Innovative Food Science and Emerging Technologies 43 (2017) 68–76

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

# Inactivation of bacterial pathogens on lettuce, sprouts, and spinach using hurdle technology

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ARTICLE INFO

*Keywords:*
Bacterial pathogens
Fresh produce
Hurdle technology
Inactivation
Shelf-life

ABSTRACT

Effects of chemical treatment using slightly acidic electrolyzed water (SAEW), fumaric acid (FA), or calcium oxide (CaO) and physical treatment using ultrasonication (US), micro-bubbles (MB), or ultraviolet (UV) to inactivate bacterial pathogens *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, and *Salmonella* spp. on lettuce, spinach, and sprouts were determined. Fresh produce inoculated with bacterial pathogens (~9 log CFU/mL) was immersed in distilled water (DW), SAEW, FA (0.5%), or CaO (0.2%) alone or in combination at 23 ± 2 °C for 3 min followed by treatment with US, MB for 3 min, or UV for 10 min. Effects of combined treatment on shelf-life of lettuce at 4 °C and 23 ± 2 °C were also determined in this study. Results revealed that the use of a combination of CaO + SAEW + FA + US exhibited significant reduction ($p < 0.05$) for bacterial pathogen on fresh produce compared to individual treatment or other combinations. CaO + SAEW + FA + US treatment exhibited highest reduction of *E. coli* O157:H7, *S. aureus*, *L. monocytogenes* and *Salmonella* spp. by 4.7, 4.9, 4.84 and 5.08 log CFU/g, respectively on lettuce as compared to spinach and sprouts. Microbial count reducing capability for combined treatment methods were ranked in the following order: SAEW + FA < CaO + SAEW + FA < CaO + SAEW + FA + US. However, introduction of US to CaO + SAEW + FA treatment resulted in little detrimental effect on the overall quality of lettuce. Moreover, CaO + SAEW + FA treatment effectively enhanced the shelf-life of lettuce stored at 4 °C and 23 ± 2 °C by about 6 days and 3 days, respectively as compared to control (DW treatment), with longer lag time (23.11 h on lettuce) for naturally occurring bacteria on fresh produce. These findings suggest that significant synergistic benefit could be obtained from combined sanitizer treatment to eliminate bacterial pathogens from fresh produce.

## 1. Introduction

Consumption of fresh produce has continued to increase over the last decade. Such increase in consumption may be due to the higher nutritional value of fresh produce, changes in social eating habits, and accessibility to fresh produce (Garrett et al., 2003; Khan, Ullah, & Oh, 2016). Fresh produce such as lettuce, spinach, cabbage, and sprouts are minimally processed. They serve as key vectors for *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* (Alegre, Abadias, Anguera, Usall, & Viñas, 2010; Issa-Zacharia, Kamitani, Muhimbula, & Ndabikunze, 2010; Khan, Khan, MisKeen, et al., 2016). Fresh produce has been increasingly implicated as a vehicle for transmission of foodborne illnesses around the world. These foodborne illnesses are estimated to cause 48 million illnesses, 128,000 hospitalizations, and 3000 deaths alone in United States (CDC, 2017), resulting in economic loss of billions of dollars due to reduced productivity and increased medical expenses.

Therefore, developing effective disinfectants to reduce pathogens in food and agricultural products is one of the most important steps in the food industry for ensuring product safety (Al-Haq, Sugiyama, & Isobe, 2005; Issa-Zacharia, Kamitani, Morita, & Iwasaki, 2010; Rahman, Khan, & Oh, 2016). The food industry has adopted various decontamination techniques throughout the food chain, ranging from chemical washing (such as chlorine-base components, acid compounds, and ozonated water) to current emerging treatments such as high hydrostatic pressure, dielectric heating, ohmic heating, ultrasonication, irradiation, and use of bacteriocins, and bacteriophages (Du, Han, & Linton, 2002; Khan & Oh, 2016; Trinetta, Vaidya, Linton, & Morgan, 2011). Although these decontamination technologies are effective in reducing microbial load, many of them are expensive

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http://dx.doi.org/10.1016/j.ifset.2017.07.033
Received 28 March 2017; Received in revised form 26 July 2017; Accepted 27 July 2017
Available online 29 July 2017

and technically difficult to be applied in a field environment.

The Hurdle concept, commonly referring to application of combined preservative methods, has become a potential technology that can decrease losses of nutritional and sensory quality while enhancing food safety (Khan, Tango, Miskeen, Lee, & Oh, 2017). The goal of hurdle technology is to improve total quality of foods and reduce treatment concentrations of chemicals (Leistner, 1985). Moreover, hurdle technology exhibits synergistic effects due to different mechanisms involved in the inhibition or inactivation of microorganisms in foods (Rahman et al., 2016).

Electrolyzed water (EW) is an oxidant sanitizer with free chlorine as its main antimicrobial agent. It is produced by electrolysis of a dilute salt solution (generally NaCl or HCl) through an electrolytic cell. It has been highlighted as one emerging alterative of sodium hypochlorite sanitizer because of its on-site and simple production, cheap and easy-to-find raw materials, low operational costs, and low trihalomethanes generation (Gil, Gómez-López, Hung, & Allende, 2015; Gómez-López, Gil, & Allende, 2017). There are two types of EW producing machines, those that contain membrane and produce acidic electrolyzed water (pH 2.5) and basic electrolyzed water (pH 10–13) in a 2-cell chambers and others that do not contain a membrane and produce neutral electrolyzed water (NEW; pH 7) and slightly acidic electrolyzed water (SAEW; pH 5.0–6.5) in a single-cell chamber. SAEW has many advantages over other types of EW. SAEW reduces the environmental damage and corrosive impact of the food industry (Tango et al., 2017). In addition, the sensory quality of food products is not negatively affected by the use of SAEW (Rahman et al., 2016).

To further enhance SAEW efficacy, it can be used in combination with chemical sanitizers such as fumaric acid (FA), calcium oxide (CaO) or physical treatments such as ultrasonication, micro-bubble, and ultraviolet irradiation (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Tango, Wang, & Oh, 2014). Therefore, the objective of the current study was to evaluate different decontamination treatments to establish an optimal hurdle treatment with synergistic antimicrobial effect to improve the quality and safety of fresh produce.

## 2. Materials and methods

### 2.1. Microbial culture and inoculum preparation

The following bacteria were used in this study: *L. monocytogenes* (ATCC 19111, 19118, 49594), *E. coli* O157:H7 (ATCC 23150, 946, 938), *S. aureus* (ATCC 13150, 6538, 23235), and *Salmonella enterica* serovars (*S.* Typhimurium ATCC 19585 and *S. enteritidis* ATCC 13076). These strains were obtained from College of Agriculture and Life Sciences, Kangwon National University, Chuncheon, Gangwon-do, South Korea. Each strain was individually cultivated in 10 mL of Tryptic Soy Broth (TSB, Becton Dickinson Diagnostic Systems, Sparks, MD, USA) supplied with 2% NaCl at 35 °C for 20 h to obtain a cell density of 9 log CFU/mL. Cell suspension was washed twice with 0.1% sterile Buffered Peptone Water (BPW). Cell pellet was obtained after centrifugation at 4000 × *g* for 10 min at 4 °C and resuspended in 10 mL of BPW. Four different cocktails were made by mixing bacterial suspension in same proportion (10 mL for each strain) according to each bacterium. Bacterial population in each inoculum was confirmed by spreading 100 μL of the inoculum from desired dilution onto two Tryptic Soy Agar (TSA, Difco, Becton, Dickinson and Company Sparks, USA) plates. These plates were incubated at 37 °C for 24 h and the number of colonies was counted.

### 2.2. Samples preparation and inoculation

Fresh spinach (*Spinacia oleracea* L.), iceberg lettuce leaves (*Lactuca sativa* L.), and vacuum-packaged alfalfa sprouts (*Medicago sativa* L.) were purchased from supermarkets at Chuncheon city, South Korea. These samples were transported to the laboratory using ice bags. They were stored at 4 °C until further experimental. Debris and other particles of leaves (spinach and lettuce) were washed with running tap water to reduce natural microbiota load, dried inside a laminar flow safety cabinet, and exposed to UV for 40 min. These samples were divided into two groups. One group was processed for decontamination treatment. The other group was processed for the shelf-life study. These leaves were inoculated with 1 mL of each bacterial cocktail on the abaxial-side of each leaf surface. Approximately 50 g of sprouts were separately immersed in each bacterial cocktail (2 mL of bacteria cocktail was individually diluted in 200 mL 0.1% BPW) for 45 min using a shaking incubator at room temperature to allow bacteria to equitably distribute on food surface. After bacterial inoculation, sprouts were removed using a sterile metal sieve, dried in a laminar flow safety cabinet for 1 h, divided into 10 g portions in a stomacher bag, and stored at 4 °C for 24 h. After bacterial inoculation, 10 g of each inoculated vegetable was set aside for microbiological analysis to determine the initial population of each bacterium.

### 2.3. Sanitizer solution preparation

SAEW (30 ppm) was generated by electrolysis of a combined solution of HCl (5%) and NaCl (2 M) in an electrolytic cell without membrane. This was a self-developed electrolyzed water generator. The flow rate of incoming tap water was adjusted to 4 L/min. The amperage and voltage were set at 10 A and 3.2 V, respectively. Electrolyte flow rate was set at 2 mL/min. The EW was collected at 30 min after starting the generator to allow amperage and voltage to reach steady state. After preparation, SAEW was either kept in polypropylene containers (BioTank, Komax, Korea) or immediately used for experimentation. Fumaric acid (FA) was directly diluted with sterile distilled water (DW) using a magnetic stirrer to obtain a final concentration of 0.5%. A solution of CaO (0.2%) was provided by Eco-Biotech Company (Hwaseong-Si, Gyeonggi-Do, Korea). The pH (5.3–5.5) and oxidation reduction potential of 818–854 mV of all SAEW were measured with a dual – scale pH meter (Accumet model 15, Fisher Scientific Co., Fair Lawn, NJ, USA). Available chlorine concentration (ACC) was determined using a digital chlorine test kit (RC-3F, Kasahara Chemical Instruments Corp., Saitama, Japan) with a colorimetric method. Combined FA + SAEW was prepared by adding FA to SAEW to obtain desired concentration.

### 2.4. Physical treatment

Six liters of sanitizer (FA + SAEW) was filled into a bench-top ultrasonic cleaner (JAC-4020, KODO Technical Research Co., Ltd., Hwaseong, Gyeonggi-do, South Korea). The device was set at a frequency of 40 kHz with acoustic energy density of 400 W/L. For microbubbles treatment, ultrasonic cleaner was filled with 6 L of FA + SAEW and connected to cable with micro-bubbles generators (Moming spa DC 40 V, Moming Co., Ltd., Seoul, Korea) to produce FA + SAEW microbubbles (FA + SAEW + MB). Ultrasonication and micro-bubbles treatments were performed by immersing samples at room temperature (23 ± 2 °C) for 3 min after CaO washing. Ultraviolet treatment was carried after sanitizers treatment (CaO + SAEW + FA) using ultraviolet light (TUV 15 W; Philips Lighting, Roosendaal, Netherlands) at a distance of 30 cm in a ultraviolet cabinet (Entkeimung schrank, 220 V, Ernst Schuttjun Laborgerotebau, 3400, Gottingen, Germany) for 10 min.

### 2.5. Decontamination procedure

For single treatments, samples (uninoculated and inoculated) were washed with 200 mL solutions (DW, SAEW, FA, and CaO). All treatments were performed at room temperature (23 ± 2 °C) for 3 min. For combined treatment, samples were divided into four groups (Table 1). For the first group, samples were washed with SAEW + FA for 3 min.