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Efficacy of washing with hydrogen peroxide followed by aerosolized antimicrobials as a novel sanitizing process to inactivate *Escherichia coli* O157:H7 on baby spinach

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ABSTRACT

Aerosolization was investigated as a potential way to apply allyl isothiocyanate (AIT), hydrogen peroxide (H_2O_2), acetic acid (AA) and lactic acid (LA) on fresh baby spinach to control *Escherichia coli* O157:H7 during refrigeration storage. In this study, baby spinach leaves were dip-inoculated with *E. coli* O157:H7 to a level of 6 log CFU/g and stored at 4 °C for 24 h before treatment. Antimicrobials were atomized into fog-like microparticles by an ultrasonic nebulizer and routed into a jar and a scale-up model system where samples were treated. Samples were stored at 4 °C for up to 10 days before the survival of the cells was determined. A 2-min treatment with 5% AIT resulted in a >5-log reduction of *E. coli* O157:H7 on spinach after 2 days refrigeration regardless if the samples were pre-washed or not; however, this treatment impaired the sensory quality of leaves. Addition of LA to AIT improved the antimicrobial efficacy of AIT. In the jar system, washing with 3% H_2O_2 followed by a 2-min treatment of 2.5% LA + 1% AIT or 2.5% LA + 2% AIT reduced *E. coli* O157:H7 population by 4.7 and >5 log CFU/g, respectively, after 10 days refrigeration. In the scale-up system, up to 4-log reduction of bacterial population was achieved for the same treatments without causing noticeable adverse effect on the appearance of leaves. Thus, this study demonstrates the potential of aerosolized AIT + LA as a new post-washing intervention strategy to control *E. coli* O157:H7 on baby spinach during refrigeration storage.

1. Introduction

The United States is the world's second-largest producer of spinach, accounting for 4% of world output (Richter, 2004). Driven by increasing fresh-market use, production of spinach for the fresh produce market in the United States has been dramatically increasing from 61.1 million lb. in 1970 to 623.9 million lb. in 2009 (Economic Research Service, 2010). However, there has been a great concern among consumers about the safety of fresh produce due to several large high-profile outbreaks. In 2006, a multi-state outbreak of *Escherichia coli* O157:H7 infection associated with contaminated bagged baby spinach resulted in 205 confirmed cases of illness, 31 cases of hemolytic-uremic syndrome and three deaths (CDC, 2006).

Although many studies have demonstrated that washing with chlorine cannot eliminate pathogens on fresh produce, it is still an important step in the industry to clean soil, dust and insects from fresh produce and to prevent cross-contamination. Due to possible formation of carcinogenic organochlorine compounds when chlorine is used in the presence of organic materials, numerous studies have investigated various types of alternative aqueous sanitizers such as electrolyzed water, chlorine dioxide, hydrogen peroxide (H_2O_2), organic acids, and ozone (Huang and Chen, 2011; Keskinen et al., 2009; Kim et al., 2008; Wei et al., 2005; Yuk et al., 2006). Generally, these sanitizers could only achieve a <2-log reduction of the population of microorganisms, although some studies reported higher reductions depending on the target microorganism, type of produce, inoculation and enumeration methods, and treatment methods (Deza et al., 2003; Karapinar and Conul, 1992; Park et al., 2008). The ineffectiveness of aqueous sanitizers on fresh produce stems largely from inefficiency in delivering lethal aqueous chemical components to access pathogen cells lodged at protected sites on the surface or sub-surface of fresh produces (Burnett and Beuchat, 2001), and thus more effective decontamination methods are needed.

Aerosolization, which can disperse liquid as a fine mist in air, has been widely used as a drug delivery system for respiratory diseases. Recently, aerosolized antimicrobials have shown promise in improving the safety of fresh produce due to their greater penetration ability and effectiveness than their aqueous counterparts. Oh et al. (2005b) reported that aerosolized antimicrobials diffuse like gas and that diffusion of aerosolized antimicrobials in a chamber was not affected by the height or orientation of the chamber, which is not the case for a trigger spray system. The same group also assessed the efficacy of aerosolized peroxyacetic acid as a sanitizer for lettuce leaves (Oh et al., 2005a). The populations of *E. coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* were reduced by 3.4, 4.5 and 3.8 log, respectively after a 60-min treatment. Fiser (1978) used aerosolized

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lactic acid (LA) to disinfect chicken house and reported that continuous disinfection by aerosolized LA resulted in an improved health of chickens. Thus, aerosolization, with its high penetration ability and broad spectrum of applicable sanitizers, has the potential to be used as an alternative sanitizer delivery system for fresh produce. However, very few studies have investigated the effectiveness of aerosolization of sanitizers against pathogens on fresh produce.

Allyl isothiocyanate (AIT), a natural compound present in all plants belonging to the family Cruciferae, has strong antimicrobial activity in both liquid and vapor forms (Lin et al., 2000a). AIT is a Generally Recognized as Safe (GRAS) substance and is exempted from the requirement for a residue tolerance in or on all raw agriculture commodities by the Environmental Protection Agency (Code of Federal Regulations, 1996). It can be commonly found in shredded cabbage and coleslaw (West et al., 1977) and food condiments such as mustard, wasabi and mayonnaise (Delaquis and Sholberg, 1997). In Japan, purified AIT is permitted for use as a food preservative and is used for packaging of raw oysters, pickled vegetables, ham and cheese slices (Cha and Chinnan, 2004). Lin et al. (2000a) reported that AIT vapor at 76.0 to 101.3 mg/L eliminated 10^4 to 10^5 CFU/g of *E. coli* O157:H7 and *Salmonella* Montevideo inoculated on lettuce in 2 days, and a lower concentration of AIT could be used as a processing aid to help control potential pathogens on fresh fruits and vegetables. H_2O_2, LA and AA also have GRAS status (CFR, 2010a, 2010b, 2010c). Our previous study showed that washing with these antimicrobials can achieve better inactivation of *E. coli* O157:H7 on baby spinach than chlorine washing (Huang and Chen, 2011). H_2O_2 has broad antimicrobial activity and improves the sensory quality and shelf life of some fresh produce (Lin et al., 2002; McWatters et al., 2002; Sapers et al., 2001), and thus is a potential alternative to chlorine. LA and AA were reported to inhibit the growth of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. on fresh produce (Akbas and Ölmez, 2007; Beuchat, 1998; Venkitanarayanan et al., 2002).

Therefore, the objective of this study was to evaluate the efficacy of aerosolized antimicrobials (AIT, H_2O_2, LA and AA) alone or in combination with H_2O_2 washing against *E. coli* O157:H7 on baby spinach as a new postharvest intervention strategy.

2. Materials and methods

2.1. Bacterial strains

Two strains of *E. coli* O157:H7 (250, sprout outbreak isolate, courtesy of Dr. Kniel, University of Delaware; DD 3795, Dupont culture collection, courtesy of Dr. Joerger, University of Delaware) were used in this study. They were adapted to grow on tryptic soy agar (Difco Laboratories, Sparks, MD, USA) supplemented with 0.6% yeast extract (Difco Laboratories), 100 μg/mL of nalidixic acid (Fisher Scientific, Hampton, NH, USA) and 100 μg/mL of streptomycin (streptomycin sulfate salt, Sigma-Aldrich, MO, USA) (TSAYE-NS). Briefly, single colonies of each parental strain were inoculated into 10 mL of tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) and grown for 24 h at 35 °C. Then, 1 ml of each individual culture was spread plated on TSAYE supplemented with 100 μg/mL nalidixic acid (TSAYE-N). The plates were incubated at 35 °C for 3–5 days. Single colony of each strain formed on TSAYE-N were then selected and inoculated into TSBYE supplemented with 100 μg/mL nalidixic acid (TSBYE-N), and sub-cultured in TSBYE-N for 2 consecutive 24-h intervals. One ml of each individual culture was then spread plated on TSAYE-NS. The plates were incubated at 35 °C for 3–5 days and typical *E. coli* O157:H7 colonies were picked and streaked on both TSAYE-NS and Sorbitol Macconkey (Difco Laboratories) supplemented with 100 μg/mL of nalidixic acid and 100 μg/mL streptomycin (SMAC-NS).

Each strain was also examined for its potential to inhibit growth of the other test strain following the method described by Lang et al. (2004). Briefly, each strain was grown in 10 mL of tryptic soy broth (Fisher Scientific) supplemented with 0.6% yeast extract, 100 μg/mL nalidixic acid and 100 μg/mL streptomycin (TSBYE-NS) at 35 °C for 24 h. Cultures of each strain were cross-streaked onto TSAYE-NS and incubated at 35 °C for 24 h. Plates were then examined for inhibition of growth at junctions of cross-streaks of test strains.

2.2. Preparation of inoculums

Single colonies of each stain were inoculated into 10 mL of TSBYE-NS and grown at 35 °C for 24 h. The individual cultures were then transferred to fresh tubes of TSBYE-NS and incubated at 35 °C for another 24 h. 15 mL of each culture was mixed to form a two-strain cocktail, harvested by centrifugation at 2450 *g* for 10 min (Centra CL2, Centrifuge, Thermo Scientific, USA). The pellet was re-suspended in 30 mL of sterile 0.1% peptone water (Fisher Scientific) to form cell suspension with a final concentration of 10^8 to 10^9 CFU/mL.

2.3. Inoculation of baby spinach

Boxed baby spinach was purchased from local grocery stores, stored at 4 °C and used within 2 days. Since washing treatment was reported to be less effective on dip inoculated lettuce than on drop (spot) inoculated samples (Singh et al., 2002), the dip-inoculation method was used in our study to mimic the worst case scenario of contamination. Briefly, a 30-mL cocktail of *E. coli* O157:H7 was mixed with 1 L of sterile 0.1% peptone water in a sterile stomach bag. Intact and un-wilted spinach leaves (40 g) were submerged in the cell suspension and the bag was heat-sealed. The bag was then gently massaged for 2 min, cut open and the liquid was gently drained. The spinach leaves were placed on a sterile wire screen and dried inside a bio-safety hood for 10–15 min at room temperature (22 ± 1 °C). This drying time was found to be optimal since longer drying time could cause wilting of the leaves. Leaves with an approximate inoculation level of 10^6 CFU/g of *E. coli* O157:H7 were stored in an alcohol-sterilized plastic box at 4 °C for 24 h to facilitate the attachment of bacteria. Inoculated spinach without treatment was used to determine the initial counts.

2.4. Effectiveness of aerosolized antimicrobials against E. coli O157:H7 on baby spinach stored at 4 °C for 2 days — screening study

A 1-L clear tall wide mouth glass jar (I-Chem, Thermo Scientific) was used as a prototype model system to test the efficacy of aerosolized antimicrobials. The jar was sealed, and aerosolized antimicrobials were routed from an ultrasonic nebulizer (Ultrasonic aromatherapy nebulizer, Hubmar, Quebec, Canada) through a flexible tygon tube as shown in Fig. 1. The tube entered the cap of the jar

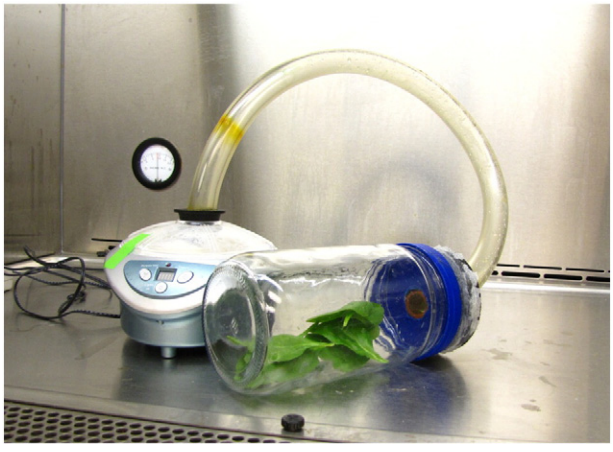

Fig. 1. Treatment of aerosolized antimicrobials in a prototype model system.