18±0.31 Ba
6	6.76±0.08 Aa	6.88±0.08 Aa	6.60±0.34 Ba	7.03±0.19 BCa	6.22±0.15 Ca	6.52±0.10 Ca	6.02±0.32 Ca	6.45±0.11 Ca	5.42±0.14 Ca	5.75±0.22 Ca
8	6.76±0.09 Aa	6.86±0.06 Aa	6.50±0.27 Ba	6.96±0.19 BCa	5.96±0.15 Da	6.16±0.14 Da	5.75±0.23 Ca	6.12±0.13 Da	4.84±0.29 Da	5.23±0.33 Da
10	6.74±0.07 Aa	6.86±0.06 Aa	6.38±0.26 Ba	6.82±0.21 Ca	5.49±0.18 Ea	5.87±0.16 Ea	5.08±0.18 Db	5.69±0.29 Ea	4.16±0.16 Ea	4.48±0.13 Ea

[a] Data represent means ± standard deviations of three replications.
[b] Means with the same capital letter in the same column are not significantly different ($P > 0.05$).
[c] Means with the same lowercase letter in the same row are not significantly different ($P > 0.05$).
[d] OAB, Oxford Agar Base; OV OAB, overlay OAB agar on TSA.

3.2.4. The effect of HPV treatment on lettuce quality

Tables 7 and 8 show the color and texture parameters of lettuce stored at 4℃ for 7 days following treatments with 0% (vaporized distilled water) or 1%, 3%, 5%, and 10% HPV for 10 min, compared with non-treated samples. There were no significant differences ($P < 0.05$) in L*, a*, and b* color measurement values and maximum load values of texture measurements among all tested samples. These results indicate that treatment with up to 10% HPV did not significantly affect visual color quality and texture of lettuce following HPV treatment and during 7 days storage.

Table 7. L*, a*, and b* values[a] changes for lettuce stored at 4°C for 7 days following treatment with vaporized distilled water (0%) or 1%, 3%, 5% and 10% HPV for 10 min compared with non-treated samples.

Parameter[b]	Treatment concentration (%)	Storage time (day)				
		0	1	3	5	7
L*	Non-treated	72.70 ± 0.76 [NS]	72.36 ± 0.45	72.55 ± 0.23	72.36 ± 0.77	72.16 ± 0.24
	0	72.73 ± 0.29	72.23 ± 0.28	72.38 ± 0.32	72.29 ± 0.31	72.45 ± 0.69
	1	72.32 ± 0.48	72.43 ± 0.47	72.49 ± 0.16	72.35 ± 0.80	72.33 ± 0.41
	3	72.72 ± 0.84	72.45 ± 0.30	71.94 ± 0.78	71.98 ± 0.38	72.35 ± 0.62
	5	72.75 ± 0.82	72.47 ± 0.08	72.59 ± 0.79	72.09 ± 0.68	72.30 ± 0.07
	10	72.10 ± 0.41	72.55 ± 0.19	72.09 ± 0.31	71.96 ± 0.87	71.88 ± 0.06
a*	Non-treated	-18.47 ± 0.31	-18.33 ± 0.13	-18.46 ± 0.44	-18.42 ± 0.29	-18.39 ± 0.12
	0	-18.01 ± 0.40	-18.24 ± 0.16	-18.46 ± 0.31	-18.39 ± 0.52	-18.45 ± 0.30
	1	-18.44 ± 0.50	-18.36 ± 0.27	-18.49 ± 0.23	-18.17 ± 0.06	-18.46 ± 0.28
	3	-17.80 ± 0.22	-17.96 ± 0.50	-17.91 ± 0.40	-17.94 ± 0.12	-17.96 ± 0.51
	5	-18.38 ± 0.45	-18.02 ± 0.24	-18.45 ± 0.12	-18.25 ± 0.26	-18.30 ± 0.42
	10	-18.06 ± 0.03	-18.21 ± 0.16	-17.96 ± 0.29	-18.05 ± 0.24	-17.89 ± 0.17
b*	Non-treated	35.85 ± 0.68	35.94 ± 0.48	35.76 ± 1.00	36.07 ± 0.48	36.03 ± 0.54
	0	35.68 ± 0.85	36.02 ± 0.63	36.39 ± 0.42	36.12 ± 0.46	36.38 ± 0.68
	1	36.69 ± 0.18	36.84 ± 0.87	36.27 ± 0.20	36.51 ± 0.58	36.58 ± 0.59
	3	36.05 ± 0.32	36.76 ± 0.16	36.79 ± 0.49	36.62 ± 0.29	36.21 ± 0.78
	5	35.62 ± 0.83	35.74 ± 0.76	35.78 ± 0.63	35.95 ± 0.54	35.93 ± 0.64
	10	36.33 ± 0.30	36.51 ± 0.28	36.63 ± 0.58	36.71 ± 0.70	36.62 ± 0.14

[a] Mean of three replications ± standard deviation.
[b] Color parameters are L* (lightness), a* (redness), and b* (yellowness).
[NS] : no significant statistical difference within L*, a*, b* values.

Table 8. Maximum load values[a] for texture of lettuce stored at 4°C for 7 days following treatment with vaporized distilled water (0%) or 1%, 3%, 5% and 10% HPV for 10 min compared with non-treated samples.

Treatment concentration (%)	Maximum load (N)[b]				
	Storage time (day)				
	0	1	3	5	7
Non-treated	78.53 ± 3.94 [Ns]	78.59 ± 3.15	78.70 ± 4.30	78.03 ± 3.25	77.47 ± 3.41
0	78.21 ± 1.06	77.03 ± 4.80	76.91 ± 4.68	76.89 ± 4.24	76.92 ± 3.14
1	77.63 ± 4.88	79.23 ± 5.19	78.48 ± 4.63	77.26 ± 1.76	77.65 ± 1.74
3	77.75 ± 3.28	78.08 ± 5.26	77.87 ± 3.17	77.01 ± 5.04	77.57 ± 2.49
5	78.30 ± 5.84	77.31 ± 4.45	77.52 ± 1.93	77.39 ± 5.51	76.01 ± 4.38
10	77.80 ± 5.18	78.03 ± 3.85	78.20 ± 6.20	77.05 ± 3.94	77.69 ± 1.89

[a] Mean of five replications ± standard deviation.
[b] Maximum load is load at rupture point.
[NS] : no significant statistical difference.

3.2.5. Influence of HPV on ascorbic acid content

Table 9 shows the effects of HPV treatment on the ascorbic acid content of lettuce. The Initial ascorbic acid concentration of lettuce was about 33.97 ± 0.38 mg/100g. Following treatment with 0% (vaporized distilled water) or 1%, 3%, 5%, and 10% HPV for 10 min, the ascorbic acid content of lettuce was 33.17 ± 1.81, 32.41 ± 2.01, 32.71 ± 3.00, 32.96 ± 2.46, and 33.22 ± 1.55 mg/100g, respectively. There were no significant differences in ascorbic acid content in lettuce among all tested samples ($P < 0.05$). These results showed that HPV treatment had not influence on the degradation rate of ascorbic acid content of lettuce.

Table 9. Ascorbic acid content[a] (mg/100g) of lettuce treated with 0% (vaporized distilled water) or 1%, 3%, 5% and 10% HPV for 10 min compared with non-treated samples.

Treatment concentration (%)	Ascorbic acid (mg/100g)	
Control	33.97 ± 0.38	a[b]
0	33.17 ± 1.81	a
1	32.41 ± 2.01	a
3	32.71 ± 3.00	a
5	32.96 ± 2.46	a
10	33.22 ± 1.55	a

[a] Mean of three replications ± standard deviation.
[b] Values followed by same letters within the column per parameter are not significantly different ($P > 0.05$).

3.2.6. Residual of hydrogen peroxide

Hydrogen peroxide residues were detected immediately after exposure in all treated samples (Table 10). Following treatment with 1%, 3%, and 5% HPV for 10 min, the concentration of hydrogen peroxide on lettuce was 100±0.00, 250±0.00, and 500±0.00 mg/liter, respectively. At 10% HPV, the concentration of hydrogen peroxide exceeded the detection limit (1000 mg/liter). The residues declined over time. After 24 hr, samples treated with vaporized 5% and 10% hydrogen peroxide had detectable residues. Hydrogen peroxide residues at all exposure levels declined to undetectable levels 36 hr after treatment.

Table 10. Hydrogen peroxide residues[a] in distilled water leachate from lettuce treated with 1%, 3%, 5% and 10% HPV for 10 min during different storage times.

Hydrogen peroxide concentration (%)	H_2O_2 (mg/liter) after storage (hr)			
	1	12	24	36
1	100±0.00	16.67±5.78	<0.5	-
3	250±0.00	36.67±11.55	<0.5	-
5	500±0.00	66.67±28.87	4.33±1.15	<0.5
10	>1000	83.33±28.87	6.67±2.89	<0.5

[a] Mean of three replications ± standard deviation.

IV. DISCUSSION

To date, in the food industry, treatment with aqueous chlorinated water is one of the most widely used to control pathogenic microorganisms on fresh produce (21, 22). However, chlorinated water is of limited efficacy, resulting in microbial reductions of less than 2 $\log_{10}$ CFU/g on fresh produce. There have been several research studies which demonstrate that chlorinated water have limited effect in reducing populations of pathogenetic microorganisms on fruits and vegetables. Lang et al. (32) reported that levels of *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 on lettuce were reduced by 1.1 to 1.8 $\log_{10}$ CFU/lettuce sample when treated with 200 μg/ml chlorine. Treatment with 120 or 200 mg/liter of free chlorine solution for 40 s reduced populations of *Salmonella baildon* on lettuce and tomatoes by less than 1 $\log_{10}$ CFU/g (58). Zhang and Farber (60) observed less than 2 $\log_{10}$ CFU/g reductions in *L. monocytogenes* on shredded lettuce treated with various concentration of chlorine (25 to 200 ppm) solution at 4 and 22℃ for

10 min. Most of these studies indicate that using chlorinated water was not sufficient to control levels of pathogens on vegetables and fruits. This is because aqueous sanitizers may not permit sufficient contact between attached bacteria on fresh produce surfaces and the washing and sanitizing agents (49).

For sanitizers to be effective in reducing pathogenic microorganisms, there must be direct contact between the sanitizer and the target microorganisms (43). So, gaseous sanitizers which have good penetration ability may overcome the limitations associated with aqueous sanitizers (22, 33). Additionally, gaseous sanitizers provide advantages over liquids, sprays, and wipes as they can be easily dispersed within an extensive volume such as rooms or buildings, and offer coverage over the corresponding large surface areas.

HPV is effective against a wide range of micro-organisms such as vegetative bacteria (17) and fungi (19), spores of *Bacillus* spp. (30) and exotic animal viruses (23). Furthermore, HPV breaks down to insignificant

amounts of oxygen and water, hence there are effectively no residues and the system is environmentally benign (28, 31, 38). In this study, therefore, we designed an HPV treatment system to control foodborne pathogens, including *S.* Typhimurium, *E. coli* O157: H7 and *L. monocytogenes* on spinach and lettuce. Our results indicate that HPV treatment lead to effective inactivation of *S.* Typhimurium, *E. coli* O157: H7 and *L. monocytogenes* on spinach and lettuce surface. Following HPV treatment, sub-lethally injured foodborne pathogens could assume added significance because injured microorganisms in food are potentially as dangerous as their uninjured counterparts, since injured organisms are able to repair themselves to normalcy, grow, and regain their pathogenicity under suitable conditions (24, 59). Therefore, the cell populations enumerated on selective media following treatment are probably not representative of the total surviving populations in the food stuffs. So, it is important to enumerate injured microorganisms in many applications, such as the preservation and spoilage of foods, consumer protection, the manufacture of safe foods, and evaluating the effectiveness of

processing for reduction of microorganisms. In this study, the occurrence of sub-lethally injured pathogens on lettuce was assessed by plating on selective agars with and without a resuscitation step. These results show that that HPV treatment effectively inactivated *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* on fresh produce without generating many injured cells which could recover and grow. The HPV treatment could potentially affect the quality of lettuce during storage following treatment. To investigate this possibility, the quality of treated spinach and lettuce leaves were evaluated by colorimetric and texture analysis. However, no significant influences on the color and texture were observed after treatments with HPV during 7 day storage. These results suggest that the HPV treatment could be applied to control *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* on the surface of spinach and lettuce without affecting the quality.

HPV treatment might be applied as an alternative intervention during transportation and storage. Because HPV dispersed in air can penetrate remote surfaces of fresh produce enclosed in a confined area. If pathogenic

microorganisms attach to biofilms during transportation or storage, there resistant to antibacterial compounds may be enhanced compared to planktonic bacteria (42). Therefore, it is important to sanitize fruits and vegetables during transport and storage, and a HPV system can easily be applicable to this and other industrial applications.

In conclusion, the HPV treatment was effective way to control pathogenic microorganisms on spinach and lettuce. Additionally, HPV treatment did not significantly ($P > 0.05$) quality changes (color and texture) were observed after HPV treatments during 7 day storage, as well as ascorbic acid contents were no significant differences after HPV treatments. Hydrogen peroxide residues were not detected after 36 hr storage in any of the treated samples. Therefore, the results of this study suggest that the HPV treatment could be used as an alternative to traditional fumigation treatments, such as formaldehyde, ethylene oxide and chlorine dioxide to control foodborne pathogens on fresh produce.

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Ⅵ. 국문초록

본 연구에서는 시금치와 양상추에 접종된 *Salmonella* Typhimurium, *Escherichia coli* O157:H7 그리고 *Listeria monocytogenes*에 대한 과산화수소 증기의 식중독 균 저감화 효과 및 처리에 따른 식품의 품질 변화를 살펴보았다. 이를 위해 시험 제작된 시스템을 이용하여 과산화수소 증기의 효과를 확인하였고 이 후 시뮬레이션 프로그램을 통해 식품을 처리하는데 최적화된 시스템을 제작하여 과산화수소 증기의 효과 및 이에 따른 식품의 품질변화를 조사하였다. 또한 과산화수소 증기가 세포의 형태적인 변화에 미치는 영향을 알아보기 위해 Transmission Electron Microscopy (TEM)을 이용하였다. 각 식중독 균의 3가지 균주를 시금치와 양상추에 접종 시킨 후 과산화수소 증기를 0, 2, 4, 6, 8 그리고 10분 동안 처리하였다. 처리시 사용된 과산화수소수의 농도

는 0, 1, 3, 5, 10%였다. 저감화 효과는 처리시간과 사용된 과산화수소수의 농도가 증가할수록 대조군(0%, 증류수 증기 처리)과의 유의적인 차이(P<0.05)를 보였다. 특히, 사용된 3가지 식중독 균 모두 10% 과산화수소 증기를 10분간 처리한 샘플에서 저감화 효과가 가장 높게 일어났다. 이 조건을 시험 제작된 시스템에 적용하였을 때 시금치에 접종된 *S.* Typhimurium, *E. coli* O157: H7 그리고 *L. monocytogenes*는 각각 4.40, 3.99 그리고 4.08 $\log_{10}$ CFU/g 수준의 감소를 보였으며, 최적화된 시스템에서는 양상추에 접종된 *S.* Typhimurium, *E. coli* O157: H7 그리고 *L. monocytogenes*가 각각 3.12, 3.15 그리고 2.95 $\log_{10}$ CFU/g 수준의 감소를 보였다. 또한, 과산화수소 증기 처리는 injured cell을 발생시키지 않고 효과적으로 균을 저감화 시키는 것으로 나타났다. 처리에 따른 유의적인(P<0.05) 품질변화(색, 텍스처 그리고 비타민 C)는 시금치와 양상추 모두 나타나지 않으며, 과산화수소수의 잔류

물은 36시간 이내 검출한계 이하로 낮아졌다. 과산화수소 증기가 식중독 균의 형태적인 변화에 미치는 영향을 TEM을 이용하여 확인한 결과 세포질과 세포막에 대한 피해가 관찰 되었다. 본 연구 결과, 과산화수소 증기를 통하여 신선채소류에서 품질변화 없이 *S.* Typhimurium, *E. coli* O157: H7 그리고 *L. monocytogenes*를 효과적으로 제어할 수 있다고 사료된다.

주요어: 과산화수소 증기; 식중독 균; 신선채소; 식품안전; 살균

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