INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON BABY SPINACH BY AQUEOUS AND AEROSOLIZED ANTIMICROBIALS

by

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ABSTRACT

An increasing number of outbreaks associated with fresh produce such as baby spinach have been reported in the last two decades with *Escherichia coli* O157:H7 being one of the most frequent causative agents. Chlorinated water has been widely used by food industry to wash these fresh commodities, but this washing procedure has limited efficacy and can lead to the formation of carcinogenic substances. Thus, more effective and safer treatment methods are needed to control pathogens in fresh produce. In our first study, several generally recognized as safe organic acids and hydrogen peroxide in combination with mild heat (40 and 50 °C) was tested for their efficacies against *E. coli* O157:H7 on baby spinach. Chlorinated water (200 ppm free chlorine) only reduced the population of *E. coli* O157:H7 on baby spinach by 1.2-1.6 log CFU/g, which was not significantly different from DI water washing. Washing with 1% lactic acid (LA) alone or in combination with citric acid or hydrogen peroxide (H_2O_2) at 40 °C for 5 min was the most effective treatment achieving a 2.7 log reduction of *E. coli* O157:H7. In the second study, aerosolization was investigated as a potential way to apply allyl isothiocyanate (AIT), H_2O_2, acetic acid (AA) and LA on fresh baby spinach to control *E. coli* O157:H7 during 10 days of storage at 4 °C. Treatment of aerosolized 5% AIT resulted in > 5 log reduction of *E. coli* O157:H7 on spinach regardless if the samples are pre-washed or not; however, this treatment impaired the sensory quality of leaves. Addition of LA to AIT improved the efficacy of AIT against *E. coli* O157:H7 during the storage. Treatment of 3% H_2O_2 washing followed by a 2-min treatment of aerosolized 1% AIT + 2.5% LA reduced *E. coli* O157:H7 on spinach leaves by 4.8 log CFU/g after 10 days storage at 4 °C

without causing noticeable adverse effects on the appearance of samples. Therefore, low dose application of AIT in combination with LA has the potential to control *E. coli* O157:H7 on fresh produce.

Chapter 1

INTRODUCTION

Fresh fruit and vegetables are an indispensable part of a healthy diet. The United States consumption of fresh fruits and vegetables per capita increased from 83.1 and 164.1 pounds in 1976 to 103.0 and 221.2 pounds in 2006, respectively (Cook, 2008). Driven by increasing fresh-market use, production of spinach for the fresh market in the US has been dramatically increasing from 61.1 million lb. in 1970 to 623.9 million lb. in 2009 (Economic Research Service, 2010), which also make the United State the world's second-largest producer of spinach, accounting for 4 percent of world output (Richter, 2004). However, fruits and vegetables, in particular leafy greens that are consumed raw, are increasingly being recognized as important vehicles for transmission of human pathogens. In the last two decades, there has been an increase in the frequency of outbreaks of foodborne illnesses associated with consumption of these foods. From 1982 to 2002, 49 states reported 350 outbreaks, representing 8,598 cases, in which 52% were foodborne and 21% of the foodborne outbreaks were due to fresh produce such as lettuce, spinach, grapes and sprouts (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005).These outbreaks have caused heightened concern about the safety of fresh produce (Altekruse, Cohen, & Swerdlow, 1997; Beuchat, 1996).

Escherichia coli O157:H7 has emerged as an important public health concern in the North America since it was implicated in two outbreaks of a distinctive

bloody diarrheal syndrome in 1982 (Griffin & Tauxe, 1991; Riley et al., 1983). This pathogen is very hardy, since it can grow at pH levels ranging from 4.4 – 9.0, a_w of > 0.95 and NaCl levels of < 8.5% (Riemann & Cliver, 2006). The infectious dose of *E. coli* O157:H7 was estimated to be in the range of 4 to < 40 CFU/g of food (Strachan, Ogden, & Fenlon, 2001; Teunis, Takumi, & Shinagawa, 2004). It has been estimated that *E. coli* O157:H7 causes 73,480 cases of illness and 61 deaths each year in the United States (Mead et al., 1999). *E. coli* O157:H7 is also the most common cause of hemolytic uremic syndrome (HUS) and the leading cause of kidney failure among children in the United States (Slutsker, Altekruse, & Swerdlow, 1998). In 2006, a multi-state outbreak of *E. coli* O157:H7 infection associated with contaminated bagged baby spinach resulted in 205 confirmed cases of illness, 31 cases of HUS and three deaths (CDC, 2006; FDA, 2009).

Although every processor may have different procedures, leafy greens such as baby spinach usually go through a triple-wash process (Sapers, Solomon, & Matthews, 2009), in which chlorinated water of 50 – 200 ppm is widely used as the primary postharvest sanitizing agent because of its broad antimicrobial activity and low cost. However, this sanitation procedure can only achieve a < 2-log reduction in the counts of *E. coli* O157:H7 (Beuchat, Nail, Adler, & Clavero, 1998). In addition, chlorine compounds can be inactivated by organic materials on fresh produce and possibly form various carcinogenic organochlorine compounds (CDC, 2009; Richardson et al., 1998) Therefore, food industry has been looking for alternatives to chlorine to control pathogens in fresh produce.

Most of organic acids such as lactic acid, acetic acid, citric acid, malic acid and tartaric acid are natural compounds that are generally recognized as safe

(GRAS). They are known to have antimicrobial activity and are potential disinfectants for fruits and vegetables (Beuchat et al., 1998). Lactic acid, acetic acid and citric acid inhibit growth of *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. on fresh produces (Akbas & Ölmez, 2007; Hwang & Beuchat, 1995; Laury et al., 2009; Rhee, Lee, Dougherty, & Kang, 2003; Venkitanarayanan, Zhao, & Doyle, 1999). Malic acid and tartaric acid have bactericidal effects against *E. coli* O157:H7 (Conner & Kotrola, 1995). Hydrogen peroxide (H_2O_2) is also used as an antimicrobial compound due to its strong oxidative activity. Sapers & Sites (2003) reported that 1% hydrogen peroxide had an equal or better effect against *E. coli* O157:H7 than 200 ppm chlorine when applied on apples. Treatment of lettuce with 2% H_2O_2 at 50°C yielded a 4-log reduction of *E. coli* O157:H7 (Lin, Moon, Doyle, & McWatters, 2002). Recently, it has been reported that exposure to mild heat of 40 – 50°C could reduce browning and improve the quality of lettuce and spinach (Delaquis, Stewart, Toivonen, & Moyls, 1999; Gomez et al., 2008; Murata, Tanaka, Minoura, & Homma, 2004; Roura et al., 2008; Roura, Valle, & Pereyra, 2008), and that it enhanced the antimicrobial effect of sanitizers and sensory of packaged leafy greens during storage (Delaquis, Stewart, Cazaux, & Toivonen, 2002; Venkitanarayanan et al., 1999).

Gaseous sanitizers have showed some promises on improving the safety of fresh produce due to their greater penetration ability and effectiveness than aqueous sanitizers (Delaquis, Sholberg, & Stanich, 1999; Han, Sherman, Linton, Nielsen, & Nelson, 2000; Himathongkham, Nuanualsuwan, Riemann, & Cliver, 2001; Lee, Costello, & Kang, 2004; G. M. Sapers, Walker, Sites, Annous, & Eblen, 2003; Simmons, Smilanick, John, & Margosan, 1997); however, the need for sophisticated equipment and the low number of applicable sanitizers limited it use. Recently, it has

been reported that aerosolized sanitizers diffuse like gaseous sanitizers and that, in contrast to sprays generated with a spraying system, diffusion of aerosolized sanitizers in a chamber was not affected by height or orientation (Oh et al., 2005). Thus, aerosolization, with its high penetration ability and broad spectrum of applicable sanitizers, has the potential to be an alternative sanitizer delivery system. Aerosolized lactic acid has been used to disinfecting chicken house. Fiser (1978) reported that the continual disinfection by aerosolized lactic acid resulted in an improved state of health of chickens. However, very few studies have investigated the effectiveness of aerosol of these sanitizers against pathogens on fresh produce.

Allyl isothiocyanate (AIT), a natural compound present in all plants belonging to the family Cruciferae, has anticancer (Zhang, 2010) and strong antimicrobial activity in both liquid and vapor forms (Lin, Preston, & Wei, 2000). It is commonly found in food such as shredded cabbage and coleslaw (West, McLaughlin, & Badenhop, 1977) and in food condiments such as mustard, wasabi and mayonnaise (Delaquis & Sholberg, 1997). Lin, Kim, Du, & Wei (2000) showed that AIT vapor at 76.0 – 101.3 mg/L eliminated *E. coli* O157:H7 and *Salmonella* Montevideo inoculated at 10^4 – 10^5 CFU/g on lettuce within 2 days, and that a lower application rate of AIT could be used as a processing aid to help control potential pathogens on fresh fruits and vegetables.

Therefore, the objectives of our research were to (i) evaluate the effectiveness of various organic acids and hydrogen peroxide alone or in binary combinations on the inactivation of *E. coli* O157:H7 on baby spinach, (ii) investigate possible synergistic antimicrobial effects between these sanitizers and mild heat, (iii) determine the effectiveness of aerosolized antimicrobials alone, and (iv) develop a

new hurdle sanitizing process to control *E. coli* O157:H7 on baby spinach.

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Chapter 2

LITERATURE REVIEW

2.1 *Escherichia coli* O157:H7

2.1.1 History

E. coli O157:H7 is a relatively new pathogen. It was first described in 1977 by Konowalchuk, Speirs, & Stavric (1977) who found that certain diarrheagenic *E. coli* strains produce a cytotoxin that can kill Vero cells, thus they named it verotoxin (VT). In 1982, *E. coli* O157:H7 was recognized for the first time as a human pathogen when two outbreaks occurring in Oregon and Michigan were associated with eating undercooked hamburgers from a fast food restaurant chain (Riley et al., 1983). This outbreak was characterized by a distinctive hemorrhagic colitis, and a rare isolate of O157:H7 serotype of *E. coli* was implicated as the agent of disease. Searches of culture collections in the USA dating from 1973, and in Canada and the UK dating from 1978, found only eight *E. coli* O157 isolates deposited before 1982 (Griffin & Tauxe, 1991). Unfortunately, it was not until 1993, following a large outbreak with more than 700 cases infected by eating contaminated fast food hamburgers, that *E. coli* O157:H7 was recognized as a major food safety issue (Bell et al., 1994). During the past 30 years, an increasing number of *E. coli* O157:H7 outbreaks have gained a worldwide niche as a formidable public-health concern. In 1994, *E. coli* O157:H7 became a nationally notifiable infection and by 2000, reporting was mandatory in 48

states (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). The US Department of Agriculture (USDA) has established a 'zero tolerance' policy for *E. coli* O157:H7 on ground beef. However, this policy has not resolved the problem, as an estimated 73,480 illnesses due to *E. coli* O157:H7 infection occur in the United States every year, leading to an estimated 2,168 hospitalizations and 61 deaths annually (Mead et al., 1999).

2.1.2 Taxonomy

E. coli is a Gram-negative, non-spore-forming facultative anaerobe in the family of *Enterobacteriaceae*. Serotyping and serogrouping of *E. coli* is used for subdividing the species into serovars. Serotyping in *E. coli* involves serological identification of three surface antigens: O (somatic lipopolysaccharide), K (capsular) and H (flagellar). Orskov and Orskov (1992) estimated that 173 O antigens, 80 Kantigens and 56 H antigens existed. Based on the presence of certain virulence factors and their interaction pattern with mammalian cells or tissues and toxin production, pathogenic *E. coli* are also classified in six virotypes (1) enterotoxigenic *E. coli* (ETEC), (2) enteropathogenic *E. coli* (EPEC), (3) enterohemorrhagic *E. coli* (EHEC), (4) enteroinvasive *E. coli* (EIEC), (5) enteroaggregative *E. coli* (EAEC), (6) diffusely adhering *E. coli* (DAEC) (Bhunia, 2008). The first documented and most well studied EHEC strain is *E. coli* O157: H7 which is also considered the prototypical serotype of EHEC.

2.1.3 Biology

Most of *E. coli* are generally considered to be harmless as members of the indigenous microbiota in the gut of humans and warm-blooded animals; however,

there are also some serotypes that are responsible for causing severe foodborne disease such as bloody diarrhea and hemolytic uremic syndrome (HUS), and the disease is prevalent in developed countries such as the United America, Japan and the United Kingdom. As opposed to other commensal strains, *E. coli* O157: H7 generally does not ferment sorbitol and does not have β-glucuronidase activity, which is a very useful marker for bacterial identification. It grows rapidly at 30 – 42°C, does not grow at 10 °C or below and does not grow or grows poorly at 44°C or above with an optimum temperature of 37 °C (Bhunia, 2008). The organism is destroyed by thorough cooking of foods when all parts reach a temperature of 70 °C or higher, however, it can survive for weeks at 4 °C or even -20°C. Although there is some variability among strains, this pathogen is relatively acid-tolerant and can grow at pH levels ranging from 4.4 – 9.0, a_w of > 0.95 and NaCl levels of < 8.5%. In foods at pH levels of 3.5 – 5.5, it can survive for extended periods (Riemann & Cliver, 2006).

E. coli O 157:H7 can produce two different Shiga-like toxins, namely SLT1 and SLT2. These Shiga-like toxins are very similar, if not identical, to the toxins produced by *Shigella dysenteriae.* Due to their toxic effects on Vero (African green monkey kidney) cells, *E. coli* O157:H7 is also known as verotoxin-producing *E. coli* (VTEC). The growth of this pathogen in the human body produces a large quantity of these toxins that cause severe damage to the lining of the intestine and may cause additional damage to kidneys, pancreas and brain. In addition to Shiga-like toxins, *E. coli* O157:H7 can produce other virulence factors that may increase the severity of human illnesses. These virulence factors include intimin and enterohemolysin, which are responsible for intimate attachment to the intestinal surface and enterocyte damage, respectively (Riemann & Cliver, 2006).

2.1.4 Prevalence of *E. coli* O157:H7

Generally, ruminants, particularly cattle are a major reservoir for human infection with *E. coli* O157:H7. Because cattle lack the receptor for the Shiga-like toxin, *E. coli* O157:H7 can persist in cattle without causing disease. As many as half of all cattle carry this pathogen at some time in their lives, and some are 'super spreaders' of the organism (Matthews et al., 2006). In addition to cattle, the organism has been isolated from deer, sheep, goats, horses, dogs, birds and flies (Chapman, Siddons, Malo, & Harkin, 1997; Hancock et al., 1998). Milnes et al. (2008) reported that intestinal contents of 4.7% of cattle, 0.7% of sheep, and 0.3% of pigs tested positive for *E coli* O157 at slaughter.

Many studies have measured the prevalence of *E coli* O157:H7 in cattle. Hussein & Bollinger (2005b) reviewed published reports in the past 3 decades and concluded that the prevalence rates of *E. coli* O157:H7 ranged from 0.3 – 19.7% in feedlot cattle, from 0.7 – 27.3% in cattle on irrigated pasture, and from 0.9 – 6.9% in cattle grazing rangeland forages. Global testing of beef showed wide ranges of prevalence rates of *E. coli* O157:H7 (from 0.01 – 54.2%) (Hussein & Bollinger, 2005a). For dairy cattle, the prevalence estimated by testing feces ranged from 0.4 – 40% in the USA and Canada (Pennington, 2010). Although the result of these surveys may vary, these observations all indicated that cattle are an important source of *E. coli* O157:H7. In addition, a seasonal influence is clear, with higher shedding of the pathogen in warmer months than in cooler months (Joseph, Ingram, & Kaper, 2002). McGee et al. (2002) suggested that cattle do not harbor the organism on a continuous basis, but are susceptible to re-infection. Other vectors could also exist to reintroduce *E. coli* O157:H7 to cattle, and so maintain its persistence on-farm.

Environmental studies have shown that *E. coli* O157:H7 can persist in

manure, water troughs, and other places on farms (Hancock et al., 1998). Jiang, Morgan, & Doyle (2002) reported that this pathogen survived for 77, > 226 and 231 days in manure-amended autoclaved soil stored at 5, 15 and 21°C, respectively. A survey conducted in four major feeder-cattle states in the USA showed that 10.2% of fecal samples (out of 10,662 samples tested) and 13.1% of water or water-tank sediment samples were positive for *E. coli* O157:H7, with over 60% of feedlots having at least one positive water or water sediment sample (Sargeant, Sanderson, Smith, & Griffin, 2003). LeJeune JT, Besser TE, & Hancock DD (2001) suggested that contaminated troughs can act as long-term reservoirs of *E. coli* O157:H7 with a potential for infection of cattle weeks or months later. The study of Lynn TV et al. (1998) revealed that *E. coli* O157:H7 can survive in water trough sediments for at least 4 months and persists even though troughs are cleaned at intervals of > 6 months on most dairy farms. *E. coli* O157:H7 can also grow in acidic conditions such as corn silage since they are acid-resistant. A study conducted in Midwest showed that the prevalence of *E. coli* O157:H7 in feedlot was as high as 14.9% (75 out of 504 samples) (Dodd CC et al., 2003).

2.2 Food association and outbreaks of *E. coli* O157:H7

2.2.1 Fresh produce production

In the USA, leafy vegetable production moves from California to Arizona and Mexico as the seasons change, ensuring a constant supply of production throughout the year (Sapers, Solomon, & Matthews, 2009). The United States is the world's second-largest producer of spinach, accounting for 4 percent of world output, following China, which contributes 76 percent of the world output (Richter, 2004).

Driven by increasing fresh-market use, production of spinach for the fresh market in the US has been dramatically increasing from 61.1 million lb. in 1970 to 623.9 million lb. in 2009 (Economic Research Service, 2010). Much of the growth over the past decades has been due to sales of triple-washed cello-packaged spinach and, more recently, baby spinach. These packaged products have been one of the fastest-growing segments of the packaged salad industry (Economic Research Service, 2007).

Fresh market spinach destined for bagged spinach or salad mixes is usually mechanically harvested. The leaves are lifted by conveyor belt into bins on trailers and transported to the processing plant for sorting, flume washing, centrifugation or forced-air drying and packaging into a variety of different bagged or boxed products. All produce intended for processing may be stored for a short period before processing. Cooling is generally achieved under forced air or vacuum, but more gradual reduction in temperature in passive storage is still common (Delaquis, Bach, & Dinu, 2007; Koike, 2011).

2.2.2 Contamination sources of *E. coli* O157:H7

Fresh produce can become contaminated with pathogenic microorganisms while still in fields, or at various points during harvesting, postharvest handling, processing, and distribution in the production chain. Some factors may contribute to pre-harvest contamination of fresh produce, which include use of improperly composted manure as fertilizer, use of contaminated irrigation water, flooding of fields, wild animals and insects, etc.

All spinach fields in California are sprinkler irrigated to germinate the seed and most growers water the entire crop with sprinklers to minimize labor (Koike, 2011); however, continued use of overhead water favors contamination with various

pathogenic and spoilage bacteria. A study designed to track *E. coli* O157:H7 in a major produce production region of California suggests that the pathogen, when found in water, is generally close to a point source (Cooley et al., 2007). The incidence of *E coli* O157:H7 increased significantly when heavy rain caused an increased flow rate in the rivers. The authors also pointed out that in periods of high water-flow, often associated with flooding, the pathogen may be transported over 30 km. Okafo, Umoh, & Galadima (2003) reported that unregulated release of untreated sewage into river and streams can result in contamination of water source, which place any crops irrigated with the water at risk of being contaminated.

Animal manure is also considered as one of the most important source of contamination as animal manure is often used as soil amendments. *E. coli* O157:H7 may be present in these manure, if not composted or thermo-treated properly, and contaminate leafy greens (Sapers et al., 2009). Mootian, Matthews, & Wu (2009) showed that about 30% of the lettuce samples initially irrigated with or grown in contaminated soil (including manure-amended soil) for 15 days were positive for *E. coli* O157:H7. In order to decrease the risk of "manure-borne" pathogens, the USDA requires that application of non-composted manure into the soil should be more than 120 days prior to the harvest of a product whose edible portion has direct contact with the soil surface or soil particles (USDA, 2000).

Post-harvest contamination may occur from cross-contamination of washing water, human handling, transport containers and processing equipments. The handling of fresh produce during and immediately postharvest could have great impact on microbial safety of the product. In a study that simulated handling practices in a restaurant-associated outbreak, Wachtel & Charkowski (2002) reported that *E. coli*

O157:H7 can be transferred between cut lettuce slices by both mixing under dry conditions and submersion in water. This observation suggest that a few contaminated leaves can cross-contaminate a large mass of cut lettuce. Pathogens attached to contaminated leaf surfaces can remain viable for long period and can even grow depending on the storage temperature, moisture content and nutritional availability (Delaquis et al., 2007; Khalil RK & Frank JF, 2010). Although there is no evidence to show that enteric pathogens, such as *E. coli* O157:H7, can become established in the processing environment, Delaquis et al. (2007) suggest that it is distinctly possible that cross-contamination within discrete lots via contact with contaminated equipment surfaces can occur. Gagliardi, Millner, Lester, & Ingram (2003) also implicated process water used for cooling and washing melons as a source of contamination. As a result of the concerns regarding food production, safety and quality, the concept of Good Agricultural Practices (GAP) has evolved in recent years and efforts are underway by governments and industry to develop and apply GAPs, Good Manufacturing Practices (GMPs) and Hazard Analysis & Critical Control Points (HACCP) throughout the food chain.

2.2.3 Foodborne diseases and outbreaks of *E. coli* O157:H7

E. coli O157:H7 infections are principally transmitted via consumption of contaminated food or water, although direct transmission from one person to another and occupational exposure may also lead to infections of this pathogen. The clinical manifestations of *E. coli* O157 infection range from symptom-free carriage to non-bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and death. The average interval between exposure and illness is 3 days; incubation periods as short as 1 day and as long as 8 days have been reported (Mead & Griffin, 1998). HC

is a distinct clinical syndrome that is characterized by sudden abdominal pain, followed within 24 h by the onset of non-bloody diarrhea and leading to subsequent bloody diarrhea within several days (S. S. Park, Worobo, & Durst, 2001). Most patients with HC recover spontaneously within 7 days. Over 70% of patients report bloody diarrhea in most outbreaks (Slutsker L et al., 1997). Vomiting occurs in 30 – 60% of cases, and fever, usually low grade, can be documented in only 30%. HUS is the most common cause of acute renal failure in children. In HUS, the patient suffers form bloody diarrhea, hemolytic anemia, kidney disorder and renal failure. Central nervous disease may develop which can lead to seizures, coma and death (Forsythe, 2010). About 10 – 15% of patients infected with *E. coli* O157:H7 develop HUS 5 – 13 days after the onset of diarrhea (Pennington, 2010). Mortality is approximately 5% and approximately 10% of survivors are left with severe sequelae (Slutsker, Altekruse, & Swerdlow, 1998).

Because of the extensive critical disease syndromes caused by *E. coli* O157:H7, human volunteer studies have not been performed. According to the Food and Drug Administration (FDA) of the United States, the infectious dose for *E. coli* O157:H7 is not known. However, the estimated infectious dose of *E. coli* O157:H7 in contaminated food has been estimated to range from 4 to < 40 CFU/g of food (Strachan, Ogden, & Fenlon, 2001; Teunis, Takumi, & Shinagawa, 2004). The incidence of *E. coli* O157:H7 infections varies by age, with the highest incidence of reported cases occurring in children aged under 15 years (0.7 cases per 100,000 in the USA) (WHO, 2005). It was concluded that several factors could be attributed to the reason for the low *E. coli* O157:H7 infectious dose. Acid tolerance resulting in better survival through the indigestive system, quorum sensing and various virulence factors

likely contribute to this organism's infectivity (Forsythe, 2010).

Results from a study of 90 outbreaks confirmed microbiologically in the UK, Ireland, Denmark, Norway, Finland, USA, Canada, and Japan, occurring between 1982 and 2006, showed that food was the source of transmission in 42.2% of the outbreaks (Snedeker, Shaw, Locking, & Prescott, 2009). In the United States, foodborne illness has been estimated to cause approximately 47.8 million illnesses 128,000 hospitalizations, and 3,000 deaths per year (Scallan et al., 2011; Scallan, Griffin, Angulo, Tauxe, & Hoekstra, 2011).

Since the first notable incidents of E. coli O157:H7 in the United States reported in 1982, when two outbreaks of hemorrhagic colitis occurred in Oregon and one in Michigan, *E. coli* O157:H7 has become one of the most prevalent pathogens in the world. During 1982 –2002, 49 states reported 350 *E. coli* O157:H7 outbreaks representing 8,598 cases, 1,493 hospitalizations, 354 HUS cases, and 40 deaths, in which over half cases were foodborne (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005).

In the past few years, fresh fruits and vegetables have accounted for a growing number of recognized outbreaks. Radish sprouts have been implicated in several outbreaks in Japan, including the massive Sakai city outbreaks in 1996, with 7,966 cases (2,764 microbiologically confirmed, 106 with HUS) (Michino et al., 1999). In the USA, fresh produce such as lettuce, baby spinach, apple cider, unpasteurized apple juice and alfalfa sprouts have been implicated (Rangel et al., 2005). Consumption of prepackaged raw cookie dough was strongly linked to a multistate outbreak in the USA in 2009, with 72 cases of *E coli* O157 infection, ten with HUS (CDC, 2009). The 2006 multi-state outbreak of *E. coli* O157:H7 infection, which is

associated with contaminated bagged baby spinach, resulted in 205 confirmed cases of illnesses, 31 cases of HUS and three deaths (CDC, 2006). Recently, a multistate outbreak of *E. coli* O157:H7 associated with Lebanon bologna was reported (CDC, 2011). As of March 22, 2011, 14 persons infected with the outbreak strain of *E. coli* serotype O157:H7 were reported from Maryland (3 cases), New Jersey (2 cases), North Carolina (1 case), Ohio (2 cases) and Pennsylvania (6 cases).

2.3 Current sanitizing methods for fresh produce

2.3.1 Chlorine

Although every processor may have different procedures, leafy greens usually go through a triple-wash process (Sapers et al., 2009). The first wash occurs when product goes into an agitating tank containing weakly chlorinated water. This step is intended to remove gross physical debris such as bugs, soil and dirt, from the produce. The major cleaning step occurs in the second step. Chlorinated water is used in a tank of flume, which is intended to control bacterial numbers in the wash water and prevent cross-contamination. The final wash is actually a rinse step that is intended to remove residual chlorine from the product. The total exposure time may range from 60 – 120 s.

At present, chlorine (as sodium or calcium hypochlorite or Cl_2 gas) of 50 – 200 ppm is still the primary postharvest sanitizing agent used by fresh produce industry because of its broad antimicrobial activity and low cost. Free chlorine, which is the main component that has antimicrobial effect, is defined as the concentration of residual chlorine in water present as dissolved gas (Cl_2), hypochlorous acid (HOCl), and/or hypochlorite ion (OCl^-). At a pH of 5 – 7, free chlorine is most effective as

HOCl is the predominant form. The effectiveness declines with increased pH. Typically, since metal containers and processing equipments are often susceptible to corrosion at low pH, a pH of 6.0 – 7.5 is usually used to protect equipments and yield acceptable chlorine efficacy.

However, chlorine as a sanitizer has some drawbacks. A concern of using chlorine is that prolonged exposure to chlorine vapors can cause irritation to the skin and respiratory tract (Beuchat, 1998). Since chlorine is highly reactive with leaves, soil, and any plant or vegetable matter whenever oxygen is present, resulting in rapid chlorine depletion, the concentration of free chlorine needs to be frequently monitored and replenished as needed (Suslow, 2000). Although chlorine has a broad spectrum of antimicrobial activity, its efficacy against pathogens attached to the leafy greens is limited. It has been reported in the literature that washing with chlorinated water of up to 200 ppm free chlorine can only achieve < 2 log reduction (Baert et al., 2009; Beuchat, Nail, Adler, & Clavero, 1998; Lee & Baek, 2008). In some studies, chlorine is only as effective as de-ionized water or marginally better (Li, Brackett, Chen, & Beuchat, 2001). Unfortunately, due to the low infectious dose of some human pathogens such as *E. coli* O157:H7, chlorine treatment cannot guarantee to assure safety of fresh produce.

2.3.2 Electrolyzed water

Acid electrolyzed water (AEW) and neutral electrolyzed water (NEW) have been studied as alternative sanitizers to chlorinated water. Electrolyzed oxidizing (EO) water is generated through the electrolysis of a dilute NaCl solution (ca. 0.1%) in an electrolyzing chamber where the anode and cathode are separated by a septum (Park, Costa, Kang, Alexander, & Taylor, 2008b). AEW only passes through the

anode chamber and its antimicrobial effect is mainly due to its low pH (2 – 4), high oxidation – reduction potential (ORP > 1000 mV), and hypochlorous acid in it. NEW is generated by passing the NaCl solution through both the anode and cathode chambers without a septum. Since HCl formed at the anode site neutralizes the NaOH at the cathode site, NEW has a pH of around 6.8 and is less corrosive to processing equipment or irritating to hands (Izumi, 1999). The main bactericidal agents of NEW are HOCl, ClO^-, HO_2 and O_2 radical (Abadias, Alegre, Vi©łas, Usall, & Oliveira, 2008).

Many studies have investigated the effectiveness of EO water on pathogens associated with fresh produce and mixed results have been reported. Abadias et al. (2008) showed that the bactericidal effect of NEW (50 ppm of available chlorine, pH 8.6) against *E. coli* O157:H7, *Salmonella*, *Listeria innocua* and *Erwinia carotovora* on lettuce was similar to that of chlorinated water (120 ppm of free chlorine) with reduction of 1 – 2 log units. A 2 log reduction of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* on lettuce was reported by Yang, Swem, & Li (2003a) who treated fresh-cut lettuce with 300 ppm EO water at 30 °C for 5 min. Park, Ezeike, Kim, Hung, & Doyle (2001) reported that treatment with EO water containing 45 ppm of available chlorine for 3 min significantly decreased mean populations of *E. coli* O157:H7 and *L. monocytogenes* by 2.4 and 2.7 log CFU per lettuce leaf, respectively. Some other studies have yielded > 3.5 log unite population reduction, depending on the commodity, method of inoculation, inoculation site, interval between inoculation and treatment, media used for enumeration, strength of EO water, etc (Deza, Garrido, & Araujo, 2003; Koseki, Yoshida, Kamitani, Isobe, & Itoh, 2004; Park, Costa, Kang, Alexander, & Taylor, 2008a). The efficacy of EO water is also

affected by many factors such as organic load, bio-film formation, and etc (Park, Costa, Kang, Alexander, & Taylor, 2008b; Yang, Swem, & Li, 2003b). Thus, whether these larger reductions in attached microbial population could be achieved in a real processing plant still needs to be further investigated.

2.3.3 Chlorine Dioxide

Chlorine dioxide (ClO_2), as a strong oxidizing agent that has broad and high biocidal effectiveness, has been used as an alternative to chlorine. It has approximately 2.5 times the oxidation capacity of chlorine (Benarde, Israel , Olivieri, & Granstrom, 1965). According to FDA, ClO_2 may be used as an antimicrobial agent in water used to wash fruits and vegetables that are not raw agricultural commodities in an amount not to exceed 3 ppm residual chlorine dioxide. This treatment must be followed by a potable water rinse or by blanching, cooking, or canning (Code of Federal Regulations). Since ClO_2 is explosive at concentrations above 10 percent or at temperatures above 130 °C, it is usually generated on-site by combining chlorine gas and sodium chlorite or sodium hypochlorite, hydrochloric acid, and sodium chlorite. ClO_2 can also be generated with a ClO_2 sachet kit with a mixture of chemicals and activated in water (Lee, Kang, & Costello, 2004).

The pathogen reductions achieved by aqueous ClO_2 have varied from study to study (Huang et al., 2006; Keskinen, Annous, & Burke, 2009; Kim et al., 2008; Rodgers, Ryser, Siddiq, & Cash, 2004) and are are highly dependenton the targeted pathogen, inoculation method, recovery methods, treatment time and temperature, but are usually in the range of 1-3 logs. Generally, the effectiveness of aqueous ClO_2 is similar to that of chlorine (Keskinen et al., 2009). A greater reduction of pathogens have been reported with ClO_2 gas (Lee et al., 2004; Mahmoud & Linton,

2008; Sy, Beuchat, Harrison, & Murray, 2005); however, negative impacts on produce appearance were also indicated when higher reductions were achieved.

2.3.4 Hydrogen peroxide (H_2O_2)

H_2O_2 is a well established antimicrobial agent used as an antiseptic on wounds, for dental and medical instrument disinfection, and as a sterilizing agent for aseptic packaging containers (Fan, 2009). H_2O_2 has been investigated as a potential alternative to chlorine for sanitizing fresh produce. It is approved by the FDA as generally recognized as safe (GRAS) (Code of Federal Regulations), but its use in the food industry is limited only to some products (milk, dried egg, starch, tea and wine) as an antimicrobial or bleaching agent in the range of 0.04 – 1.25% unless it is used at low concentrations and combined with acetic acid to form peroxyacetic acid. The use of H_2O_2 for raw agricultural commodities is exempt from the requirements of a tolerance if the concentration used is 1% or less by the U.S. Environmental Protection Agency (EPA) (Code of Federal Regulations).

Many studies have demonstrated the efficacies of dilute H_2O_2 and peroxyacetic acid in sanitizing fresh produce including mushrooms (Sapers, Kamp, Pilizota, & Miller, 2001), apples (Sapers, Miller, Jantschke, & Mattrazzo, 2000), melons (Ukuku, Sapers, & Pilizota, 2001), tomatoes (Sapers & Jones, 2006; Venkitanarayanan, Lin, Bailey, & Doyle, 2002) and leafy greens (Lin, Moon, Doyle, & McWatters, 2002; McWatters, Doyle, Lin, Chinnan, & Walker, 2002a; Wei, Hammes, & Wolf, 2005). One of the main advantages of using H_2O_2 as a disinfecting agent is that it produces no residue as it is decomposed into water and oxygen by the enzyme catalase which is naturally found in plants (Ölmez & Kretzschmar, 2009). Although some studies also showed that treatment of H_2O_2 could cause browning and

adverse impacts on some types of fresh produce (Sapers et al., 2001), other reports indicated that H_2O_2 treatment improved sensory quality and shelf life of some fresh produce (Lin et al., 2002; McWatters, Doyle, Lin, Chinnan, & Walker, 2002b; G. M. Sapers, Miller, Pilizota, & Mattrazzo, 2001).

2.3.5 Organic acids

A number of organic acids such as citric, lactic and acetic acids are GRAS and are commonly used as antimicrobials in food preservation. The mode of action of these acids is attributed to direct pH reduction, depression of the internal pH of microbial cells by ionization of the undissociated acid molecule, or disruption of substrate transport by alteration of cell membrane permeability (Beuchat, 1998). Citric, malic and acetic acids are widely used in various types of food and beverage as a preservative. Acetic and lactic acid has been successfully used in the meat industry as a post-evisceration carcass rinse (Huffman, 2002). Organic acids are very stable in the presence of organic material and generally present no objectionable odor. The major drawback of using organic acids is the relatively high cost. Additionally, a possible disadvantage is that the treatments of organic acid may change the flavor and aroma of treated products depending on the type of product and treatment condition.

Some organic acids have been extensively studied for their ability to reduce natural microflora populations as well as pathogens. Nascimento, Silva, Catanozi, & Silva (2003) reported that the population reductions of aerobic mesophilic bacteria, yeast and mold, and total coliforms were 3.9, >3.6, and >2.3 log CFU/g, respectively, after washing for 15 min in 4% acetic acid solution. When samples were washed in 2 % acetic acid for 15 min, populations of aerobic mesophilic bacteria, yeast and mold, and total coliforms were reduced by 3.4, >3.5, >2.3, and >0.3 log

CFU/g, respectively. Wright, Pierson, Zoecklein, Sumner, & Hackney (2000) reported that a 2-min dip in 5% acetic acid at room temperature was the most effective of several treatments investigated in reducing populations of *E. coli* O157:H7 on apple surfaces, achieving >3 log reductions. Karapinar & Conul (1992) demonstrated that washing parsley leaves in a solution containing 2% acetic acid for 15 min reduced counts of *Yersinia enterocolitica* by >7 log CFU/g. Escudero et al., (1999) also reported a > 6 log reduction of *Y. enterocolitica* on fresh lettuce after treatment of 0.5 % lactic acid plus 100 ppm of chlorine. Akbas & Ölmez (2007) studied various type of organic acid for their effectiveness on inactivation of *E. coli* and *L. monocytogenes* on iceberg lettuce, and showed that dipping of iceberg lettuce in 0.5% citric acid or 0.5% lactic acid solution for 2 min could be as effective as chlorine washing.

2.3.6 Allyl isothiocyanate

Allyl isothiocyanate (AIT) is derived from glucosinolates, a group of natural compounds called glycosides stored within cell vacuoles of all plants belonging to the family *Cruciferae* (Delaquis & Mazza, 1995). AIT is a GRAS substance and is exempted from the requirement for a residue tolerance in or on all raw agriculture commodities by the US EPA (EPA, 1996). Some foods were reported to contain significant amounts of AIT. West, McLaughlin, & Badenhop (1977) found 3 and 17 ppm AIT in shredded cabbage and coleslaw, respectively. In Japan, purified AIT is permitted for use as a food preservative provided that the compound is extracted from natural source as synthetically produced AIT may be contaminated with chloride compounds (Isshiki, Tokuoka, Mori, & Chiba, 1992). 'Wasaouro' is a brand of AIT being used in Japan for the packaging of raw oyster, pickled vegetables, ham and cheese slices (Cha & Chinnan, 2004). In the USA, AIT can be found

commonly in food condiments such as mustard, wasabi and mayonnaise (Delaquis & Sholberg, 1997). In spite of its GRAS status, its low solubility and stability in water limited its use as antimicrobial in food system. As an electrophile, AIT is unstable in aqueous solution (Ohta, Takatani, & Kawakishi, 1995); however, the study of Pechacek, Hrabcova, & Velisek (1997) showed that AIT is more stable and effective in solutions with lower pH. Tsao et al. (2000) reported that AIT had higher stability at acid pH values. Several other approaches to improve the stability of AIT in long term storage have also been developed (Chacon, Buffo, & Holley, 2006; Liu & Yang, 2010).

AIT was reported to have strong antimicrobial activity in both liquid and vapor forms (Delaquis & Sholberg, 1997; Lin, Preston, & Wei, 2000; Rhee, Lee, Dougherty, & Kang, 2003). Obaidat & Frank (2009) reported that 4µl/L AIT vapor inactivated > 4 log of *E. coli* O157:H7 on intact lettuce leaves at 0 and 4 °C in 4 days and at 10 °C in 2 days. Lin, Kim, Du, & Wei (2000) showed that AIT vapor at 76.0 – 101.3 mg/L eliminated *E. coli* O157:H7 and *S.* Montevideo inoculated at 10^4 – 10^5 CFU/g on lettuce within 2 days, and a lower application rate of AIT could be used as a processing aid to help control potential pathogens on fresh fruits and vegetables. The antimicrobial mechanism of AIT is not well understood. It was proposed that AIT could alter protein structures at a concentration that inhibited microbial growth (Kawakishi & Kaneko, 1985; Kawakishi & Kaneko, 1987). Proteins were shown to be attacked by AIT at disulfide bonds (Kawakishi & Kaneko, 1987). Lin et al. (2000) revealed that AIT vapor could cause metabolite leakage, measurable increases in β–galactosidase activity, and reduction of viable bacteria.

2.3.7 Other intervention technologies

Irradiation is a penetrating nonthermal process in which high-energy

gamma rays, X-rays, or electrons are applied to foods, resulting in the inactivation of associated pathogens (Arvanitoyannis, Stratakos, & Tsarouhas, 2009). In 2008, the FDA approved the use of irradiation up to 4.0 kGy on fresh lettuce and spinach to inactivate human pathogens such as *E. coli* O157:H7 and *Salmonella* (FDA, 2008). Foods treated with ionizing radiation must be labeled with the Radura symbol or with the statement "Treated by irradiation" or "Treated with radiation" (FDA, 2008). Irradiation has been shown to be very effective in eliminating internalized *E. coli* O157:H7 from baby spinach and various types of lettuce (Romaine, Iceberg, Boston, green leaf, red leaf) where traditional sodium hypochlorite was generally ineffective (Niemira, 2008; Niemira, 2007). Neal et al. (2010) reported that e-beam irradiation can effectively reduce counts of spoilage bacteria, extend the shelf life of fresh spinach and keep good sensory characteristics. Therefore, irradiation could be a practical treatment to complete eliminates pathogens on leafy greens without cause undue sensory damage.

Cold plasma is a relatively new sanitizing technology for fresh produce. Although plasma may be considered for practical purposes to be an energetic form of gas, it is technically a distinct state of matter. As energy is added to materials, they change state, going from solid to liquid to gas, and large-scale intermolecular structures break down. When additional energy is added, the intra-atomic structures of the components of the gas break down, yielding plasmas – concentrated collections of ions, radical species and free electrons (Niemira & Sites, 2008). Deng et al. (2007) reported a 5-log reduction of *E. coli* on almonds by using nonthermal plasma for 30 sec. Critzer, Golden, South, & Kelly-Wintenberg (2007) showed that treatment with cold plasma for 2 min reduced *E. coli* O157:H7 on Red Delicious apples by about 3

logs, reduced *S. Enteritidis* on cantaloupe by about 3 logs, and reduced *L. monocytogenes* on iceberg lettuce by about 2 logs. They also found that 5-min treatment of cantaloupes and lettuce with cold plasma could reduce *L. monocytogenes* by 5 logs. Therefore, cold plasma may hold additional promise with respect to rough and difficult-to-sanitize surfaces.

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Chapter 3

EFFECT OF ORGANIC ACIDS, HYDROGEN PEROXIDE AND MILD HEAT ON INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON BABY SPINACH

ABSTRACT

Minimally processed baby spinach contaminated with *Escherichia coli* O157:H7 has been associated with multiple outbreaks of foodborne illnesses recently. Chlorinated water is widely used to wash vegetables commercially, but this washing procedure has limited efficacy and can lead to the formation of carcinogenic substances. This study was conducted to determine the 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. 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. Individual washing solutions (1% and 2 % lactic acid [LA], citric acid [CA], malic acid [MA], tartaric acid [TA], acetic acid [AA], hydrogen peroxide [H_2O_2] as well as binary combinations of LA, CA, MA and H_2O_2 at final concentrations of 1% were used to decontaminate spinach leaves at 22, 40 or 50 °C for 2-5 min to test their efficacy in reducing *E. coli* O157:H7. Chlorinated water (200 ppm free chlorine) decreased the population of *E. coli* O157:H7 on baby spinach by only 1.2-1.6 log CFU/g, which was not significantly different from DI water washing. Washing with 1% LA at 40 °C for 5 min was the most effective treatment achieving a 2.7 log reduction of *E. coli* O157:H7 which is significantly higher than chlorine washing. Washing with LA + CA or LA + H_2O_2 at 40 °C for 5 min was equally effective against *E. coli* O157:H7, resulting in a 2.7 log reduction of *E. coli* O157:H7. The application of mild heat significantly enhanced the efficacy of washing solutions on the inactivation of *E. coli* O157:H7. There was, however, no significant difference between treatments at 40 °C for 5 min and 50 °C for 2 min. The

results suggested that the use of organic acids in combination with mild heat can be a potential intervention to control *E. coli* O157:H7 on spinach.

3.1 Introduction.

Fresh fruit and vegetables are an indispensable part of a healthy diet. Since the publication of the 1995 dietary guidelines for Americans (Kennedy, Meyers, & Layden, 1996), the United States consumption of fresh fruits and vegetables per capita increased from 83.1 and 164.1 pounds in 1976 to 103.0 and 221.2 pounds in 2006, respectively (Cook, 2008). Unfortunately, as a result of an increased demand for minimally processed and ready-to-eat fresh produce, there has also been an increase in the frequency of outbreaks of foodborne illnesses associated with consumption of these foods. These outbreaks have caused heightened concern about the safety of fresh produce (Altekruse, Cohen, & Swerdlow, 1997; Beuchat, 1996).

Escherichia coli O157:H7 has emerged as an important public health concern in the North America since it was implicated in two outbreaks of a distinctive bloody diarrheal syndrome in 1982 (Griffin & Tauxe, 1991; Riley et al., 1983). This pathogen has been estimated to cause 73,480 cases of illness and 61 deaths each year in the United States (Mead et al., 1999). From 1982 to 2002, 49 states reported 350 outbreaks, representing 8,598 cases, in which 52% were foodborne and 21% of the foodborne outbreaks were due to fresh produce such as lettuce, spinach, grapes and sprouts (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). *E. coli* O157:H7 is also the most common cause of hemolytic uremic syndrome (HUS) and the leading cause of kidney failure among children in the United States (Slutsker, Altekruse, & Swerdlow, 1998). In 2006, a multi-state outbreak of *E. coli* O157:H7 infection associated with contaminated bagged baby spinach resulted in 205 confirmed cases of illness, 31 cases of HUS and three deaths (CDC, 2006; FDA, 2009). Although contributing factors have not been determined in all cases, several notable causes have

been proposed, including contaminated water, untreated manure or sewage, and fecal matters of both domestic and wildlife animals (Brackett, 1999).

Currently, washing vegetables with chlorinated water containing 50 to 200 ppm chlorine is widely used in food processing plants to remove dirt and contamination, but this sanitation procedure can only achieve a < 2-log reduction in the counts of *E. coli* O157:H7 (Beuchat, Nail, Adler, & Clavero, 1998). In addition, chlorine compounds can be inactivated by organic materials on fresh produce and form various carcinogenic organochlorine compounds (CDC, 2009; Richardson et al., 1998) Therefore, it is necessary to seek for more effective and safer alternatives to control *E. coli* O157:H7 in fresh produce.

Various sanitizers have been proposed and studied (Akbas & Ölmez, 2007; Delaquis, Stewart, Cazaux, & Toivonen, 2002; Kondo, Murata, & Isshiki, 2006; Koseki & Isobe, 2006; Li, Brackett, Chen, & Beuchat, 2001; Lin, Moon, Doyle, & McWatters, 2002). Organic acids are natural compounds that are generally recognized as safe (GRAS). They are known to have antimicrobial activity and were reported to have potential as disinfectants for fruits and vegetables (Beuchat et al., 1998). Inhibition of growth of microorganisms by weak acids has been proposed to be due to a number of actions including membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis and the accumulation of toxic anions (Brul & Coote, 1999). Lactic acid, acetic acid and citric acid have been shown to inhibit the growth of *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. on fresh produces (Akbas & Ölmez, 2007; Hwang & Beuchat, 1995; Laury et al., 2009; Rhee, Lee, Dougherty, & Kang, 2003; Venkitanarayanan, Zhao, & Doyle, 1999). Malic acid and tartaric acid have been reported to have bactericidal effects against *E.*

coli O157:H7 (Conner & Kotrola, 1995). However, the antimicrobial activities against *E. coli* O157:H7 vary across organic acids (Over, Hettiarachchy, Johnson, & Davis, 2009). Hydrogen peroxide is also used as an antimicrobial compound due to its strong oxidative activity. Sapers & Sites (2003) reported that 1% hydrogen peroxide had an equal or better effect against *E. coli* O157:H7 than 200 ppm chlorine when applied on apples. Treatment of lettuce with 2% H_2O_2 at 50°C was reported to yield a 4-log reduction of *E. coli* O157:H7 (Lin et al., 2002). However, the studies on the effects of these sanitizers on baby spinach are very limited (Lee & Baek, 2008).

Recently, it was reported that exposure to mild heat at 40 to 50°C reduced browning and improved the quality of lettuce and spinach (Delaquis, Stewart, Toivonen, & Moyls, 1999; Gomez et al., 2008; Murata, Tanaka, Minoura, & Homma, 2004; Roura et al., 2008; Roura, Valle, & Pereyra, 2008). Mild heat also enhanced the antimicrobial effect of sanitizers and sensory of packaged iceberg lettuce during storage (Delaquis et al., 2002; Venkitanarayanan et al., 1999). Thus, we hypothesized that the combination of sanitizers with mild heat would enhance the inactivation of *E. coli* O157:H7 while maintaining the sensory quality of baby spinach.

The objectives of this study were to 1) evaluate the effect of various organic acids and hydrogen peroxide alone or in binary combinations on the inactivation of *E. coli* O157:H7 on baby spinach, and 2) determine whether mild heat could enhance the bactericidal effectiveness of these sanitizers.

3.2 Material and Methods

3.2.1 Bacterial strains

Five nalidixic acid-resistant strains of *E. coli* O157:H7 (250, 251, 1730,

J58, cider) were maintained at 4 °C on tryptic soy agar (Difco Laboratories, Sparks, MD, USA) supplemented with 0.6% yeast extract (Difco Laboratories) and 50 µg/mL of nalidixic acid (Fisher Scientific, Hampton, NH, USA) (TSAYE-N) as described by Neetoo, Ye, & Chen (2008). Each strain of *E. coli* O157:H7 was examined for its potential to inhibit growth of other test strains following the method described by Lang, Harris, & Beuchat (2004a). Briefly, each strain was grown in 10 mL of tryptic soy broth (Fisher Scientific) supplemented with 0.6% yeast extract and 50 µg/mL nalidixic acid (TSBYE-N) at 35 °C for 24 h. Cultures of each strain were cross-streaked onto TSAYE-N and incubated at 35 °C for 24 h. Plates were examined for inhibition of growth at junctions of cross-streaks of all combinations of test strains.

3.2.2 Preparation of inoculums

Single colonies of each stain were inoculated into TSBYE-N and grown at 35 °C for 24 h. The individual cultures were then transferred to fresh tubes of TSBYE-N and incubated at 35°C for another 24 h. Five mL of each culture was mixed to form a five-strain cocktail with a concentration of approximately 10^9 CFU/mL.

3.2.3 Inoculation of baby spinach

Boxed baby spinach was purchased from local grocery stores, stored at 4 °C and used within two days. Twenty-five mL of the cocktail of *E. coli* O157:H7 were mixed with 1 liter of sterile 0.1% peptone water (Fisher) in a sterile stomach bag. Intact and un-wilted spinach leaves (50g) were submerged in the cell suspension. The bag was heat-sealed and gently massaged for 5 min. Cell suspension was drained and spinach leaves were placed on sterile cheese cloth supported by a wire screen and dried inside a bio-safety hood at room temperature (22 ± 1 °C) for 15 min. 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 as recommended by Lang, Harris, & Beuchat (2004a). Inoculated spinach without treatment was used to determine the initial counts. To determine the counts of *E. coli* O157:H7, 95 mL of sterile 0.1% peptone water was added to 5 g of the inoculated spinach samples in a stomacher bag. The mixture was pummeled in a laboratory stomacher (Seward 400 Stomacher; Seward Medical Co., London, United Kingdom) for 2 min. The spinach slurry was then serially diluted in sterile 0.1% peptone water and spread-plated onto TSAYE-N plates. Plates were incubated at 35 °C for 72 h before typical *E. coli* O157:H7 colonies were enumerated.

3.2.4 Effect of leaf size on the attachment of *E. coli* O157:H7

Experiments were conducted to determine whether the number of *E. coli* O157:H7 cells attached to spinach was dependent on the size and weight of spinach leaves. Based on the weight of individual spinach leaves, leaves were divided into three groups, 0.5 ± 0.1, 0.7 ± 0.1 and 1 ± 0.1g/leave. The leaves in these three groups were then inoculated with *E. coli* O157:H7 and stored at 4 °C for 24 h as described in section 2.3. The counts of *E. coli* O157:H7 in these samples were determined.

3.2.5 Preparation of treatment solutions

All treatment solutions were prepared within 1 h before experiment. Lactic acid (LA) (Purac FCC88, Purac America, USA), citric acid (CA) (Fisher), malic acid (MA) (Acros, NJ, USA), tartaric acid (TA) (Sigma Aldrich, St Louis, MO), acetic acid (AA) (Fisher) and hydrogen peroxide (H_2O_2) (Onpoint, Clifton, NJ) were added to de-ionized (DI) water to prepare solutions containing 1% and 2% (v/v) LA, 1% and 2%

(w/v) CA, 1% and 2% (w/v) MA, 1% and 2% (w/v) TA, 1% and 2% (v/v) AA, 1% and 2% (v/v) H_2O_2. Solutions containing binary combinations of selected organic acids and hydrogen peroxide were prepared in a similar way by dissolving these chemicals in DI water to the final concentration of 1% for each component. Chlorinated water (CW) was prepared by adding commercial bleach into DI water to obtain a concentration of 200 ppm of free chlorine. Chlorine concentration was determined by free chlorine micro check test strips (HF Scientific, Ft. Myer, FL). The pH values of solutions were measured using a digital pH meter (Basic pH meter UB-10, Denver Instrument Co., NY, USA).

3.2.6 Procedures for treatment

To determine the appropriate holding times for the mild heat treatments, spinach leaves (5 g) were exposed to 40 or 50 °C in a circulating water bath (Neslab EX-35 Digital One Heating Circulators, VWR, USA) for 1-10 min. The treated leaves were then kept at 4 °C in a plastic box for 5 days during which color and texture of the spinach leaves were checked by visual inspection and compared with untreated leaves. The highest temperatures and holding times that did not lead to deterioration of leaves were 5 min at 40 °C and 2 min at 50 °C. Spinach leaves were inoculated with *E. coli* O157:H7, dried and kept at 4 °C for 24 h as described above. Before being subjected to the treatments below, inoculated leaves were kept at room temperature (22 ± 1 °C) for 30 min to equilibrate to room temperature. Five g of inoculated leaves were immersed in 200 mL of treatment solutions at room temperature for 5 min, 40 °C for 5 min or 50 °C for 2 min and gently stirred by a spatula. For the mild heat treatment, the treatment solutions were preheated to the targeted temperature (40 and 50 °C) in the circulating water bath. After treatment, all treatment solutions were decanted and the

samples were immediately immersed in ice water for 30 s. The spinach leaves were added to a stomacher bag containing 95 mL of sterile 0.1% peptone water and pummeled in the stomacher. Counts of *E. coli* O157:H7 in the samples were determined as described in Section 2.3.

3.2.7 Statistical analysis

All experiments were replicated three times. Colony counts were converted to log CFU/g and means and standard deviations were calculated. Statistical analyses were conducted using JMP (SAS Cary, NC, USA). One-way analysis of variance and Student's t one-way multiple comparisons were used to determine significant differences among treatments ($P<0.05$).

3.3 Results

3.3.1 Test of cross-strain inhibition

Cross-strain inhibition tests showed that none of the 5 strains inhibited the growth of other strains. Thus the use of a 5-strain cocktail in inoculums would not present a concern with regard to loss of viability or inhibition caused by strain interaction or serotype interaction.

3.3.2 Effect of leaf size on the attachment of *E. coli* O157:H7

In this study, we used dip inoculation instead of spot inoculation to simulate a worst case scenario of contamination. The number of *E. coli* O157:H7 detected on inoculated spinach was not significantly ($P > 0.05$) influenced by leaf size. Leaves ranging from 0.5 to 1 g in weight contained a mean population of 6.0 ± 0.1 log CFU/g. Thus spinach leaves of different sizes were randomly selected in the following

experiments.

3.3.3 Effect of individual washing solutions with or without mild heat on inactivation of *E. coli* O157:H7 on baby spinach

Table 1 shows the effect of different treatments on reducing *E. coli* O157:H7 on baby spinach. The initial population of *E. coli* O157:H7 inoculated on spinach was 6.0 ± 0.1 log CFU/g. At 22 °C, the two washing controls, DI water and 200 ppm CW only reduced *E. coli* O157:H7 counts by 1.1 and 1.2 log CFU/g, respectively and there was no significant difference between them ($P>0.05$). Except for 1% AA, all of the organic acids and 2% H_2O_2 resulted in significantly higher reductions of *E. coli* O157:H7 than the two washing controls ($P<0.05$) while 1% H_2O_2 showed no advantage over the control treatments. LA (1%) was shown to be the most effective treatment solution (1.9 log CFU/g reduction). Our results also showed that there was no significant difference in the effectiveness of high and low concentration of the washing solutions ($P>0.05$).

At 40 °C, washing spinach with DI water and 200 ppm CW resulted in 1.1and 1.4 log CFU/g reduction of *E. coli* O157:H7, respectively; however, no significant difference was observed for the effectiveness of these two treatments ($P>0.05$). Meanwhile, significant higher reductions in the number of *E. coli* O157:H7 were obtained for samples treated with selected organic acids and H_2O_2 solutions compared with the washing controls ($P<0.05$). Mild heat (40 °C for 5 min) enhanced the effectiveness of all the washing solutions and there were significantly higher reductions of *E. coli* O157:H7 for most of the treatments compared to treatments at 22 °C ($P<0.05$). Washing baby spinach in 1% LA at 40 °C for 5 min significantly reduced *E. coli* O157:H7 counts by 2.7 log CFU/g ($P<0.05$). Compared with 1%

selected treatment solutions, a higher concentration of each solution (2%) did not bring about a significantly higher reduction of *E. coli* O157:H7 ($P>0.05$).

At 50 °C, both DI water and 200 ppm CW washing reduced *E. coli* O157:H7 counts by 1.6 log CFU/g, which were significantly higher than washing with DI water and 200 ppm CW at 22 °C ($P<0.05$). Compared with controls, populations of *E. coli* O157:H7 decreased significantly when spinach was washed with selected washing solutions ($P<0.05$). The highest log reduction of *E. coli* O157:H7 was obtained using 2% MA (2.5 log CFU/g) followed by 1% LA (2.3 log CFU/g) but there was no significant difference between these two treatments ($P>0.05$). Similar to the treatments at 40°C, various treatments conducted at 50°C for 2 min also promoted the inactivation of *E. coli* O157:H7 compared to the same treatments at 22°C. However, there was no significant difference in log reduction between the treatment of 40 °C for 5 min and the treatment of 50°C for 2 min when the same washing solutions were used ($P>0.05$). In addition, when the concentration of the selected treatment solutions was increased from 1% to 2%, no significant reductions were observed ($P>0.05$).

Table 3.1 Inactivation of *E. coli* O157:H7 on baby spinach in the presence of different antimicrobials at 22, 40 and 50 °C.

Treatment	Concentration	pH	Log reduction at washing temperatures of		
			22 °C	40 °C	50 °C
LA	1%	2.3	1.9 ± 0.2^{Ab}	2.7 ± 0.2^{Aa}	2.3 ± 0.3^{ABab}
	2%	2.1	1.7 ± 0.1^{ABb}	2.4 ± 0.3^{ABa}	2.0 ± 0.3^{BCab}
CA	1%	2.3	1.5 ± 0.0^{BCb}	2.3 ± 0.2^{ABCa}	2.2 ± 0.2^{ABCa}
	2%	2.1	1.5 ± 0.3^{BCb}	2.0 ± 0.2^{BCDa}	2.1 ± 0.1^{BCa}
MA	1%	2.3	1.7 ± 0.2^{ABb}	2.3 ± 0.2^{ABCa}	2.2 ± 0.2^{ABCa}
	2%	2.2	1.8 ± 0.3^{ABb}	2.3 ± 0.4^{ABCab}	2.5 ± 0.4^{Aa}
TA	1%	2.2	1.5 ± 0.1^{BCb}	2.2 ± 0.4^{BCa}	2.0 ± 0.3^{BCab}
	2%	2.0	1.7 ± 0.3^{ABCb}	2.1 ± 0.2^{BCDab}	2.1 ± 0.1^{BCa}
AA	1%	2.7	1.4 ± 0.0^{CDb}	1.8 ± 0.2^{DEFa}	2.0 ± 0.2^{BCa}
	2%	2.5	1.5 ± 0.2^{BCb}	1.7 ± 0.2^{DEFab}	2.0 ± 0.2^{BCa}
H_2O_2	1%	4.5	1.1 ± 0.2^{Db}	1.6 ± 0.2^{EFGa}	1.8 ± 0.1^{CDa}
	2%	4.3	1.5 ± 0.2^{BCb}	1.9 ± 0.3^{CDEab}	2.2 ± 0.1^{ABCa}
CW	200 ppm	10.0	1.2 ± 0.1^{Db}	1.4 ± 0.2^{FGab}	1.6 ± 0.2^{Da}
DI water	-	7.2	1.1 ± 0.1^{Db}	1.1 ± 0.2^{Gb}	1.6 ± 0.2^{Da}

* Data represent mean population reduction (log10 CFU/g) of three replicates ± standard deviations;
* Data in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$) and data in the same column followed by the same capital letter are not significantly different ($P > 0.05$);
* LA = lactic acid, CA =citric acid, MA = malic acid, TA = tartaric acid, AA = acetic acid, H_2O_2 = hydrogen peroxide, CW = chlorinated water.

3.3.4 Effect of binary combination of organic acids and hydrogen peroxide with or without mild heat

The effect of binary combination of organic acids and hydrogen peroxide with or without mild heat on inactivating *E. coli* O157:H7 on baby spinach is shown in Table 2. Since there was no significant difference between the bactericidal effect of 1% and 2% organic acid and H_2O_2, all the organic acids and H_2O_2 solutions were used at a final concentration of 1% when used individually or in binary combinations. Washing with organic acids, H_2O_2 and DI water alone served as comparisons for binary combination treatments. In this part, three organic acids (LA, CA and MA) were selected since their effectiveness against *E. coli* O157:H7 was generally better than the other two organic acids (Table 1). Hydrogen peroxide was also selected because it has a different antibacterial mechanism. The selected organic acids and H_2O_2 alone or in binary combinations resulted in significantly higher inactivation of *E. coli* O157:H7 than DI water washing ($P<0.05$) which only reduced the counts of *E. coli* O157:H7 by 0.9-1.3 log CFU/g at 22, 40 and 50 °C (Table 2). At 22 °C, 1% LA was shown to be the most effective treatment solutions which resulted in 2.1 log CFU/g reduction of *E. coli* O157:H7. The binary combinations of LA and CA, MA or H_2O_2 did not increase the inactivation of *E. coli* O157:H7. At 40 °C, 1% LA, LA+ H_2O_2 and LA+CA were the most effective treatments against *E. coli* O157:H7 (2.7 log CFU/g reduction). Compared with treatments at 22 °C, treatments at 40 °C significantly enhanced the effectiveness of these washing solutions ($P<0.05$); however, no additional bactericidal effect was observed when using binary combination of these washing solutions. At 50 °C, the binary combination of MA and H_2O_2 was the most effective treatment (2.6

log CFU/g reduction) followed by 1% LA alone and LA+MA (both 2.5 log CFU/g reduction). Our result suggest that these washing solutions showed higher efficacy when applied at 40 or 50 °C than 22 °C; however, no further benefit was provided by using a binary combination of these treatment solutions.

Table 3.2 Inactivation of *E. coli* O157:H7 on baby spinach in the presence of different binary combinations of antimicrobials at 22, 40 and 50 °C.

Treatment	pH	Log reduction at washing temperatures of		
		22 °C	40 °C	50 °C
LA	2.3	2.1 ± 0.1^{Ab}	2.7 ± 0.1^{Aa}	2.5 ± 0.3^{Aa}
CA	2.3	1.3 ± 0.1^{EFb}	2.1 ± 0.2^{Ba}	2.2 ± 0.1^{ABa}
MA	2.3	1.7 ± 0.0^{CDb}	2.4 ± 0.4^{ABa}	2.2 ± 0.2^{ABab}
H_2O_2	4.5	1.1 ± 0.0^{Fb}	1.5 ± 0.1^{Cab}	1.9 ± 0.4^{Ba}
LA+CA	2.1	1.8 ± 0.2^{BCDb}	2.7 ± 0.2^{Aa}	2.4 ± 0.3^{Aa}
LA+MA	2.2	1.9 ± 0.2^{ABCb}	2.3 ± 0.4^{ABab}	2.5 ± 0.2^{Aa}
LA+ H_2O_2	2.3	2.0 ± 0.2^{ABb}	2.7 ± 0.1^{Aa}	2.4 ± 0.2^{Aa}
CA+MA	2.1	1.7 ± 0.2^{CDb}	2.6 ± 0.5^{Aa}	2.3 ± 0.2^{ABab}
CA+ H_2O_2	2.2	1.7 ± 0.2^{CDb}	2.1 ± 0.3^{Bab}	2.3 ± 0.1^{ABa}
MA+ H_2O_2	2.3	1.5 ± 0.2^{DEb}	2.6 ± 0.2^{Aa}	2.6 ± 0.1^{Aa}
DI water	7.2	0.9 ± 0.1^{Gb}	1.2 ± 0.2^{Cab}	1.3 ± 0.2^{Ca}

* Data represent mean population reduction (log10 CFU/g) of three replicates ± standard deviations;
* Data in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$) and data in the same column followed by the same capital letter are not significantly different ($P > 0.05$);
* The concentrations of all the organic acids and H_2O_2 in the solution are 1%, respectively;
* LA = lactic acid, CA =citric acid, MA = malic acid, H_2O_2 = hydrogen peroxide.

3.4 Discussion

The use of antibiotic resistant bacterial strains has been used in the creation of identifiable marker for a number of foodborne pathogens (Gündüz, Gönül, & Karapinar, 2009; Wang, Zhao, & Doyle, 1996; Northcutt, Smith, Ingram, Hinton, & Musgrove, 2007). Lang, Harris, & Beuchat (2004b) reported that the use of nonselective media could facilitate the resuscitation of injured cells and significantly higher or equal populations of *E. coli* O157:H7 were recovered from nonselective media (TSA supplemented with nalidixic acid and sodium pyruvate) than selective media (sorbitol McConkey agar supplemented with nalidixic acid and sodium pyruvate). In our study, a nalidixic acid-resistant mutant of *E. coli* O157:H7 was used as a model indicator and plated onto non-selective media incorporated with nalidixic acid for the recovery of injured cells. This approach was simple and effective for enumeration of *E. coli* O157:H7.

This study demonstrated that there was no significant difference between the bactericidal effect of 200 ppm CW and DI water at different temperatures (22, 40 and 50 °C), which was consistent with the results reported by Beuchat (1999), who found that treatments of lettuce with 200 ppm chlorine solution or DI water were equally effective in killing or removing *E. coli* O157:H7 on lettuce. Lee & Baek (2008) reported that 100 ppm of sodium hypochlorite reduced levels of *E. coli* O157:H7 on spinach only by 1.1 CFU/g which might be due to a loss of free chlorine during washing in the presence of organic material on spinach. Baert et al. (2009) reported that sodium hypochlorite at an initial concentration of 200 or 20 ppm contained only 112 ppm or 0.5 ppm of free chlorine after washing, indicating a substantial loss of free chlorine during washing and there was no significant difference between CW and tap water on the inactivation of *E. coli* O157:H7.

In our study, there were no visible differences in appearance and texture between mild heat-treated and untreated leaves observed after treatment and during a 5-day refrigeration in a plastic box. This result was in general agreement with previous studies. Delaquis et al. (1999) showed that a 3-min dip in chlorinated water at 47 °C provide optimum retention of appearance in stored, packaged iceberg lettuce. McWatters, Doyle, Lin, Chinnan, & Walker (2002) reported that treatment with 2% hydrogen peroxide at 50 °C for 60 s was effective in maintaining sensory quality over 15 days of storage.

Mild heat could enhance the antimicrobial effect of sanitizers. Warm chlorinated water (47 °C for 3 min) has been reported to result in more than 2 log reductions of *E. coli* O157:H7 on lettuce (Delaquis et al., 2002). Inatsu, Bari, Kawasaki, Isshiki, & Kawamoto (2005) reported sanitation efficacy of acidified sodium chlorite coupled with mild heat (50 °C) significantly increased the reduction of *E. coli* O157:H7 compared with room temperature and 4 °C. Lin et al. (2002) reported that more than 4 log reduction of *E. coli* O157:H7 per leaf was obtained by treatment of 1.5% LA and 1.5% H_2O_2 at 40 °C for 15 min. Recently, it was also reported that heat shock in water at 50 °C for 2 min was efficient to control vegetable browning by reducing phenylalanine ammonia lyase activity (Roura et al., 2008). Murata et al. (2004) reported that organoleptic quality of cut lettuce treated by heat shock was significantly better than that of the untreated lettuce. Heat treatment also has promise in delaying leaf senescence and might be used to extend the postharvest shelf life of spinach (Gomez et al., 2008). Delaquis et al. (1999) reported that microbial flora was reduced by approximately 3 log CFU/g on lettuce washed in chlorinated water at 47 °C, and 1 log CFU/g at 4 °C. However, some studies showed that mild heat

treatment could favor the growth of bacteria on fresh produce stored at abusive temperature (Delaquis et al., 2002; Li et al., 2001), which indicates the importance of temperature control during extended storage.

The results in this study suggested that the use of mild heat significantly enhanced the effectiveness of various washing solutions. In our study, the maximum reduction (2.7 log CFU/g) of *E. coli* O157:H7 was obtained when spinach leaves were washed with 1% LA, LA+CA or LA+ H_2O_2 at 40 °C for 5 min, which was 0.6, 0.9 and 0.7 log CFU/g higher compared with room temperature treatments, respectively. However, very limited enhancement in effectiveness was found with CW or DI water at 40 °C (0-0.3 log CFU/g), indicating that mild heat of 40 °C alone might not be sufficient to kill *E. coli* O157:H7 since the growth temperature of *E. coli* O157:H7 was reported to be as high as 41 °C (Raghubeer & Matches, 1990). However, 50 °C DI water was able to result in significantly higher reduction of *E. coli* O157:H7 than 22°C DI water. This was similar to the result obtained by Kondo et al. (2006) who found that the combination of 200 ppm CW and mild heat at 50 °C for 1 min reduced the number of *E. coli* O157:H7 by 1.5 CFU/g in lettuce. Fukuyama et al. (2009) indicated that the combination of mild heat (50 °C for 1 min) and 100 ppm CW did not bring about bacterial inactivation in shredded cabbage compared to CW alone; however, they used a much longer treatment time (10 min) at room temperature than at 50 °C. There might be variations of results obtained from different studies. These differences in the results may also be due to different experimental conditions such as produce type, concentration of sanitizers and bacteria strains.

In the 1st part of our study, 1% and 2% of organic acids and H_2O_2 were tested for their effectiveness in reducing *E. coli* O157:H7. The results indicated that all

the washing solutions were capable of reducing microbial population to some extent; however, at each temperature, a higher concentration (2%) had little influence on the effectiveness of these sanitizers. These results are consistent with the data reported by Akbas & Ölmez (2007), who found that increasing the concentration of LA, CA, ascorbic acid, and AA from 0.5% to 1% did not result in any further decrease of the population of *E. coli* O157:H7 on lettuce at room temperature. The inhibitory activities of organic acids and H_2O_2 were proposed to be pH-dependent (Beuchat, 1998; Brul & Coote, 1999). Acids have optimal inhibitory activity at low pH because it favors the uncharged, undissociated state of the molecule which is responsible for the bactericidal activity. In our study, we found that the pH values of 1% and 2% of each washing solutions were very close to each other (Tables 1 and 2), which may explain why a higher concentration of washing solution did not result in a significant higher reduction of *E. coli* O157:H7.

In the 2nd part of our study, 1% organic acids and H_2O_2 were used for binary combination treatments. The results suggested that binary combination of these sanitizers did not significantly enhance the effectiveness of these sanitizers compared with individual sanitizers. Inatsu et al. (2005) reported that combination of acidified sodium chlorite and various organic acids did not result in higher level of inactivation of *E. coli* O157:H7 in Chinese cabbage. Lin et al. (2002) reported that a larger reduction of *E. coli* O157:H7 population was obtained on lettuce treated with a combination of LA and H_2O_2 than H_2O_2 alone, which agrees with our current results. Moreover, in this study, we also found LA+H_2O_2 had no more killing effect than 1% LA alone, which indicates that LA is the main component that causes the inactivation. In our study, 1% H_2O_2 was the least effective washing solution, which might be due to

the loss of antimicrobial efficacy in high level of organic material on spinach. Similar results were also reported by Sapers, Miller, Pilizota, & Kamp (2001) who found that a 1% H_2O_2 pre-wash solution lost strength due to reaction with the mushrooms and suspended soil.

3.5 Conclusion

Our results suggest that organic acids in conjunction with mild heat is a more effective way to inactivate *E. coli* O157:H7 on spinach than the traditional chlorine treatment; however, it was impossible to completely eliminate *E. coli* O157:H7 inoculated on spinach by washing with sanitizers. The populations of *E. coli* O157:H7 inoculated on baby spinach decreased significantly ($P<0.05$) after washing with organic acids and hydrogen peroxide compared with washing at 200 ppm chlorinated water or DI water. There was no significant ($P > 0.05$) difference between the effectiveness of 200 ppm chlorinated water and DI water. There was an additive effect between temperature and washing solutions. No significant difference was found between treatments at 40 °C for 5 min and 50 °C for 2 min. The binary combination of organic acids or H_2O_2 did not demonstrate any synergistic effect at each temperature level. Our results showed that 1% LA was the most effective solution among the tested treatment solutions at all three temperatures, achieving the highest reduction of *E. coli* O157:H7 (2.7 log CFU/g) at 40 °C for 5 min.

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Chapter 4

EFFICACY OF WASHING WITH HYDROGEN PEROXIDE FOLLOWED BY AEROSOLIZED ANTIMICROBIALS AS A NOVEL SANITIZING PROCESS TO INACTIVATE *ESCHERICHIA COLI* O157:H7 ON BABY SPINACH

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. Antimicrobials were atomized into fog-like micro-particles by an ultrasonic nebulizer and routed into a jar or a fume tank model system. 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. Treatment with aerosolized 5% AIT resulted in a > 5-log reduction of *E. coli* O157:H7 on spinach 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 effectiveness of AIT. Washing with 3% H_2O_2 followed by a 2-min treatment of aerosolized 1% AIT + 2.5% LA reduced *E. coli* O157:H7 by 4.8 and 3.1 log CFU/g in the jar and scale-up system, respectively, after 10 days storage at 4 °C without causing noticeable adverse effect on the appearance of leaves. Thus, H_2O_2 washing followed by aerosolized AIT could be a new and effective way to sanitize leafy greens during storage.

4.1 Introduction

The United States is the world's second-largest producer of spinach, accounting for 4 percent of world output, following China, which contributes 76 percent of the world output (Richter, 2004). Driven by increasing fresh-market use, production of spinach for the fresh market in the US has been dramatically increasing from 61.1 million lb. in 1970 to 623.9 million lb. in 2009 (Economic Research Service, 2010). However, since the exposure of several large foodborne outbreaks related to consumption of fresh produce, there has been a great concern among consumers about the safety of these foods. Some factors may explain the plausible reasons for increased incidence of foodborne outbreaks such as increased surveillance and reporting, changes in the food manufacturing and agricultural practices, changes in consumption habits, increased at-risk populations, improved detection methods and emerging pathogens with survivability in stressed conditions (Bhunia, 2008).

Although many studies have demonstrated that washing with chlorine alone cannot eliminate pathogens on fresh produce, it is still an important step in industry to clean soil, insects from fresh produce and prevent cross-contamination. Due to the possible formation of various carcinogenic organochlorine compounds when using chlorine in the presence of organic materials, food industry has been looking for alternatives to chlorine for produce washing. Numerous studies have demonstrated the efficacy of various types of aqueous sanitizers such as electrolyzed water, chlorine dioxide, hydrogen peroxide, organic acids, ozone, etc (Keskinen, Annous, & Burke, 2009; Kim et al., 2008; Wei, Hammes, & Wolf, 2005; Yuk et al., 2006). Generally, these sanitizers could only achieve a < 2-log reduction of the population of microorganism, although some studies (Deza, Garrido, & Araujo, 2003;

Karapinar & Conul, 1992; Park, Costa, Kang, Alexander, & Taylor, 2008) reported higher reductions depending on the target microorganism, type of produce, inoculation and enumeration methods, treatment methods, etc. The ineffectiveness of aqueous sanitizers in reducing high numbers of pathogens on fresh produce stem 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 & Beuchat, 2001). Recently, gaseous sanitizers have shown some promise in improving the safety of fresh produce due to their greater penetration ability and effectiveness. Gaseous chemicals such as acetic acid (AA), ammonia, hydrogen peroxide (H_2O_2), and chlorine dioxide have been shown as potential means to inactivate microorganisms on the surface of fruits and vegetables (Delaquis, Sholberg, & Stanich, 1999; Han, Sherman, Linton, Nielsen, & Nelson, 2000; Himathongkham, Nuanualsuwan, Riemann, & Cliver, 2001; Lee, Costello, & Kang, 2004; Sapers, Walker, Sites, Annous, & Eblen, 2003; Simmons, Smilanick, John, & Margosan, 1997). However, one disadvantage of vapor was that the treatment duration tended to be long (0.5 – 24 h), which might limited its application in the food industry. Other factors that limited its application include the need for a sophisticated equipment and a limited numbers of applicable gaseous sanitizers.

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, Preston, & Wei, 2000). AIT is a GRAS substance and is exempted from the requirement for a residue tolerance in or on all raw agriculture commodities by the Environmental Protection Agency (Environmental Protection Agency, 1996). It can be commonly found in food condiments such as mustard, wasabi and mayonnaise

(Delaquis & Sholberg, 1997). In Japan, purified AIT is permitted for use as a food preservative and is used in Japan for the packaging of raw oysters, pickled vegetables, ham and cheese slices (Cha & Chinnan, 2004). Based on the study of Lin, Kim, Du, & Wei (2000), AIT vapor at 76.0 to 101.3 mg/L eliminated *E. coli* O157:H7 and *Salmonella* Montevideo inoculated at 10^4 to 10^5 CFU/g 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, lactic acid (LA) and AA are all Generally Recognized as Safe (GRAS) antimicrobials. H_2O_2 has broad antimicrobial activity and improves the sensory quality and shelf life of some fresh produce (Lin, Moon, Doyle, & McWatters, 2002; McWatters, Doyle, Lin, Chinnan, & Walker, 2002; Sapers, Miller, Pilizota, & Kamp, 2001). LA and AA were reported to inhibit the growth of *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. on fresh produce (Akbas & Ölmez, 2007; L. R. Beuchat, 1998; Venkitanarayanan, Lin, Bailey, & Doyle, 2002).

Aerosolization is defined as the dispersion of liquid as a fine mist in air. Aerosol has been reported to have better penetration than a trigger spray in assessment of surface bio-burden during hospital aseptic processing (Hiom et al. 2003). Recently, Oh, Gray, Dougherty, & Kang (2005) reported that aerosolized sanitizers diffuse like gaseous sanitizers and that diffusion of aerosolized sanitizers in a chamber was not affected by height or orientation, which is not the case for a spraying system. The same group also assessed the efficacy of aerosolized peroxyacetic acid as a produce sanitizer (Oh, Dancer, & Kang, 2005). *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* were reduced by 3.4, 4.5 and 3.8 log, respectively after a 60-min treatment. Thus, aerosolization, with its high penetration ability and broad spectrum of

applicable sanitizers, has the potential to be an alternative sanitizer delivery system. Aerosolized LA has also been used to disinfect chicken house, and Fiser (1978) reported that continuous disinfection by aerosolized LA resulted in an improved state of health of chickens. However, very few studies investigated the effectiveness of aerosol of these sanitizers against pathogens on fresh produce (Oh et al., 2005).

This study was conducted to evaluate the efficacy of aerosolized antimicrobials (AIT, H_2O_2, LA and AA) alone or in combination with washing against *E. coli* O157:H7 on baby spinach as a post-harvest intervention strategy.

4.2 Materials and Methods

4.2.1 Bacterial strains

Two strains of *E. coli* O157:H7 (250, dd 3795) (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 streptomycin (streptomycin sulfate salt, Sigma-Aldrich, MO, USA) (TSAYE-NS). Each strain was examined for its potential to inhibit growth of the other test strain following the method described by Lang, Harris, & Beuchat (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.

4.2.2 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. Fifteen mL of each culture was mixed to form a two-strain cocktail, harvested by centrifugation at 2,450 g for 10 min (Centra CL2, Centrifuge, Thermo Scientific, USA). The pellet was re-suspended in sterile 0.1% peptone water (Fisher Scientific) with a final concentration of approximately 10^8 to 10^9 CFU/mL.

4.2.3 Inoculation of baby spinach

Boxed baby spinach was purchased from local grocery stores, stored at 4 °C and used within two days. 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. The bag was heat-sealed and gently massaged for 2 min, the liquid was drained from the bag, and 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). 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.

4.2.4 Effectiveness of aerosolized antimicrobials against *E. coli* O157:H7 on baby spinach stored at 4 °C for 2 days – Screening study

A glass jar (I-Chem, 1 L clear tall wide mouth jars, 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 Figure 4.1. The tube entered the cap of the jar through a rubber stopper. Aqueous 2.5 – 5 % AA (Fisher Scientific), LA (Purac FCC88, Purac America, USA), H_2O_2 (30%, Fisher Scientific), AIT (95% allyl isothiocyanate, Sigma-Aldrich, USA), were atomized into extremely fine micro-particles in the nebulizer. Inoculated spinach leaves (5 g) were placed in the jar and treated with aerosolized antimicrobials for 2 min in a biosafety hood at room temperature. The concentrations of the sanitizers were shown in Table 4.1. After treatment, the jar was immediately sealed with a cap and refrigerated at 4 °C for 2 days before microbiological analysis.

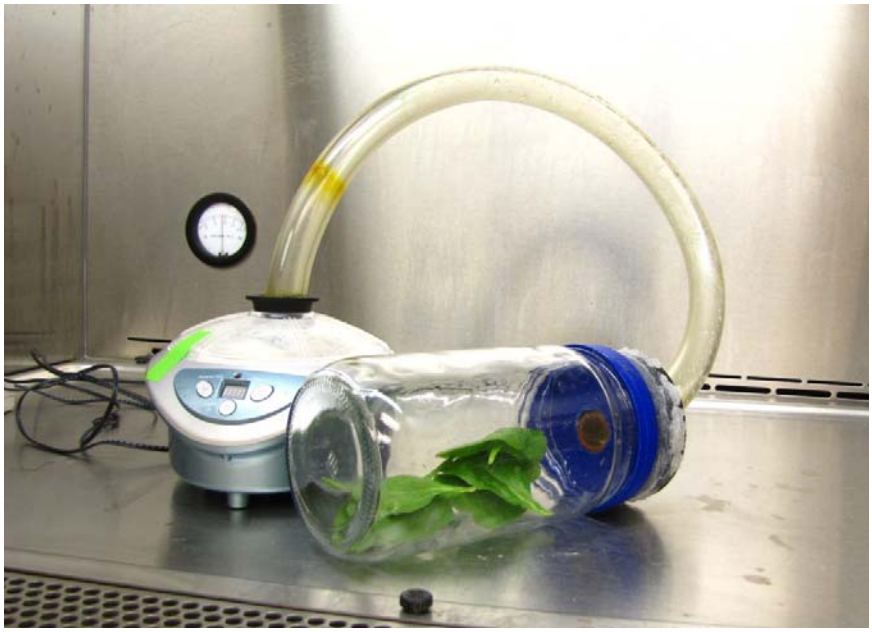

Figure 4.1 Treatment of various aerosolized antimicrobials in a prototype model system

4.2.5 Effectiveness of washing followed by treatment of aerosolized antimicrobials against *E. coli* O157:H7 on baby spinach stored at 4 °C for 10 days – prototype study

3% H_2O_2 and 200 ppm chlorinated water (CW) was prepared by adding de-ionized (DI) water in to H_2O_2 (30%, Fisher Scientific) and commercial bleach, respectively. Free chlorine concentration was determined by free chlorine micro-check test strips (HF Scientific, Ft. Myer, FL). The pH values of washing solutions were measured using a digital pH meter (pHTestr 20, Eutech Instruments, Thermo Scientific). Inoculated spinach leaves (35 g) were immersed in 1 L of 3% H_2O_2, 200 ppm chlorinated water or DI water and gently stirred with a spatula for 5 min at 22 ± 1°C. Spinach samples were dried in a salad spinner before treatment with aerosolized antimicrobials or enumeration.

Aqueous H_2O_2, LA, and AIT alone and combination of LA and AIT were prepared and aerosolized in a jar model system as described in section 2.4. Washed or unwashed spinach leaves (5 g) were placed in a jar and treated with aerosolized antimicrobials for 2 min in a bio-safety hood at room temperature. The concentrations of the antimicrobials were shown in Table 4.2 and 4.3. After treatment, the jar was sealed with cap immediately and refrigerated at 4 °C for 10 days before microbiological analysis.

4.2.6 Inactivation of *E. coli* O157:H7 on baby spinach packaged in a commercial PET box during 10 days of refrigeration storage – scale-up study

Inoculated spinach leaves (35 g) were immersed in 1L of 3% H_2O_2 and gently stirred with a spatula for 5 min at 22 ± 1°C. Spinach samples were then dried in a salad spinner before treatment of aerosolized antimicrobials.

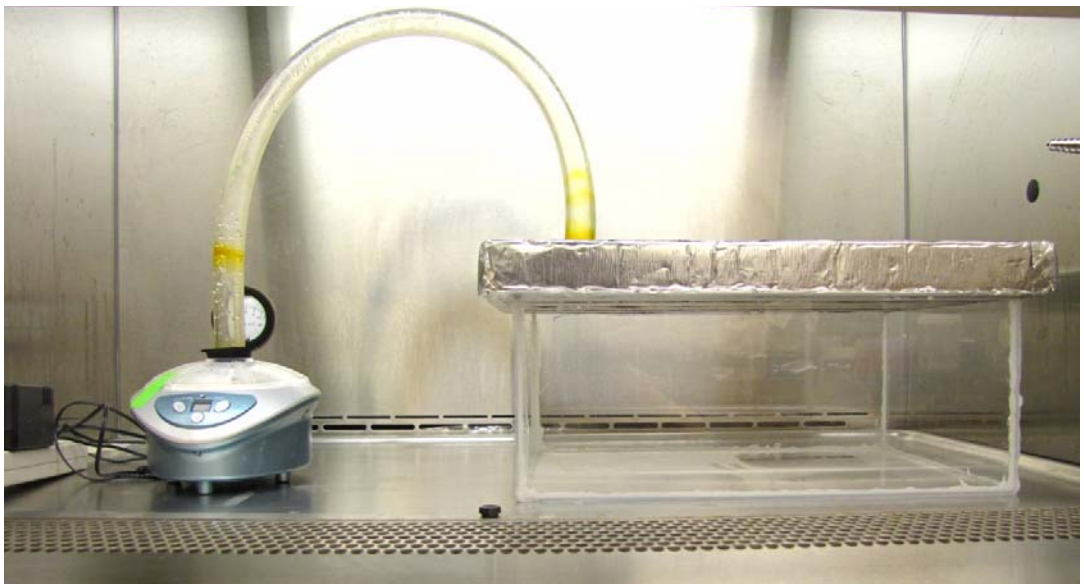

Figure 4.2 Treatment of aerosolized antimicrobials in a fume tank model system

A fume tank (18cm*28cm*14cm = 7 L) was used to test the efficacies of aerosolized antimicrobials in a scale-up model system (Figure 4.2). The tank was sealed, and aerosolized antimicrobials were routed from an ultrasonic nebulizer using a flexible tygon tube. The tube entered the lid of the fume tank through a rubber stopper, which was sealed around the edges with silicon glue. Solution of 2.5% LA in combination with 1% AIT was atomized into extremely fine micro-particles in the nebulizer and routed into the tank for 5 min to let sanitizer mist visually saturate in the tank. Washed spinach samples (5 g) were placed in a polyethylene terephthalate (PET) box (Safe-T-Fresh TS16, inline plastics corp., Shelton, CT) with lid uncovered. Three boxes of samples were treated per batch in the fume tank for 2 min at room temperature. In addition, 0 – 5 µl of AIT (equivalent to 0, 5, 10 µl of AIT per liter of

air in the PET box) were deposited on a filter paper (filter paper, Q5, 55 mm diameter, Fisher Scientific) attached to the cover of PET box to supplement additional AIT. Treated samples were immediately sealed in the PET box and refrigerated at 4°C for up to10 days before microbiological analysis.

4.2.7 Microbiological analysis

To determine the counts of *E. coli* O157:H7, 95 mL of sterile 0.1% peptone water was added to 5 g of spinach samples in a sterile stomacher bag. The mixture was pummeled in a laboratory stomacher (Seward 400 Stomacher; Seward Medical Co., London, United Kingdom) at high speed for 4 min. The spinach slurry was then serially diluted in sterile 0.1% peptone water and spread-plated onto TSAYE-NS and Sorbitol Macconkey (Difco Laboratories) supplemented with 100 µg/mL of nalidixic acid and 100 µg/mL streptomycin (SMAC-NS) plates for the counts of injured and uninjured cells. Plates were incubated at 35 °C for 48 – 72 h before typical *E. coli* O157:H7 colonies were enumerated.

4.2.8 Statistical analysis

All experiments were replicated three times. Colony counts were converted to log CFU/g and means and standard deviations were calculated. Statistical analyses were conducted using JMP (SAS Cary, NC, USA). One-way analysis of variance and Student's t one-way multiple comparisons were used to determine significant differences among treatments ($P < 0.05$).

4.3 Results and discussion

4.3.1 Test of cross-strain inhibition

Cross-strain inhibition tests showed that neither of the 2 strains inhibited the growth of the other strain. Thus the use of a 2-strain cocktail in inoculums would not present a concern with regard to loss of viability or inhibition caused by strain interactions. In addition, the use of antibiotic resistant bacterial strains and medium supplemented with antibiotic facilitated the enumeration of *E. coli* O157:H7 without being influenced by background microflora on baby spinach.

4.3.2 Effectiveness of aerosolized antimicrobials against *E. coli* O157:H7 on baby spinach stored at 4 °C for 2 days.

H_2O_2 (2.5 and 5%), LA, AA and AIT were atomized into extremely fog-like fine particles and tested for their effectiveness against *E. coli* O157:H7 on baby spinach during 2-day refrigeration storage in a glass jar (Table 4.1). The rate of aerosolized antimicrobials generated from the nebulizer was about 639 μL/min. In the absence of antimicrobials, the population of *E. coli* O157:H7 on the spinach decreased about 0.6 logs after 2 days refrigeration storage. A 0.3 – 1.2 log reduction of *E. coli* O157:H7 on spinach was observed on day 0 after treatments of aerosolized antimicrobials, among which H_2O_2 was the most effective. 5% AIT only reduced *E. coli* O157:H7 by 0.3 logs on day 0; however, after 2 days refrigeration, the *E. coli* O157:H7 population on samples decreased by about 2 log units. There was no significant difference between the counts on SMAC-NS or TSAYE-NS on Day 2 for all the treatments, except for 2.5% AA, indicating that these treatment killed cells without causing substantial number of sub-lethally injured cells after 2 days storage at 4 °C.

Table 4.1 Effect of various aerosolized antimicrobials against *E. coli* O157:H7 on baby spinach during 2-day storage at 4 °C.

		Day 0		Day 2	
Treatment	pH	SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS
Untreated		6.4 ± 0.1 ^{Aa}	6.5 ± 0.1 ^{Aa}	5.8 ± 0.1 ^{Ab}	5.9 ± 0.1 ^{Ab}
DI Water	6.3	6.1 ± 0.1 ^{ABa}	6.2 ± 0.1 B^{a}	5.7 ± 0.1 ^{ABb}	5.8 ± 0.0 ^{Ab}
2.5% AA	2.8	5.8 ± 0.1 ^{Ba}	5.9 ± 0.2 ^{BCa}	5.2 ± 0.1 ^{BCc}	5.5 ± 0.2 ^{ABb}
2.5% LA	2.4	5.5 ± 0.2 ^{Cab}	5.7 ± 0.2 ^{CDa}	5.0 ± 0.4 ^{Cb}	5.2 ± 0.3 ^{BCb}
2.5% H_2O_2	5.3	5.2 ± 0.2 ^{Cab}	5.4 ± 0.1 ^{Ea}	4.8 ± 0.4 ^{CDb}	4.9 ± 0.3 ^{CDb}
2.5% AIT	6.3	5.9 ± 0.3 ^{Ba}	6.0 ± 0.3 ^{Ba}	4.6 ± 0.5 ^{CDb}	4.9 ± 0.3 ^{BCDb}
5% AA	2.6	5.4 ± 0.2 ^{Cab}	5.5 ± 0.1 ^{DEa}	5.0 ± 0.3 ^{Cb}	5.2 ± 0.4 ^{BCab}
5% LA	2.3	5.3 ± 0.2 ^{Cab}	5.6 ± 0.4 ^{CDEa}	4.9 ± 0.4 ^{Cb}	4.9 ± 0.3 ^{CDb}
5% H_2O_2	4.9	5.3 ± 0.3 ^{Ca}	5.3 ± 0.3 ^{Ea}	4.8 ± 0.2 ^{CDb}	4.9 ± 0.1 ^{CDb}
5% AIT	6.3	6.1 ± 0.2 ^{Ba}	6.2 ± 0.1 ^{Ba}	4.3 ± 0.6 ^{Db}	4.6 ± 0.5 ^{Db}

* Data represent mean population (log10 CFU/g) of three replicates ± standard deviations;
* Data in the same row followed by the same lowercase letter are not significantly different (P > 0.05) and data in the same column followed by the same capital letter are not significantly different (P> 0.05).

Fumigation with AA has been reported to protect grape and strawberry from spoilage for up to 2 months or 2 weeks, respectively, in MAP at refrigeration temperatures (Moyls, Gaunce, & Sholberg, 1996). AA vapor has also been used to control postharvest decay of pear without causing adverse effect on appearance (Sholberg, Moyls, Randall, & Shephard, 2004). However, in our study, brown spots were observed on the leaves after treatment with aerosolized AA (5%) and storage at 4 °C for 2 days. A similar result was reported by Sapers et al. (2003) who found that AA vapor may cause discoloration on apple. They also reported up to 1.7 log reduction of *E. coli* on apple after treatment of H_2O_2 vapor. Our results indicated that 2.5% H_2O_2 aerosolization was as effective as 5% H_2O_2, which resulted in >1 log

reduction of *E. coli* O157:H7 on the spinach after only a 2-min treatment. Based on the effectiveness of these antimicrobial tested, 2.5% H_2O_2, 5% LA, 2.5 and 5% AIT were selected for a 10-day storage study.

4.3.3 Effectiveness of washing with hydrogen peroxide followed by treatment of aerosolized antimicrobials against *E. coli* O157:H7 on baby spinach stored at 4 °C for 10 days.

A pre-washing step was combined with treatment of aerosolized antimicrobial at this stage, followed by 10 days refrigeration storage at 4 °C (Table 4.2). DI water and 3% H_2O_2 were used to wash samples before treatment of aerosolized antimicrobials. Samples treated with aerosolized antimicrobials without a pre-washing step and samples washed with 200 ppm chlorinated water were used as a comparison. The initial inoculation level was 6.3 log CFU/g. During the 10-day storage at 4 °C, the population of *E. coli* O157:H7 on control samples gradually decreased by 0.8 logs. For samples prewashed with DI water or 3% H_2O_2, a similar reduction of *E. coli* O157:H7 was found during the subsequent 10-day storage, although a slightly lower reduction (0.4 log CFU/g) of *E. coli* O157:H7 was found for samples treated with 200 ppm chlorine. These results were in general in agreement with previous studies. Obaidat & Frank (2009) reported that the population of *E. coli* O157:H7 decreased by 1 log on lettuce stored at 0 or 4 °C for 4 days. Li, Brackett, Chen, & Beuchat (2001) reported a 1-log reduction of *E. coli* O157:H7 on lettuce stored at 5 °C for 18 days. Abdul-Raouf, Ammar, & Beuchat (1993) found that the population of *E. coli* O157:H7 decreased by 0.8 on shredded lettuce stored at 5 °C for 14 days. All of these studies showed storage temperature is of great importance to the safety of post-harvest fresh produce.

Table 4.2 Effect of various aerosolized antimicrobials against *E. coli* O157:H7 on baby spinach during 10-day storage at 4 °C in the jar system.

Aerosolized antimicrobials	Day 0		Day 2		Day 4	
	SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS
200 ppm chorine washing followed by treatment of aerosolized antimicrobials						
Untreated	4.6 ± 0.2	4.7 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	4.2 ± 0.2	4.3 ± 0.2
With 3% H_2O_2 pre-washing (pH = 5.0)						
2.5% H_2O_2	4.7 ± 0.2 ^{A}	4.8 ± 0.2 ^{A}	4.5 ± 0.3 ^{A}	4.6 ± 0.3 ^{A}	4.1 ± 0.1 ^{A}	4.3 ± 0.1 ^{A}
2.5%AIT	4.7 ± 0.5 ^{A}	4.8 ± 0.4 ^{A}	4.0 ± 0.2 ^{C}	4.1 ± 0.2 ^{B}	2.1 ± 0.9 ^{B}	2.7 ± 1.2 ^{B}
5%AIT	4.0 ± 0.6 ^{B}	4.3 ± 0.6 ^{A}	< 1.3 ^{D}	< 1.3 ^{D}	< 1.3 ^{B}	< 1.3 ^{C}
5%LA	4.6 ± 0.3 ^{AB}	4.7 ± 0.3 ^{A}	4.0 ± 0.2 ^{BC}	4.3 ± 0.2 ^{B}	3.9 ± 0.2 ^{A}	4.0 ± 0.1 ^{A}
Untreated	4.6 ± 0.1 ^{AB}	4.7 ± 0.2 ^{A}	4.3 ± 0.1 ^{AB}	4.4 ± 0.1 ^{AB}	4.1 ± 0.5 ^{A}	4.3 ± 0.4 ^{A}
With DI water pre-washing (pH = 6.3) followed by treatment of aerosolized antimicrobials						
2.5% H_2O_2	4.9 ± 0.2 ^{A}	5.1 ± 0.2 ^{A}	4.4 ± 0.2 ^{B}	4.6 ± 0.3 ^{B}	4.3 ± 0.3 ^{A}	4.4 ± 0.3 ^{A}
2.5%AIT	5.1 ± 0.2 ^{A}	5.2 ± 0.2 ^{A}	4.9 ± 0.2 ^{A}	5.1 ± 0.1 ^{A}	3.2 ± 0.3 ^{B}	3.6 ± 0.3 ^{B}
5%AIT	5.1 ± 0.2 ^{A}	5.3 ± 0.2 ^{A}	< 1.3 ^{C}	< 1.3 ^{C}	< 1.3 ^{C}	< 1.3 ^{C}
5%LA	4.9 ± 0.3 ^{A}	5.0 ± 0.4 ^{A}	4.6 ± 0.4 A^{B}	4.8 ± 0.4 ^{AB}	4.3 ± 0.1 ^{A}	4.5 ± 0.1 ^{A}
Untreated	5.1 ± 0.2 ^{A}	5.2 ± 0.2 ^{A}	4.6 ± 0.3 ^{AB}	4.8 ± 0.3 ^{AB}	4.5 ± 0.2 ^{A}	4.7 ± 0.1 ^{A}
Treatment of aerosolized antimicrobials with no pre-washing						
2.5% H_2O_2	5.2 ± 0.1 ^{B}	5.2 ± 0.1 ^{D}	5.1 ± 0.2 ^{B}	5.1 ± 0.1 B	4.8 ± 0.3 B	4.9 ± 0.3 ^{B}
2.5%AIT	5.9 ± 0.2 ^{A}	6.1 ± 0.1 ^{AB}	5.4 ± 0.1 ^{AB}	5.5 ± 0.1 AB	5.0 ± 0.1 B	5.4 ± 0.2 ^{B}
5%AIT	5.8 ± 0.3 ^{A}	5.9 ± 0.2 ^{B}	< 1.3 ^{C}	< 1.3 C	< 1.3 C	< 1.3 ^{C}
5%LA	5.5 ± 0.2 ^{B}	5.6 ± 0.2 ^{C}	5.0 ± 0.5 ^{B}	5.2 ± 0.5 ^{B}	4.8 ± 0.5 ^{B}	4.9 ± 0.4 ^{B}
Untreated	6.1 ± 0.1 ^{A}	6.3 ± 0.1 ^{A}	5.7 ± 0.1 ^{A}	5.9 ± 0.1 ^{A}	5.6 ± 0.1 ^{A}	5.8 ± 0.1 ^{A}

Table 4.2 (Continued)

Aerosolized	Day 6		Day 8		Day 10	
antimicrobials	SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS
200 ppm chorine washing						
Untreated	4.3 ± 0.1	4.4 ± 0.1	4.3 ± 0.1	4.3 ± 0.1	4.2 ± 0.0	4.3 ± 0.1
With 3% H_2O_2 pre-washing (pH = 5.0)						
2.5% H_2O_2	4.0 ± 0.3 ^{A}	4.1 ± 0.2 ^{A}	4.1 ± 0.4 ^{A}	4.2 ± 0.3 ^{A}	3.4 ± 0.4 ^{A}	3.5 ± 0.6 ^{A}
2.5%AIT	2.2 ± 0.8 ^{B}	3.0 ± 0.1 ^{B}	1.5 ± 0.5 ^{B}	2.0 ± 0.8 ^{B}	< 1.3 ^{B}	2.1 ± 0.7 ^{B}
5%AIT	< 1.3 ^{C}	< 1.3 ^{C}	< 1.3 ^{B}	< 1.3 ^{B}	< 1.3 ^{B}	< 1.3 ^{B}
5%LA	3.6 ± 0.5 ^{A}	4.1 ± 0.3 ^{A}	3.5 ± 0.4 ^{A}	3.7 ± 0.5 ^{A}	3.1 ± 0.9 ^{A}	3.3 ± 0.6 ^{A}
Untreated	4.1 ± 0.1 ^{A}	4.3 ± 0.2 ^{A}	3.6 ± 0.3 ^{A}	4.0 ± 0.3 ^{A}	3.8 ± 0.6 ^{A}	3.9 ± 0.6 ^{A}
With DI water pre-washing (pH = 6.3) followed by treatment of aerosolized antimicrobials						
2.5% H_2O_2	4.4 ± 0.6 ^{A}	4.5 ± 0.6 ^{A}	4.3 ± 0.5 ^{A}	4.4 ± 0.5 ^{A}	4.1 ± 0.3 ^{A}	4.3 ± 0.3 ^{A}
2.5%AIT	3.2 ± 0.2 ^{B}	3.6 ± 0.5 ^{B}	< 1.3 ^{B}	3.0 ± 0.1 ^{B}	< 1.3 ^{B}	1.9 ± 0.6 ^{B}
5%AIT	< 1.3 ^{C}	< 1.3 ^{C}	< 1.3 ^{B}	< 1.3 ^{C}	1.3 ± 0 ^{B}	< 1.3 ^{C}
5%LA	4.1 ± 0.7 ^{A}	4.2 ± 0.5 ^{AB}	4.2 ± 0.1 ^{A}	4.4 ± 0.2 ^{A}	4.0 ± 0.2 ^{A}	4.3 ± 0.2 ^{A}
Untreated	4.6 ± 0.2 ^{A}	4.7 ± 0.1 ^{A}	4.6 ± 0.3 ^{A}	4.8 ± 0.3 ^{A}	4.1 ± 0.3 ^{A}	4.4 ± 0.3 ^{A}
Treatment of aerosolized antimicrobials with no pre-washing						
2.5% H_2O_2	4.6 ± 0.4 ^{A}	4.7 ± 0.3 ^{BC}	4.3 ± 0.2 ^{B}	4.5 ± 0.2 ^{B}	4.2 ± 0.3 ^{B}	4.4 ± 0.2 ^{B}
2.5%AIT	3.1 ± 0.6 ^{B}	4.2 ± 0.1 ^{C}	2.3 ± 0.8 ^{C}	3.9 ± 0.2 ^{C}	< 1.3 ^{C}	2.8 ± 0.3 ^{C}
5%AIT	< 1.3 ^{C}	< 1.3 ^{D}	< 1.3 ^{D}	< 1.3 ^{D}	< 1.3 ^{C}	< 1.3 ^{D}
5%LA	4.6 ± 0.7 ^{A}	4.8 ± 0.6 ^{B}	4.3 ± 0.6 ^{B}	4.6 ± 0.4 ^{B}	4.2 ± 0.3 ^{B}	4.5 ± 0.3 ^{B}
Untreated	5.1 ± 0.2 ^{A}	5.3 ± 0.2 ^{A}	5.4 ± 0.2 ^{A}	5.6 ± 0.2 ^{A}	5.4 ± 0.2 ^{A}	5.5 ± 0.2 ^{A}

* The inoculated samples were pre-washed with 3% H_2O_2 or DI water and then treated with various aerosolized antimicrobials in glass jars. The treated samples in the jars were stored at 4 °C for 10 days.
* Data represent mean population (log10 CFU/g) of three replicates ± standard deviations;
* Data in the same column of each pre-washing solution category followed by the same capital letter are not significantly different (P> 0.05).

The population of *E. coli* O157:H7 on spinach was reduced by 1.0, 1.6 and 1.6 log, respectively, after washing with DI water, 3% H_2O_2 and 200 ppm chlorinated water for 5 min at room temperature. Washing with 3% H_2O_2 showed the same effectiveness as chlorine washing against *E. coli* O157:H7 on spinach. Some other studies have reported similar results (Beuchat, Nail, Adler, & Clavero, 1998; Keskinen et al., 2009), although some higher reduction of *E. coli* O157:H7 has also been reported. Lin et al. (2002) reported 4.5, 4.7, and 2.7 log reductions of *Salmonella enteric* serotype Enteritidis, *E. coli* O157:H7, and *L. monocytogenes*, respectively, by washing lettuce leaves with 2% H_2O_2 at 50 °C for 1.5 min. Sapers, Mattrazzo, & Miller (1999) revealed that inoculated apples washed with 5% H_2O_2 alone or in combination with acidic surfactants for 1 min reduced 3 – 4 log of *E. coli*. These differences may plausibly result from the different targeted produce, treatment temperature and duration, inoculation method, enumeration method and medium.

The combination of 3% H_2O_2 pre-washing and treatment of aerosolized 2.5% H_2O_2, 5% LA, 2.5 or 5% AIT followed by 10 days storage at 4 °C reduced population of *E. coli* O157:H7 on spinach by 2.8, 3.0, 4.2 and > 5.0 log CFU/g, respectively. For samples treated with aerosolized H_2O_2 or LA, 10 days storage at 4 °C resulted in an additional <1.3 log reduction of *E. coli* O157:H7 by the end of the storage compared to that of day 0. On day 0, no further reduction of *E. coli* O157:H7 was observed after pre-washed samples were treated with aerosolized H_2O_2 or LA, although the same treatment significantly reduced *E. coli* O157:H7 on unwashed samples. The population of *E. coli* O157:H7 on these treated samples gradually decrease by 0.8 to 1.3 logs which was similar to that of the control. It is likely that aerosolized H_2O_2 deposited on the spinach leaves was inactivated since it can be

decomposed into water and oxygen by catalase which is naturally found in plants (Ölmez & Kretzschmar, 2009). The inefficiency of H_2O_2 and LA could also due to the short treatment time (2 min) employed in our study. Oh et al. (2005) reported significant higher reduction of targeted pathogens when the treatment time was prolonged from 10 min to 60 min. However, such long treatment time would be impractical for the produce industry.

5% AIT showed the strongest antimicrobial effect during the storage; however, the quality of the leaves was impaired by the second day of refrigeration. 2.5 % AIT preserved the quality of leaves much better; however, noticeable browning effects also occurred on day 8 and 10. Thus, a lower concentration of AIT is needed to better preserve the quality of spinach. AIT has been reported to be more stable at acidic pH (Tsao, Potter, Chiba, Yu, & Friesen, 2000). Therefore, lower concentrations of AIT in combination with lactic acid were tested for their potential to control *E. coli* O157:H7 during refrigerated storage of packaged spinach.

Table 4.3 Effect of aerosolized AIT in combination with lactic acid against *E. coli* O157:H7 on baby spinach in glass bottles during 10-day storage at 4 °C. The inoculated samples were pre-washed with 3% H_2O_2 and then aerosolized with AIT or AIT plus lactic acid in glass jars. The treated samples in the jars were stored at 4 °C for 10 days.

Aerosolized	0		5		10	
antimicrobials	SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS
1% AIT	5.0 ± 0.2^{A}	5.0 ± 0.2^{A}	3.8 ± 0.2^{A}	4.0 ± 0.1^{A}	2.9 ± 0.3^{A}	3.8 ± 0.3^{A}
2% AIT	4.8 ± 0.3^{A}	5.0 ± 0.2^{A}	2.3 ± 0.9^{B}	3.6 ± 0.3^{AB}	$<1.3^{B}$	1.8 ± 0.8^{B}
2.5% LA+1% AIT	4.5 ± 0.7^{A}	4.7 ± 0.4^{A}	2.3 ± 0.9^{B}	2.7 ± 1.2^{BC}	$<1.3^{B}$	1.6 ± 0.6^{B}
2.5% LA+2% AIT	4.7 ± 0.3^{A}	4.8 ± 0.3^{A}	$<1.3^{C}$	1.7 ± 0.6^{C}	$<1.3^{B}$	$<1.3^{B}$
5% LA+1% AIT	4.7 ± 0.1^{A}	4.7 ± 0.1^{A}	$<1.3^{C}$	2.1 ± 0.9^{C}	$<1.3^{B}$	1.7 ± 0.7^{B}
5% LA+2% AIT	4.9 ± 0.2^{A}	4.9 ± 0.2^{A}	$<1.3^{C}$	1.6 ± 0.4^{C}	$<1.3^{B}$	$<1.3^{B}$

* Data represent mean population (log10 CFU/g) of three replicates ± standard deviations;
* Data in the same column followed by the same capital letter are not significantly different (P> 0.05).

As shown in Table 4.3, 1 or 2 % AIT was used alone or in combination with 2.5 or 5% LA to test their efficacies against *E. coli* O157:H7 during 10 days storage at 4 °C. On day 0, no significant difference on the effectiveness was observed between treatments of different aerosolized antimicrobials. On day 5, all the treatments resulted in significant reduction of *E. coli* O157:H7 compared with initial inoculation level. Treatment of 2.5% LA+2% AIT, 5% LA +1% AIT or 5% LA + 2% AIT resulted in > 4.8 log reduction of *E. coli* O157:H7 on SMAC-NS and 4.6, 4.2 and 4.7 log reduction of *E. coli* O157:H7 on TSAYE-NS, respectively. No significant difference was found between counts on SMAC-NS and TSAYE-NS plates. On day 10, all the treatments except for 1% AIT reduced counts of *E. coli* O157:H7 by > 4.5 log CFU/g. Treatment of 1% AIT resulted in 3.3 and 2.5 log reduction of *E. coli* O157:H7 in 10 days on SMAC-NS and TSAYE-NS, respectively; however, t-test showed that there is a significant difference between these two counts indicating a portion of sub-lethally injured cells after the treatment. Our results showed that 2.5% LA + 1% AIT could be as effective as 2% AIT when it is used in combination with 3% H_2O_2 pre-washing followed by storage at 4 °C for 10 days.

In our study, the rate of aerosolized antimicrobials generated from the nebulizer was about 639 μL/min, thus the final concentration of AIT in the jar was about 12.8 ppm for application of 1% AIT for 2 min, although the actual value could be lower due to degradation of AIT in water. Some other studies have also investigated the effectiveness of AIT against various pathogens. Lin et al. (2000) tested the bactericidal activity of AIT against pathogens on lettuce, tomatoes and apples in a large freezer bag loaded with various amounts of AIT and found that 100 ppm of AIT vapor killed about 5 logs of pathogen population in 24 h. However, they

also reported that the lettuce acquired a strong pungent flavor and lost crispness after treatment when a high dose of AIT was used. Obaidat & Frank (2009) reported a > 4 log reduction of *E. coli* O157:H7 on lettuce at 0 and 4 °C in 4 days and at 10 °C in 2 days by using AIT vapor of 4, 8, or 16 µl/L. In their study, they deposited AIT on a filter paper inside a septa jar; however, their sample only consisted of one inoculated leaf.

In our study, we found that the addition of 2.5% LA to 1% AIT was as effective as 2% AIT, and resulted in significant higher reductions of *E. coli* O157:H7 on spinach than 1% AIT alone. This result was in accordance with the study of Luciano & Holley (2009) who reported that AIT was a more effective antimicrobial at low pH and its degradation reduced its activity. Tsao et al. (2000) found that AIT had a half-life at pH 6.0 of 34 days, but of only 26 days at pH 9.0. Rhee, Lee, Dougherty, & Kang (2003) investigated the individual and combined effects of mustard flour (0, 10, or 20%) and acetic acid (0, 0.5, or 1%) against *Escherichia coli* O157:H7, *L. monocytogenes*, and *Salmonella enterica* serovar Typhimurium at 5 and 22°C and found the addition of 0.5% AA to the mustard flour reduced the antimicrobial effect of mustard but a higher reduction of those pathogens was obtained when 1% AA was added to the mustard flour.

Table 4.4 Effect of aerosolized AIT in combination with lactic acid against *E. coli* O157:H7 on baby spinach in PET box during 10-day storage at 4 °C. The inoculated samples were pre-washed with 3% H_2O_2 and then aerosolized with AIT plus LA in open PET boxes inside a fume tank. The treated samples in the closed PET boxes with or without AIT on a piece of filter paper were stored at 4 °C for 10 days.

Aerosolized antimicrobials	AIT on filter paper	0		5		10	
		SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS	SMAC-NS	TSAYE-NS
2.5%LA+1%AIT	0 µL	4.0 ± 0.2^{A}	4.1 ± 0.2^{A}	3.7 ± 0.2^{A}	3.7 ± 0.1^{A}	3.0 ± 0.4^{A}	3.4 ± 0.4^{A}
2.5%LA+1%AIT	2.5µL	4.0 ± 0.2^{A}	4.1 ± 0.2^{A}	3.1 ± 0.3^{AB}	3.4 ± 0.2^{A}	1.5 ± 0.3^{B}	2.5 ± 0.7^{B}
2.5%LA+1%AIT	5 µL	4.0 ± 0.2^{A}	4.1 ± 0.2^{A}	1.5 ± 0.3^{C}	2.2 ± 0.8^{B}	$<1.3^{B}$	$<1.3^{C}$
Untreated	2.5µL	4.5 ± 0.0^{B}	4.5 ± 0.1^{B}	2.9 ± 0.2^{B}	3.4 ± 0.4^{A}	1.9 ± 0.6^{B}	2.8 ± 0.6^{AB}
Untreated	5 µL	4.5 ± 0.0^{B}	4.5 ± 0.1^{B}	3.0 ± 0.7^{AB}	3.2 ± 0.8^{AB}	$<1.3^{B}$	1.6 ± 0.4^{C}

* Data represent mean population (log10 CFU/g) of three replicates ± standard deviations;
* Data in the same column followed by the same capital letter are not significantly different (P> 0.05)

4.3.4 Inactivation of *E. coli* O157:H7 on baby spinach packaged in commercial PET box during 10-day refrigeration storage.

The effectiveness of a combination of 3% H_2O_2 pre-washing and aerosolized LA and AIT followed by storage at 4 °C for 10 days in a scale-up model system is shown in Table 4.4. Combination of 2.5% LA + 1% AIT was selected based on the result in section 3.2 in light of the cost and quality of leaves after treatment. The initial inoculation level was 6.3 log CFU/g. Pre-washing with 3% H_2O_2 for 5 min reduced *E. coli* O157:H7 by 1.7 – 1.8 log CFU/g. After treatment with aerosolized LA + AIT, an additional 0.4 log reduction of *E. coli* O157:H7 was achieved on day 0. During the next 10 days, 2.9 to > 5 log reduction of *E. coli* O157:H7 on baby spinach was observed among the treatments by day 10. Pre-washing with 3% H_2O_2, and exposure to aerosolized 2.5% LA + 1% AIT and 5 µl AIT (equivalent to 10 µl/L of air) supplement on filter paper resulted in 4.1 log (TSAYE-NS) and > 5 log reduction of *E. coli* O157:H7 on day 5 and day 10, respectively. No noticeable color change was found on day 10.

Treatment of pre-washing with H_2O_2 followed by aerosolized 2.5% LA + 1% AIT, without supplementary AIT, in the scale-up system reduced *E. coli* O157:H7 on spinach by 3.1 (SMAC-NS) or 2.9 (TSAYE-NS) logs after 10 days refrigeration, while the same treatment in the prototype model system resulted in > 4.8 (SMAC-NS) and 4.6 (TSAYE-NS) log reduction of *E. coli* O157:H7. This difference may be mainly due to the different packaging materials (glass jar and PET box) used for samples since PET has a much higher permeability for gas and liquid than glass. The concentration of AIT gas in the box could have been diluted over time due to exchange of air between PET box and storage environment. When 2.5 or 5 µl AIT (equivalent to 5 or 10 µl/L of air) was added into the package, a significant higher

inactivation of *E. coli* O157:H7 was observed during the 10 days refrigeration without causing visible adverse effect on the quality of leaves. There was no pungent smell when the box was opened at day 5 and 10. Thus, the supplement of AIT on a filter paper in the PET box may help to balance the loss of AIT during the storage.

4.4 Conclusion

Our result showed a potential application of AIT as a novel sanitizing process for leafy greens. AIT showed a high antibacterial effect against *E. coli* O157:H7 during refrigeration storage when applied to baby spinach in aerosolized form. The addition of LA in AIT enhanced the antimicrobial effect of AIT. Further research on the scale-up system is needed to validate the feasibility of the proposed sanitizing method. Additional research on the influence of these treatments on sensory quality of spinach is necessary before practical application in industry.

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Chapter 5

FUTURE RESEARCH

Two intervention strategies were proposed and tested in this work and both demonstrated better effectiveness against *Escherichia coli* O157:H7 on baby spinach than chlorine washing without causing noticeable damage to the appearance. However, before these treatments can be scaled-up, a sensory evaluation with regard to the texture, color, smell, shelf life of the treated samples would have to be necessary to be done. The time that baby spinach requires for transportation, distribution and finally reaches our lab is not known but could be as long as a week. Therefore, it would be ideal to have freshly harvested baby spinach for a research project like this since there may be discrepancy regarding the freshness of the samples. Analysis of total microbial load on baby spinach was not conducted in our studies; however, it would be useful for us to better understand the effectiveness of our treatments against spoilage and microorganisms naturally present on the spinach leaves. Temperature plays a very important role in the shelf life of fresh produce. In our study, all of our samples were stored at 4 °C; however, it is very unlikely that this temperature is always maintained during handling, transportation and distribution process. Thus, from a practical standpoint, it would be useful to know the impact of storage of treated baby spinach at abusive temperature. The application of aerosolized AIT + LA on pre-washed baby spinach showed promise, in comparison with chlorine washing, as a novel processing method to inactivate *E. coli* O157:H7 on baby spinach. To better test the effectiveness of AIT, other delivery system such as a nozzle system could also be

tested. AIT could also be encapsulated or mixed with other compounds such as triglycerides to control the release of AIT during refrigeration storages. Further work is much needed to validate the effectiveness of this treatment in a scale-up system and selection of proper packaging material would be of great importance to the effectiveness of AIT as well.