EFFICACY OF ADVANCED OXIDATION TECHNOLOGY AND LACTIC ACID WASH FOR CONTROLLING *ESCHERICHIA COLI* O157:H7 IN BAGGED BABY SPINACH

By

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Abstract

Escherichia coli O157:H7 outbreaks have been linked to leafy green produce and bagged spinach. The objective of this study was to evaluate a Photohydroionization (PHI) panel (novel advanced oxidation technology) and varying concentrations of lactic acid washes for controlling *E. coli* O157:H7 on baby spinach. Leaves were dip inoculated with a five-strain cocktail of *E. coli* O157:H7 inoculum having a concentration between 5-6 log CFU/ml. Leaves were submerged in inoculum for 30 s and dried for 1 h. Non-inoculated and inoculated leaves were washed for 30 s in food grade lactic acid diluted to concentrations of 0.5, 1.0, or 2.0% and allowed to dry for 10 min. For PHI treatment, leaves were placed under the PHI panel and treated for 1, 2, or 5 min on both sides for total treatment times of 2, 4 or 10 min. Following treatments, leaves were either sealed in low-density polyethylene bags or enumerated. Samples were enumerated at 0, 3, 7, 10, and 14 days following inoculation. Ten gram samples were diluted with sterile peptone and stomached for one min, and then 0.1 ml was plated onto sorbitol MacConkey agar with cefixime and tellurite plates that were incubated at 37°C for 24 h. For lactic acid treatments, *E. coli* O157:H7 populations were different ($P < 0.05$) compared to the control. There was no difference ($P > 0.05$) due to sampling time so sampling times where pooled together for each lactic acid concentration of 0.5, 1.0, and 2.0% and resulted in 2.01, 2.78, and 3.67 log CFU/g reductions, respectively. Leaves treated with 1.0% and 2.0% lactic acid had color degradation and were organoleptically unacceptable by day 14. When leaves were treated with PHI for 1, 2, or 5 min per side, *E. coli* O157:H7 populations were reduced 1.6, 1.49, or 1.95 log CFU/g, respectively. Leaves treated with PHI were not different from one another, but were different ($P < 0.05$) from the positive control. No color change occurred in leaves treated with PHI. The PHI panel and lactic acid washes of 0.5% or higher are effective in reducing *E. coli* O157:H7 in baby spinach.

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Dedication

I would like to dedicate this achievement to my beloved maternal grandparents, Norbert and Martha Mueller. I am glad to have spent so much time with you and miss you both dearly. Your love of cooking has been an inspiration to me and I will always think of you when I step into a kitchen. I would not be the person I am today without your love. Thank you for teaching me what it means to be a family even through the toughest parts of life. We all miss you both.

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Chapter 1 - Introduction

Leafy green produce, such as baby spinach, Romaine lettuce, leaf lettuce, and Iceberg lettuce, has recently been associated with outbreaks of foodborne illness (Ackers and others 1998, CalFERT 2007, Arnade and others 2009, FDA 2012). While many pathogens are associated with these outbreaks, one of the most common in the last decade has been *Escherichia coli* O157:H7. This Gram-negative, motile, non-spore forming bacterium is commonly associated with beef products, but since 1995 *E. coli* O157:H7 has been linked to numerous outbreaks and recalls of spinach and lettuce products (Ackers and others 1998). *Escherichia coli* O157:H7 can grow in a wide variety of temperatures with a minimum temperature for growth of 8°C, a maximum temperature for growth of 44.5°C, and an optimum temperature of 37°C (ICMSF 1996). *Escherichia coli* O157:H7 can grow from pH 4.0 to 9.0 and has a minimum water activity of 0.95 (ICMSF 1996). *Escherichia coli* O157:H7 is a rare variety of *E. coli* but is extremely virulent due to the toxins (Shiga-like toxins: Stx-1, Stx-2, or both) that it produces (Murray and others 2009).

Most infections of *E. coli* O157:H7 are linked to foodborne illnesses with undercooked ground beef products, contaminated water, unpasteurized milk or fruit juices, and uncooked produce (Murray and others 2009). The infectious dose of *E. coli* O157:H7 is very small and some sources believe as few as 10 cells can cause an infection (FDA 2009). Infections with this pathogen range from uncomplicated to hemorrhagic colitis with harsh abdominal pain and bloody diarrhea (Murray and others 2009).

Produce-associated foodborne illnesses have increased over the last several decades due to a large increase in the consumption of fresh fruits and vegetables. Fresh spinach consumption grew more than 130% in the United States (U.S.) between 1999 and 2006 (Ilic and others 2008). Sivapalasingam and others (2004) looked at produce-linked outbreaks that occurred between 1973 and 1997 and found that there was an eightfold increase in the proportion of outbreaks attributed to produce. With the average consumer increasing their intake of fresh produce, such as leafy green produce, the number of outbreaks associated with produce could greatly increase in the future.

The first *E. coli* O157:H7 outbreak linked to produce occurred in 1991, and since that time produce, such as leafy green produce, is becoming a more prominent food vehicle for foodborne pathogens such as *E. coli* O157:H7. The first outbreak of *E. coli* O157:H7 linked to

lettuce occurred in 1995 in Montana (Ackers and others 1998). Thirteen people were hospitalized due to infection, 92 people reported bloody diarrhea, one person developed Hemolytic Uremic Syndrome (HUS), but no deaths were reported. In 1996, a multi-state outbreak linked to Mesclun mixed greens occurred that caused 61 illnesses and three cases of HUS (Sivapalasingam and others 2004).

One of the largest produce outbreaks in history occurred in 2006 when bagged baby spinach was contaminated with *E. coli* O157:H7 (CalFERT 2007). This outbreak caused 205 illnesses, 104 hospitalizations, 31 cases of HUS, and three deaths in 26 states and one Canadian province (Arnade and others 2009). Epidemiological studies conducted after the outbreak linked contamination to river water, cattle feces, and wild pig feces located within one mile of spinach field (CalFERT 2007). Groundwater-surface water interactions were the most likely irrigation related issue that could cause contamination of spinach (Gelting and others 2011). The recall following this outbreak caused a $201.9 million decline in the retail expenditures and even at the end of 2007 sales of bagged spinach were still down 10% compared to before the outbreak (Arnade and others 2009).

The production methods used to cultivate, harvest, and transport spinach and other leafy greens, can actually increase the bacterial load on leaves. Irrigation water and use of manure are believed to be two major sources of contamination for spinach, but conditions used to transport and store leaves can also contribute to an increase in bacterial populations (Solomon and others 2002 a, b; Moyne and others 2011; Islam and others 2004; Mukherjee and others 2003).

Leafy produce watered using overhead spray irrigation have been found to have significantly more contamination than that watered using surface irrigation (Solomon and others 2002a; Moyne and others 2011). *Escherichia coli* O157:H7 has been found to survive in the soil amended with manure. Islam and others (2004) found that *E. coli* O157:H7 could survive in soil 154 days and 217 days in lettuce and parsley fields, respectively, amended with manure. It is recommended that manure be aged more than eight months prior to being applied to vegetable fields. Spinach and other leafy green produce fertilized with *E. coli* O157:H7 tainted irrigation water or manure can take bacteria up through the root system and internalize it in the leaves, thus bacteria become resistant to traditional sanitizing treatments (Solomon and others 2002a)

While pre-harvest contamination does occur, post-harvest processing should focus on reducing the risk of *E. coli* O157:H7. Cross-contamination can occur if unchlorinated water is

used to clean food processing equipment or food contact surfaces, since *E. coli* O157:H7 can be a waterborne pathogen (ICMSF 1996). The recommended storage temperature for fresh-cut produce is 1 to 3°C to maintain quality and prolong shelf-life (Lou and others 2010). Lettuce stored at 5°C had limited outgrowth of *E. coli* O157:H7 while lettuce stored at 12°C had significant growth after eight days (Lou and others 2010). Being able to regulate the temperature of leafy green produce from harvest to retail level is one of the most important ways to control growth of pathogens.

Studies have been conducted looking at the interaction of *E. coli* O157:H7 with the phyllosphere of spinach plants. Xicohtencatl-Cortes and others (2009) found that adherence of *E. coli O157:H7* was independent of the adhesive capacity of the Shiga toxin. The ability of *E. coli* O157:H7 to attach to spinach plants is attributed to the Type 3 secretion system (T3SS) and flagella (Xicohtencatl-Cortes and others 2009). Shaw and others (2008) found that adhesion is T3SS dependent and flagella, pili, intimin, and other adhesions have a minor role in leaf adhesion. *Escherichia coli* O157:H7 is genetically complex and Deng and others (2011) found that *E. coli* O157:H7 may use different gene regulation strategies to resist different sanitizing agents.

According to Beuchat (1995), fruits and vegetables commonly arrive at processing plants or packinghouses with 10^4 to 10^6 microorganisms per gram. Despite washing produce with chlorinated water, pathogens continue to linger on the surface of produce. Illic and others (2008) looked at two minimal processing plants. Prior to washing, 53% of samples tested positive for coliforms and after processing 79% tested positive. Illic and others (2008) also found that 8.9% of samples were contaminated with *E. coli* and 2.8% and 2.4% of positive samples had higher than 10^2 and 10^3 CFU/g, respectively.

Chlorinated water is the most commonly used sanitizer for leafy green produce. Lou and others (2011) studied the effect of free chlorine levels on the reduction, survival, and transference of *E. coli* O157:H7 during washing of fresh-cut lettuce. Washing with levels of free chlorine at or above 0.5 mg/liter (0.5 ppm), resulted in no *E. coli* O157:H7 cells surviving. Transference happened when levels of free chlorine were less than 1.0 mg/liter (1 ppm). Even when 1 to 2 mg/liter (1 to 2 ppm) of free chlorine was present, transference still occurred and when levels were increased to 5.0 mg/liter (5 ppm) non-inoculated lettuce was still contaminated despite no *E. coli* O157:H7 being present in washing solution. Lou and others (2011) concluded

that 5 mg/liter (5 ppm) of free chlorine was needed to prevent pathogen survival, but 10 mg/liter (10 ppm) was necessary to prevent cross-contamination of produce.

The Food and Drug Administration (FDA) approved the use of irradiation on leafy green produce in August 2008 (CFR 21 Part 3 Section 179.26). Since then, numerous studies have evaluated different irradiation methods. Neal and others (2008) studied the effect of low-dose electron beam (e-beam) radiation on the reduction of *E. coli* O157:H7 and *Salmonella* in spinach. Radiation doses above 1.16 kGy reduced *E. coli* O157:H7 from 7.3 log CFU/g to levels at or below the 0.8 log CFU/g detection limit. Radiation doses of 1.2 and 2.1 kGy had little impact on the respiration rate of spinach leaves (Neal and others 2008). Gomes and others (2008) found that treating leaves with up to 1.0 kGy did not cause significant changes in produce quality. Niemira (2007) found that 300 ppm and 600 ppm sodium hypochlorite washing solutions were not effective against internalized *E. coli* O157:H7 from Romaine lettuce or baby spinach. However, ionizing radiation effectively reduced the viable population of pathogens.

Besides chlorinated wash water and irradiation, many other alternative methods of reducing pathogens on leafy green produce have been researched. The use of organic acids, such as lactic acid, citric acid, or ascorbic acid, has been shown to reduce the risk of *E. coli* O157:H7 by as much as 4.88 log CFU/g while still being generally regarded as safe (GRAS) compounds (Huang and Chen 2011; Ölmez 2010; Ho and others 2011). Klockow and Keener (2009) showed that a novel in-package ozone gas treatment could reduce *E. coli* O157:H7 up to 5.6 log CFU/g, but with notable color degradation. Kim and others (2009) found that treating lettuce with a TiO_2-UV photocatalytic reaction provided a 2.6 log CFU/g reduction of *E. coli* O157:H7. While there are numerous intervention strategies available, more work is needed to find effective methods that do not cause adverse nutritional or organoleptic changes.

Lactic acid has the potential to be used an alternative method because of its GRAS status and its effectiveness at reducing bacterial populations. Advanced oxidation technology, such as Photohydroionization, is being utilized in the food industry and has the potential to greatly reduce microbial populations due to numerous antimicrobial compounds produced, including vapor hydrogen peroxide and gaseous ozone. Combining different technologies has the potential to provide a hurdle effect and reduce the risk of pathogens even more. Therefore, the objective of this study was to evaluate a Photohydroionization (PHI) panel, a novel advanced oxidation

technology, and varying concentrations of lactic acid washes on controlling *E. coli* O157:H7 in bagged baby spinach throughout a 14 day shelf-life.

Chapter 2 - Literature Review

2.1 Introduction to *Escherichia coli* O157:H7

Escherichia coli is a Gram-negative, motile, facultative anaerobic, non-spore-forming short, rod (bacillus) shaped bacteria. It is catalase-positive, oxidase-negative, and most strains can ferment lactose in addition to numerous other sugars. It is considered a part of the normal flora of the intestines of mammals, including humans (FDA 2009). General *E. coli* can grow in a wide range of temperatures with a minimum of 7-8°C, a maximum of 44-46°C, and an optimum temperature for growth of 35-40°C (ICMSF 1996). Generic *E. coli* have a minimum water activity of 0.95 with 0.995 being the optimum water activity for growth (ICMSF 1996). Depending on the acid present in media, general *E. coli* can grow from pH 4.0 to 9.0. *Escherichia coli* O157:H7, for example, can grow at pH 4.5 in medium adjusted with hydrochloric acid (HCl) (ICMSF 1996). If lactic acid is used to adjust the pH, then *E. coli* O157:H7 is inactivated (ICMSF 1996).

Strains of *Escherichia coli* are classified, as with all members of Enterobacteriaceae, based on three major antigens: somatic O polysaccharides, capsular K antigens, and flagellar H proteins. There are a multitude of strains that are subdivided into five different pathogenic groups: Enterotoxigenic *E.coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Enterohemorrhagic *E. coli* (EHEC). Each of the strains in these groups has different virulence factors and can cause various diseases, such as urinary tract infections, neonatal meningitis, gastroenteritis, and bacteremia. These groups also affect various sections of the intestines; ETEC, EPEC, and EAEC primarily involve the small intestine while EIEC and EHEC usually affect the large intestine (Murray and others 2009).

Escherichia coli O157:H7 was first recognized as a human pathogen in 1982. This strain of *E. coli* is a rare variety but is extremely virulent due to the toxins (Shiga-like toxins: Stx-1, Stx-2, or both) that it produces (Murray and others 2009). *Escherichia coli* O157:H7 has a minimum temperature for growth of 8°C, a maximum temperature for growth of 44.5°C, and an optimum temperature of 37°C (ICMSF 1996). Pathogenic strains of *E. coli* are able to withstand refrigeration temperatures and *E. coli* O157:H7 populations have little to no change when stored in ground beef at -20°C for over nine months (ICMSF 1996). Thermal inactivation studies have shown that *E. coli* O157:H7 is more heat sensitive than *Salmonella* spp. and is fairly salt tolerant

and can survive in up to 6.5% salt solutions. The Centers for Disease Control and Prevention (CDC) estimates 70,000 *E. coli* O157:H7 infections and 60 deaths occur each year in the United States (NIH 2009). Most infections are attributed to contaminated food, but person-to-person infections also occur. Foods most often implicated in outbreaks are undercooked ground beef products, contaminated water sources, unpasteurized milk or fruit juices, and uncooked produce (Murray and others 2009). The infectious dose for *E. coli* O157:H7 is believed to be very small and some sources say as few as 10 cells are needed to cause an infection (FDA 2009).

Infections with *E. coli* O157:H7 range from mild, uncomplicated diarrhea to hemorrhagic colitis with severe abdominal pain and bloody diarrhea (Murray and others 2009). Fever is usually absent, but only half of patients develop vomiting. Infections are usually self-limiting and last on average only eight days (FDA 2009). Approximately 30-65% of patients develop bloody diarrhea within 2 days of infection and 5-10% of children develop hemolytic uremic syndrome (HUS) (Murray and others 2009). Hemolytic uremic syndrome is characterized by renal failure and hemolytic anemia (FDA 2009). In patients with HUS, up to 30% of patients will develop serious sequelae and 3-5% of patients die from renal failure (Murray and others 2009). Elderly patients with HUS have a mortality rate as high as 50% (FDA 2009).

Produce-associated foodborne illnesses have increased over the last several decades due to a large increase in the consumption of fresh fruits and vegetables. Looking at these outbreaks can provide information about how produce becomes contaminated and may provide insight on the effectiveness of pre-harvest prevention methods. Sivapalasingam and others (2004) looked at all produce-associated outbreaks from 1973 through 1997 and found that during that time there was an eightfold increase in the proportion of illness attributed to produce. During this time produce-associated outbreaks caused 16,058 illnesses, 598 hospitalizations, and eight deaths in 32 states in the U.S. These 103 outbreaks with known etiological agents were caused by bacteria (62 cases, 60%), viruses (21 cases, 20%), parasites (16 cases, 16%) and chemicals or poisons (4 cases, 4%). The most common bacterial pathogen identified was *Salmonella* but during the time of this study *E. coli* O157:H7 was newly implicated as a cause of foodborne illness in produce.

Outbreaks of *E. coli* O157:H7 are often difficult to track and diagnose, but the information from outbreaks can be very valuable to understand how to prevent future outbreaks. Rangel and others (2005) reviewed the outbreaks of *E. coli* O157:H7 that occurred since 1982.

Among the 183 foodborne outbreaks that occurred during that time, 38 (21%) were attributed to produce (Table 1).

Table 1 *Escherichia coli* O157:H7 foodborne outbreaks that occurred since 1982 (*n*= 183) (Rangel and others 2005).

Food outbreak was attributed to:	Outbreaks (percentage of total cases):
Ground Beef	75 (41%)
Unknown Source	42 (23%)
Produce	38(21%)
Other Beef Products	11 (6%)
Other Foods	10 (5%)
Dairy Products	7 (4%)

The first *E. coli* O157:H7 case reported in produce occurred in 1991, and since that time produce is becoming a more prominent food vehicle for *E. coli* O157:H7 outbreaks, especially leafy green vegetables such as spinach, lettuce, and sprouts. Rangel and others (2005) found that of the 38 produce-related cases of *E. coli* O157:H7, 19 cases or 50% were attributed to leafy produce (Table 2).

Table 2 Produce related cases of *Escherichia coli* O157:H7 since 1982 that occurred in the United States (*n*= 38) (Rangel and others 2005).

Outbreak attributed to specific produce	Outbreaks (percentage of total cases)
Lettuce	13 (34%)
Apple juice or cider	7 (18%)
Salad	6 (16%)
Coleslaw	4 (11%)
Melons	4 (11%)
Sprouts	3 (8%)
Grapes	1 (3%)

Produce-associated outbreaks occurred mostly in the summer and fall with 74% of the outbreak cases occurring between July and October. Of the produce-associated cases, none in the U.S. were reported due to imported produce. Fast food restaurants and salad bars were uncommon 50 years ago, but they are becoming common sites for foodborne illness in our society today (Altekruse and Swerdlow 1996). Most of the outbreaks occurred in restaurants (15 cases (39%)), and 20 (53%) of the outbreaks in produce did not involve cross-contamination in the kitchen (Rangel and others 2005). Produce-associated outbreaks did have significantly greater median number of cases per outbreak than beef-associated outbreaks, 20 vs. 8 (P <0.001) (Rangel and others 2005).

2.2 Spinach Consumption in the United States

With the average consumer increasing their intake of fresh produce (Lucier and others 2004) and with more availability of fresh produce year round, the number of outbreaks associated with produce could greatly increase in the future. Globally the harvest area for lettuce increased by 218% and spinach harvest area increased by 300% from 1986 to 2006 (World Health Organization 2008). Spinach is grown around the world and in 2006 China produced 84% of all the spinach produced globally (World Health Organization 2008). In 2011, the USA produced 33,200 acres of fresh spinach, which was valued over $2.5 million (USDA NASS 2012). Since this one commodity travels world-wide, the potential of a foodborne illness has global consequences.

The per capita consumption of spinach has quintupled since 1972 and American consumption of spinach reached 671 million pounds (fresh-equivalent) from 2000-2002 (Lucier and others 2004). Fresh spinach consumption grew more than 130% in the U.S. between 1999 and 2006 (Ilic and others 2008).

Spinach is a nutritious functional food that contains high amounts of vitamins and minerals, such as vitamins C and A, lutein, iron, folic acid, and magnesium (Lucier and others 2004). There are two major varieties of spinach available on the market. Savoy spinach has curly leaves while baby spinach has flat leaves (Ilic and others 2008). One of the fastest growing segments of the packaged salad market is the bagged spinach sector. This value added product accounted for a tenth of total supermarket sales in the $2 billion fresh-cut salad industry (Lucier and others 2004). From September 2002 to August 2003, the sale of baby spinach rose 70% to reach $116 million in sales. As the sales of bagged spinach and other leafy greens increase, the

more risk there is of major foodborne pathogen outbreaks occurring, such as the outbreaks of *E. coli* O157:H7 that have occurred in the last decade.

2.3 *Escherichia coli* O157:H7 Outbreaks Linked to Leafy Green Produce

Prior to 2000

Since 1991, there have been several outbreaks of *E. coli* O157:H7 linked to leafy green vegetables and there have been several major recalls of leafy green vegetables because of possible contamination. The first outbreak of *E. coli* O157:H7 linked to lettuce occurred in July 1995 in Montana (Ackers and others 1998). The outbreak was identified after 40 Montana residents had laboratory-confirmed *E. coli* O157:H7 infections and 52 residents had bloody diarrhea without laboratory-confirmation. Thirteen people were hospitalized due to infection, one person developed HUS, but no deaths occurred.

Epidemiological studies following the outbreak showed that consumption of leaf lettuce was the likely cause of the outbreak. Nineteen of the 27 (70%) patients reported eating purchased leaf lettuce while only eight of the 46 (17%) matched controls consumed leaf lettuce and none of them reported illness. Stool samples from 29 patients yielded *E. coli* O157:H7 and 22 of 23 isolates matched via pulsed-field gel electrophoresis (PFGE). Fifteen of the patients who recalled eating leaf lettuce provided the name of six stores where they purchased their lettuce. These stores had received their shipments from three distributors who obtained their leaf lettuce from two shippers. One of these shippers was a local produce grower and none of the 37 samples of this lettuce obtained from stores or the field or any of the 71 environmental samples obtained from the farm tested positive for *E. coli* O157:H7 (Ackers and others 1998).

To the investigators of this outbreak, this was the first reported community outbreak of *E. coli* O157:H7 linked with the consumption of leaf lettuce. One of the possible sources of cross-contamination that was identified was the practice of crisping. Crisping is the process of submerging lettuce heads in tepid water followed by refrigeration. Since lukewarm water is used to process multiple batches of lettuce, this practice could easily cause cross-contamination of the lettuce (Ackers and others 1998). Following this initial produce-associated outbreak, there were several other outbreaks of *E. coli* O157:H7 associated with leafy green produce. In 1994, 11 of 26 case-patients in Texas had culture-confirmed *E. coli* O157:H7 and patients were more likely than controls to have eaten at a salad bar but not have eaten ground beef products (Barnett and

others (1995). In 1995, there was also an *E. coli* O157:H7 outbreak caused by contaminated Iceberg lettuce in Ohio that sickened 11 people and a separate outbreak in Maine caused 30 illnesses linked back to Iceberg lettuce (Sivapalasingam and others 2004). In 1996, there was a multi-state outbreak that caused 61 illnesses and 3 cases of HUS caused by the consumption of Mesclun mix (traditionally contains chervil, arugula, lettuce, and endive) and there was also an outbreak linked to lettuce in Michigan that caused 54 people to become ill (Sivapalasingam and others 2004).

2000 to 2012

There have been several major *E. coli* O157:H7 outbreaks associated with the consumption of leafy green vegetables since 2000. Cooley and others (2007) found that up until 2006, there were 22 outbreaks of *E. coli* O157:H7 linked to produce of which nine outbreaks were traced to the Salinas Valley region, which is the major leafy green vegetable producing area in the U.S. The Salinas Valley region is often tested for *E. coli* O157:H7 and the frequency of which it is recovered from a variety of sites, lead Cooley and others (2007) to conclude that contamination of leafy green produce may be more prevalent than originally thought.

One of the largest outbreaks of *E. coli* O157:H7 associated with leafy green vegetables occurred in 2006 and was linked to the Salinas Valley region. This outbreak caused 205 illnesses, 104 hospitalizations, 31 cases of HUS, and three deaths in 26 states and one Canadian province (Arnade and others 2009). According to CalFERT (2007), on September 8, 2006 Wisconsin state health officials identified a cluster of *E. coli* O157:H7 illnesses and submitted the PFGE patterns to Pulsenet. The CDC confirmed that the PFGE patterns matched *E. coli* O157:H7 strains on September 12, 2006. On September 13, 2006 CDC alerted FDA of a multi-state outbreak that was associated with bagged baby spinach. A day later, CDC had received reports from eight states citing 50 other cases of infection. Many of the ill patients recalled consuming fresh pre-packaged spinach in the week prior to the symptoms. As the investigation continued, numerous brands were first implicated in the outbreak but it became clear that illness was most associated with Dole brand Baby Spinach. During the course of the investigation, 45 bags of spinach collected from individual households were tested for *E. coli* O157:H7. Of the 44 bags tested, 13 (29.5%) tested positive for *E. coli* O157:H7 and all 13 had a PFGE pattern that matched the outbreak strain and all 13 were Dole brand spinach (CalFERT 2007).

After the outbreak was discovered, the California Food Emergency Response Team (CalFERT) and CDC conducted studies to determine the possible source of the outbreak. CalFERT investigators looked at the processing facility but no *E. coli* O157: strains were identified in samples taken. The investigators did note conditions that would allow cross-contamination if pathogens did arrive on incoming produce. Environmental samples were taken at the four fields that were used to produce the suspected lot of spinach, with product code P227A (CalFERT 2007). *Escherichia coli* O157:H7 was found in the samples but only isolates found at one of the fields had a PFGE pattern indistinguishable from the outbreak stain. Samples that matched the PFGE pattern were taken from river water, cattle feces, and wild pig feces, which were located within one mile of the spinach field (CalFERT 2007).

Gelting and others (2011) investigated irrigation water, watershed around the area, and conditions on the farm to look for potential water-related factors contributing to the outbreak. It was determined that water from the San Benito river could have been used to recharge ground wells used to irrigate the farm and therefore could have contributed to the outbreak. Groundwater-surface water interactions were the most likely irrigation related issue for this outbreak and other ready-to-eat crops. The hydrogeologic conditions, such as a ground water table below the river, helped in understanding that surface water containing *E. coli* O157:H7 could potentially have reached irrigation wells (Gelting and others 2011). Gelting and others (2011) pointed out that by broadening the search for sources of outbreaks to include groundwater-surface water interactions beyond that of the contamination site might enhance the ability to pinpoint sources and prevent further outbreaks.

The 2006 outbreak linked to the consumption of bagged baby spinach caused consumers to change their produce purchasing habits. In a study by Arnade and others (2009), a model was used to investigate the effect of the 2006 *E. coli* O157:H7 bagged spinach outbreak. Using an announcement made by the FDA on September 14, 2006 that consumers should avoid consuming bagged spinach and the announcement made the next day to expand the warning to all fresh spinach, Arnade and others (2009) could look at the sales of bagged spinach, bulk spinach, bagged salads without spinach, Romaine hearts, bulk Iceberg lettuce and other bulk lettuce prior to the announcement and the sales of the same commodities after the outbreak. By compiling the model and the actual sales data following the outbreak, it was found that total retail expenditures on bagged spinach declined $201.9 million (20%) compared to normal sales.

Three weeks after FDA made the announcement, expenditures for bagged spinach dropped 63% and even 68 weeks after the announcement expenditures were still down 10%. Initially, bulk spinach also experienced a decline in sales, but sales of bulk spinach were above average by week 31 indicating that bulk spinach had become a replacement for bagged spinach (Arnade and others 2009). During the outbreak, other vegetables were substituted for leafy greens for a short period but the sales of leafy greens shifted as other leafy greens were sold to replace bagged spinach. Even at the end of 2007, bagged spinach sales were down 10% due to the shock of the *E. coli* O157:H7 outbreak (Arnade and others 2009).

Other than the recall of bagged spinach in 2006, there have been numerous other recalls involving leafy green produce. Between 2006 and 2011, recalls have been associated with Romaine lettuce, shredded Iceberg lettuce, bagged salad, baby spinach, bagged spinach, spinach salad, organic baby spinach from numerous companies (FDA 2012). Lettuce and spinach recalls have been due to numerous pathogens such as *Listeria monocytogenes, Salmonella* spp., *E. coli* O157:H7, and *E. coli* O145 (FDA 2012). Produce recalls due to pathogen contamination are becoming more common and research is being conducted to find sources both pre-harvest and post-harvest and methods of prevention in processing plants.

2.4 Pre-harvest Production Methods

Production methods used to grow, harvest, and transport spinach, or other leafy green produce, can actually increase the bacterial load on the leaves and therefore can increase the presence of *E. coli* O157:H7. Irrigation water and use of manure are believed to be the two major sources for contamination in spinach, but conditions used for transportation and storage of the leaves can also contribute to the increase in bacterial populations (Solomon and others 2002a,b; Moyne and others 2011; Islam and others 2004; Mukherjee and others 2003). Numerous studies have also been conducted comparing the microbiological quality of organically grown and conventionally grown leafy green produce (Mukherjee and others 2003; McMahon and Wilson 2001; Oliveria and others 2010).

Comparing bacterial levels on leafy green produce grown organically and grown using conventional methods can provide some information about the safety of these products and will assist in identifying growing conditions that may lead to greater bacterial numbers. Mukherjee and others (2003) looked at organic and conventionally grown produce in Minnesota and compared levels of coliforms, *Escherichia coli, Salmonella* spp., and *Escherichia coli* O157:H7

in each. Produce sampled included tomatoes, leafy greens (spinach, kale, amaranth, and Swiss chard), lettuce, green peppers, cabbage, cucumbers, broccoli, strawberries, apples, summer squash, bok choi, zucchini, cantaloupes, carrots, eggplant, raspberries, onions, beets, basil, and kohlrabi. Based on this study, organically grown leafy greens and broccoli had slightly greater coliform populations than other vegetables between the two types of farms (Mukherjee and others 2003). *Escherichia coli* was isolated from 8% of all produce samples analyzed, but the overall appearance of *E. coli* was approximately six times greater for organic than conventionally grown fruits and vegetables. Organic lettuce had 22.4% of samples test positive for the presence of generic *E. coli* (Mukherjee and others 2003). None of the samples tested on either type of farm tested positive for *E. coli* O157:H7.

A study done in Northern Ireland could not find *E. coli* or *E. coli* O157:H7 on any of the 86 organically grown vegetables obtained from retail stores or from producers (McMahon and Wilson 2001). Another study looked at the microbiological quality of conventionally grown and organically grown lettuce in Spain. Oliveria and others (2010) analyzed a total of 72 samples from 18 farms of both types and found that 22.2% (16 samples) of organically grown lettuce tested positive for *E. coli* O157:H7 while only 12.5% (9 samples) of conventionally grown lettuce tested positive (Oliveria and others 2010). Of the organically grown samples, 13.9% of samples had between 30 and 99 MPN 100 g^{-1} and none of the samples had more than 1,000 MPN 100 g^{-1}. The conventionally grown lettuce had 8.4% of samples with counts greater than 1,000 MPN 100 g^{-1} (Olveria and others 2010).

Based on these studies, it is clear that leafy green produce and other vegetables can easily be contaminated with *E. coli* O157:H7 and occurrence of this pathogen is determined by farming practices. Controlling the use of compost, manure, and irrigation water can have a major impact on the microbiological quality of produce whether grown organically or conventionally. The most important pre-harvest production practices that could have a major impact on the quality and safety of fresh green spinach and other leafy green produce are fecal contamination of irrigation water and the use of manure, compost tea or other methods.

Listeria monocytogenes, E. coli O157:H7, *Salmonella enterica* subsp. *enterica* Typhimurium, and *Salmonella enterica* subsp. Enteritidis were used to study possible routes of contamination of spinach leaves in a hydroponic cultivation system (Koseki and others 2011). When plants were inoculated with 10^6 bacterial cells, greater numbers of plants tested positive

when contaminated via their root system as opposed to directly on leafy tissue. When plants were inoculated via leaf tissue with 10^6 CFU/ leaf, 4 out of 20 (20%) leaves tested positive. But when inoculated with 10^6 CFU/ml in the hydroponic solution, 18 out of 27 (66%) leaves tested positive (Koseki and others 2011). Using logistic regression, Koseki and others (2011) determined that the risk of contamination from the roots was 6.93 times higher than the risk of contamination from the leaves. Koseki and others (2011) note that it is unclear whether contamination is due mainly to transmission of pathogens through the root system or from the leafy tissue and claim that if the main route of contamination were clarified more effective measures of controlling microbial pathogens would be possible.

Irrigation Water and Manure Usage

Leafy green produce, such as lettuce and spinach, requires a large quantity of water in order to grow and has high water content up to 94.9% for Romaine lettuce and 92% for spinach (USDA ARS 2011). The source of the water used to grow spinach can have an enormous impact on the safety of the final product. The Food and Drug Administration (FDA) warns that "Whenever water comes in contact with produce, its source and quality dictates the potential for contamination. Minimize the potential of microbial contamination from water used with fresh fruits and vegetables" and "…the (microbiological) quality of water in direct contact with the edible portion of produce may need to be better than the quality of water in which contact with the edible portion of the plant is minimal" (FDA 1998).

Research has shown that water contaminated with *E. coli* O157:H7 can cross-contaminate spinach. In a study by Ingram and others (2011), baby spinach plants growing in a growth chamber were irrigated repeatedly with water containing *E. coli* O157:H7 and three different levels of total organic carbon (TOC). Total organic carbon is an indicator of water quality and it measures organic molecules, such as amino acids, sugars, and fatty acids, present in water. Microbial populations use the molecules in TOC as nutrients for growth and replication. Plants irrigated with 5 to 6 logs CFU/100 ml showed a 3 log reduction within the first 24 h; regardless of the TOC levels present. Ingram and others (2011) concluded that baby spinach leaves do not support the survival of *E. coli* O157:H7 for more than 1 day post irrigation and that the TOC of irrigation has no effect on the persistence of *E. coli* O157:H7.

Mootian and others (2009) used low (10^1 to 10^4) levels of *E. coli* O157:H7 from soil, manure-amended soil, and water to contaminate young (12 days of age) and mature (30 days of

age) growing lettuce plants. Leaves were split into two sets; one set was surface sanitized and the other was not. Both sets of samples were enriched after initial testing. Approximately 22% of plants grown in *E. coli* O157:H7 tainted soil or manure-amended soil tested positive and 7% of surface sterilized plants were positive, suggesting that cells were protected at the surface level. After enrichment 2 of 42 non-surface sterilized samples grown with contaminated soil or manure-amended soil were positive 30 days after exposure. Nearly 63% of samples enriched tested positive for *E. coli* O157:H7 15 days after being irrigated with contaminated water and only 13% of surface-sterilized samples were positive. Mootian and others (2009) noted that *E. coli* O157:H7 was only detected after enrichment, indicating that the bacterial levels were extremely low. They also suggested that contamination of lettuce at times close to harvest may increase the risk of pathogens being present on leaves at harvest.

Solomon and others (2002a) showed that green ice lettuce seedling can internalize *E. coli* O157:H7 found in either irrigation water or manure used during the growth of the leafy green produce. *Escherichia coli* O157:H7 was even found internalized in plant tissue five days after being watered with contaminated water. The method of irrigation has also been found to have an effect on the levels of *E. coli* O157:H7 found on lettuce leaves. Leafy produce watered using spray irrigation was found to have significantly ($P < 0.05$) more contamination than lettuce watered using surface irrigation (Solomon and others 2002b). Even plants challenged 20 days prior to harvesting tested positive for *E. coli* O157:H7 82% (9 out of 11) of the time when irrigated with spray irrigation and only 10% (1 out of 10) of the time when irrigated with surface irrigation. The soil around plants watered with surface irrigation still contained a large amount, between 10^3 and 10^4 CFU/g, of *E. coli* O157:H7.

Moyne and others (2011) study results differed from Solomon and others (2002b) research results. In Moyne and others (2011) study, *E. coli* O157:H7 was not recovered from soil samples taken from around inoculated plants during each trial. The levels of *E. coli* O157:H7 decreased rapidly during the first hour post inoculation for all trials. When 2 week old Romaine lettuce plants were inoculated with *E. coli* O157:H7, seven days post inoculation 50% of the plants tested positive after enrichment. For 4 week old lettuce plants that were inoculated, by day seven <1 log CFU/plant was detected. For all field trials, seven days post inoculation, the level of *E. coli* O157:H7 was less than 10 cells for 82% of the samples. Like the experiments

done by Solomon (2002b), Monye and others (2011) found that plants irrigated using overhead sprinklers had significantly more positive samples than plants irrigated using drip irrigation.

Probably the most common source of contamination for leafy green produce is manure or compost used to grow leafy green produce. If manure is improperly composted, then there is a potential for pathogens to still be present and for contamination to occur. Islam and others (2004) looked at contamination of lettuce and parsley grown in a field setting using improperly composted dairy manure and poultry manure. *Escherichia coli* O157:H7 persisted in the amended soil for 154 days in the lettuce fields and 217 days in the parsley fields (Islam and others 2004). They also found that vegetable fields exposed to southern fall-winter conditions could harbor *E. coli* O157:H7 in the soil for more than seven months. Based on this study, it is recommended that manure be aged in fields for more than eight months prior to vegetables being grown in soil.

However, rainfall and water runoff could spread contaminants from composting manure or fields to water, soil, or other fields and could increase the area of *E. coli* O157:H7 contamination. The use of cattle manure as fertilizer on organic farms had a prevalence of generic *E. coli* that was 2.4 times greater than in produce grown on farms using other types of manure (Mukherjee and others 2003). Also, organic farms using materials aged 6 to 12 months were 19 times greater to test positive for *E. coli* than those from farms that aged manure for more than one year (Mukherjee and others 2003).

Patel and others (2009) study results were similar to those from Islam and others (2004) and Mukherjee and others (2003). Survival of *E. coli* O157:H7 and nonpathogenic *E. coli* on spinach leaves in organic soil were monitored while growing in a growth chamber. After inoculation of spinach plant shoots with an MPN of 2 log CFU/shoot, *E. coli* O157:H7 persisted less than 14 days (Patel and others 2009). Two strains (RM1908 and RM 304) were found 7 days post inoculation, while the other three strains were not found. No *E. coli* O157:H7 was found 14, 21, or 28 days post inoculation. In soil inoculated with an MPN of 6.5 log CFU/200 g soil declined but remained over the 28 day testing period. Populations dropped down to an MPN between 3.12 logs and 1.09 logs depending on the strain. Patel and others (2009) noted that because there is no 'kill-step' in the packaging of raw produce, identifying the source of pre-harvest contamination of fresh produce is necessary to control illness and too avoid potential produce recalls.

Wood and others (2010) found that survival of *E. coli* O157:H7 was greater in the soil than the phyllosphere of the plant. The phyllosphere is defined as the total area of a plant that is above ground. The study was conducted in a commercial production field, spinach plants were sprayed once during the eight trials with irrigation water containing between 4.90 to 7.14 log CFU/100 ml bio-tracer *E. coli*. Leaves of spinach plants were then harvested along with soil samples from test plots. Levels of bio-tracer *E. coli* decreased rapidly after application and after 72 h levels had dropped 1.63 to 4.93 logs (average = 3.49). Three to six days post inoculation culturable *E. coli* was still recovered from plant phyllosphere in 4 of 47 samples collected. Wood and others (2010) noted that survival of *E. coli* was even greater in soil than plant material.

The effect of ultraviolet (UV) light on the survival of *E. coli* was also studied. In plots that were shaded, it took more than 150 h to reach a cumulative UV radiation dose of 10-15 kJ/m^2 (level at which no culturable *E. coli* O157:H7 surrogate is detectable) (Wood and others 2010). In unshaded plots, UV radiation dose reached 10-15 kJ/m^2 in 72 to 100 h. Wood and others (2010) also found that sunlight negatively influenced the probability of recovering *E. coli* when comparing shaded and un-shaded plots.

Spinach or lettuce fertilized with *E. coli* O157:H7 contaminated irrigation water or manure can take up bacteria through the root system and internalize it in leaves, thus bacteria are resistant to traditional external sanitizing treatments (Solomon and others 2002a). As noted by Wood and others (2010) studies need to be conducted using realistic farming practices to foster a better understanding of pathogen behavior in agricultural systems. To prevent contamination, spinach and other leafy green produce growers should know the origin and distribution of irrigation water and the history of the soil in order to help limit potential *E. coli* O157:H7 outbreaks.

Compost Tea and Other Routes of Contamination

In addition to contamination due to contaminated irrigation water and use of manure to fertilize plants, there are other practices used to grow spinach and leafy greens that can lead to contamination and spread of *E. coli* O157:H7. Compost teas is a commonly used practice in organic growing and is an unheated watery extract of compost used as a spray or soil drench to promote plant growth and control plant diseases (Ingram and Millner 2007). There are two major ways of producing compost tea: aerated compost tea is made by mechanically infusing

liquid with air for the first 18 to 36 h of brewing and non-aerated compost tea is produced by steeping compost in water from several days to several weeks. Both of these methods are still used on farms today.

Ingram and Millner (2007) found no *Escherichia coli* O157:H7 was detected when no additional nutrients were added to either aerated or non-aerated compost tea. But when supplemental nutrients were added, concentration of fecal coliforms including *E. coli* O157:H7 were always greater for the aerated compost tea than the non-aerated compost tea (Ingram and Millner 2007). Depending on nutrients added to compost tea levels of bacteria increased anywhere from 100 to 1,000 fold. Based on this data, compost tea may be a possible route of contamination of leafy green produce if improperly made or if foodborne pathogens are not controlled during production of compost tea.

Besides irrigation water, manure, and compost tea other means of contamination have been studied. Talley and others (2009) collected insects from four leafy greens fields and found that 11 of 18 (61%) filth flies (Muscidae and Calliphoridae) tested positive for *E. coli* O157:H7 using polymerase chain reaction (PCR) amplification. When house flies (*Musca domestica*) were exposed to manure inoculated with *E. coli* O157:H7 labeled with green fluorescent protein (GFP) and then allowed free access to spinach leaves, fluorescing bacteria were observed on half of plant samples. When spinach plants were exposed to flies that had acquired GFP-tagged *E. coli* O157:H7 from agar plates, all of the plants had florescent bacteria present and had a greater concentration of florescent colonies on leaves. Talley and others (2009) concluded that evidence of fly defecation and number of flies in direct contact with lettuce indicated that *E. coli* O157:H7-bearing flies could be a possible route of contamination to lettuce and other leafy green produce. As more research is done with *E. coli* O157:H7 other routes of possible contamination may be discovered. Learning routes of contamination can allow growers and processors to limit the chance of *E. coli* O157:H7 in produce and find production and processing methods to help eliminate pathogens.

2.5 Post-harvest Processing and Storage Conditions

Once spinach and other leafy vegetables have been grown in fields, they have to be processed before consumers purchase them. While pre-harvest contamination often does occur, post-harvest processing should focus on reducing the risk of *E. coli* O157:H7 and other hazards whether they are biological, chemical, or physical. Processing of spinach and other leafy green

vegetables usually includes sorting spinach as it comes in from fields, then washing, drying, and packaging. While post-harvest processing is meant to help reduce the bacterial load on spinach, certain processing steps can actually cross-contaminate produce or even increase bacteria present on leaves. Certain post-harvest control points are traditionally recognized such as transport containers and vehicles, sorting, packing, and cutting (Beuchat and Ryu 1997).

Because numerous pathogens are spread through the fecal-oral route, it is important that all workers whether in field setting, post-harvest processing, retail, and food preparation know the proper method for washing hands and other proper food-handling practices. Another way to limit bacterial growth is to limit cross-contamination. One way cross-contamination can occur is if unchlorinated water is used to clean food-processing equipment or food contact surfaces, since *E. coli* O157:H7 can be a waterborne pathogen (ICMSF 1996). Cross-contamination can also occur if equipment is improperly cleaned or if rodents and other pests are in the processing area.

Since sanitizing treatments such as chlorinated water are not enough to keep produce safe, storage temperature is one of the most important factors influencing both organoleptic quality and microbial growth on fresh cut produce. Luo and others (2010) looked at the effect of storage duration and temperature on *E. coli* O157:H7 growth on two packaged fresh-cut salad brands containing Romaine and Iceberg lettuce. According to Luo and others (2010), the recommended storage temperature set by fresh-cut product processors is 1 to 3°C to maintain quality and prolong shelf-life. By storing packages of fresh-cut salad at 5°C, *E. coli* O157:H7 was able to survive, but outgrowth was limited for eight days of storage post-inoculation. After 10 days of storage, populations declined 1.9 log CFU/g. However, when bags were stored at 12°C, all three trials had significant growth during the eight day sampling period. For brand A, growth increased from 2.9 log CFU/g on day 0 to 3.7 log CFU/g on day 5, and reached 5.3 log CFU/g on day 8. For brand B, there was more significant growth going from 2.9 log CFU/g on day 0 to 4.7 log CFU/g on day 5 and finally reached 6.5 log CFU/g on day 8 of storage at 12°C. In addition, to looking at the effect of storage temperature on microbial growth, Lou and others (2010) also looked at quality of products over storage time. As expected, quality decreased more rapidly for fresh-cut lettuce stored at 12°C than lettuce stored at 5°C. However, even after *E. coli* O157:H7 growth had reached significant levels; the overall quality was still acceptable.

Similar results were found for another study by Abdul-Raouf and others (1993) investigating the influence of modified-atmosphere packaging, storage temperature, and time on

survival of *E. coli* O157:H7 in shredded lettuce, sliced cucumber, and shredded carrot. Significant growth occurred in populations of *E. coli* O157:H7 within 3 and 7 days when lettuce was stored at 12 and 21°C. When lettuce was stored at 5°C, *E. coli* O157:H7 population decreased during the 14 day storage period.

One of the most useful tools for predicting microbial deterioration of food products is the use of microbial growth and survival of pathogens. Koseki and Isobe (2005) used the growth of *E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* on lettuce at temperatures ranging from 5 to 25°C to predict growth of these pathogens under constant and fluctuating temperatures. Using data loggers, temperature of lettuce was measured as it was harvested in fields and transported to a retail store. The temperature of Iceberg lettuce after being harvested was 15-17°C and after 3 h lettuce was pre-cooled to 5°C in a vacuum refrigerator. Cooled lettuce was stored for 5 h at 5°C in a warehouse until shipping. Lettuce was then transported on non-refrigerated trucks and temperature fluctuated from 3 to 15°C during transport. At the retail store, lettuce was displayed at 7°C. Using this temperature data and the growth curve data of the bacteria grown on lettuce, Koseki and Isobe (2005) were able to model pathogen growth using real temperature data during distribution. The predicted model showed that *E. coli* O157:H7 populations would reach 5 log CFU/g, *Salmonella* spp. and *Listeria monocytogenes* populations would reach 4.5 and over 5 log CFU/g, respectively.

Being able to regulate temperature of lettuce from harvest to retail level is one of the most important ways to control growth of pathogens. Growth models for pathogens can be applied to help provide information concerning quality and safety of produce and therefore can be used to help minimize and control microbial risk. Knowing about the growth of pathogens on produce helps processors limit the chance of contamination and find methods that may help reduce populations of bacteria and pathogen present.

2.6 Attachment and Interaction with Leafy Green Produce

There are many possible sources of contamination of leafy green vegetables during growing in fields, harvesting, post-harvesting handling, processing, or distributing. Leafy greens, such as spinach, can be contaminated in the field through manure, irrigation water, wildlife, or even through farm equipment used to harvest. Numerous studies have been conducted to determine interaction of *E. coli* O157:H7 with the phyllosphere of spinach plants. These studies can provide information that could influence new intervention methods.

Xicohtencatl-Cortes and others (2009) studied environmental conditions and surface structures that influence interaction of *E. coli* O157:H7 with baby spinach and lettuce leaves in vitro. Different strains of EHEC O157:H7 were used to examine adherence to spinach leaves. Strain EDL933 and 86-64 are Shiga-toxigenic strains and strain 85-170 lacks the Shiga toxin genes, but all three of these strains are wild type *E. coli* O157:H7 strains. Using three wild type strains, the role of the Shiga toxin in colonization could be studied. The adherence of the *E. coli* O157:H7 was found to be independent of the adhesive capacity of the Shiga toxin. Isogenic strains that carried mutations in the adhesion intimin (*eae)*, flagella major subunit (*fliC*), and type 3 secretion system (T3SS) associated ATPase (*escN*) were utilized to study the role of T3SS and flagella on colonization of spinach and lettuce. Strains with mutations in the *fliC* and *escN* genes were hindered ($P < 0.0001$) in their adherence to spinach and lettuce, indicating that T3SS and flagella play a role in the ability of *E. coli* O157:H7 to attach to the phyllosphere of leafy green produce (Xicohtencatl-Cortes and others 2009).

To study if bacteria were able to penetrate or invade plant cells, Xicohtencatl-Cortes and others (2009) incubated infected leaves with gentamicin for 2 h and found that bacteria were able to penetrate leaf tissue and withstand bactericidal treatments. Strains with mutations in T3SS and flagella strains were found to be affected ($P <0.001$) in their ability to invade spinach leaves. It was also concluded that *E. coli* O157:H7 and other EHEC employ their T3SS and flagella to survive in the environment, thus making leafy green produce a vehicle for human pathogens.

Shaw and others (2008) found similar results in their study looking at specific genes of T3SS that are necessary for adherence to the plant phyllosphere. Arugula lettuce and spinach leaves were incubated with various strains of *E. coli* O157:H7 for 1 h and then observed using immunofluorescence and scanning electron microscopy (SEM). Adherent bacteria on five independent immunostained leaf sections were counted to find an adhesion level of 2.0×10^5 bacteria/cm^2 for the *E. coli* O157:H7 strain TUV 93-0. Reduced adhesion was observed when TUV 93-0 was grown at 20°C or when using another growth medium. Adhesion was localized on or around 96% (TUV 93-0 strain) and 95% (strain 85-170) of 100 counted Arugula stomatal guard cells. Mutant TUV 93-0 (Δ*escN*) bacterial cells did not adhere to leaf tissue. It was also noted that adhesion to the phyllosphere is T3SS dependent and that flagella, pili, intimin, and other adhesions have a minor role in leaf adhesion. EspA filament-like structures were observed linking wild-type bacteria to the Arugula leaf surface and when TUV 93-0 Δ*espA* were allowed

to attach to leaf surface, no adhesion was seen. EspA filaments also were observed mediating the adhesion of *E. coli* O157:H7 to spinach leaf surface. Shaw and others (2008) concluded that both *Escherichia coli* O157:H7 and other non-O157 EHEC strains adhere to lettuce and spinach leaf epidermis via EspA filaments (Shaw and others 2008).

Deng and others (2011) researched *ycfR* and *ycfQ* genes, both of which were upregulated in response to chlorine-based oxidative stress. In *E. coli* O157:H7, the function of *ycfR* is unknown but after chlorine treatment it was upregulated nearly 10 fold (Wang and others 2009). Deng and others (2011) wanted to study the function of *ycfR* during the response of *E. coli* O157:H7 to chlorine treatment. Two different strains of *E. coli* O157:H7, Sakai and TW14359, from a sprouts outbreak in Japan (1996) and a spinach outbreak in the U.S. (2006), respectively, were used for this study. In the Sakai strain, when *ycfR* was deleted the bacteria were more sensitive to chlorine treatment. The role of *ycfQ* is a possible repressor gene of *ycfR*, which is induced in the presence of chlorine. Deng and others (2011) concluded that *E. coli* O157:H7 may use gene regulation strategies to resist different sanitization agents and because chlorine disinfectants are sensitive to organic matter, light, and temperature, other sanitation methods are needed. Another conclusion made by Deng and others (2011) was that pre-harvest prevention is possibly the most effective way to minimize the risk the contamination of produce with *E. coli* O157:H7.

Berger and others (2009) looked at the interaction between enteroaggregative *E. coli* and arugula lettuce leaves. While *E. coli* O157:H7 is not an enteroaggregative species of *E. coli* O157:H7, attached cells of *E. coli* O44:H18 were found to adhere using filamentous structures resembling flagella and pili and these structures linked cells to the leaf surface and to each other (Berger and others 2009). Transgenic *E. coli* O44:H18 was found to lose its ability to adhere to stomata when missing the *fliC* gene, which is a flagella gene. The Aggregative Adherence Fimbriae (AFFs) are needed by *E. coli* to adhere to human epithelial cells and to adhere to plant tissue. Two more types of transgenic *E. coli* O44:H18 missing either *aafA* or *aafR* genes, were found to lose their ability to adhere to plant tissue (Berger and others 2009).

2.7 Processing Methods for Reducing Bacterial Load

The leafy green produce industry uses a variety of methods to reduce or eliminate the bacteria populations. According to Beuchat (1995), fruits and vegetables commonly arrive at processing plants or packinghouses with 10^4 to 10^6 microorganisms per gram. The use of

chlorinated water for washing produce is one of the most commonly practiced post-harvest processing methods. Alternative methods, such as irradiation, modified atmosphere packaging, and UV light have been studied as other possible post-harvest processing methods. Availability, cost, and efficacy of alternative processing methods determine their use by processing facilities. While the goal of processing is to eliminate or reduce potential biological hazards, measures have to be taken to ensure that systems are effective and work properly.

Ilic and others (2008) investigated effects of commercial minimal processing of spinach at two plants on coliform and *Escherichia coli* populations and prevalence of *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp., and *Listeria monocytogenes*. For 14 months, spinach samples were collected daily for a total of 1,356 samples (baby spinach, $n = 574$; savoy spinach, $n = 782$). When comparing results of processed baby from plant A and plant B spinach, no difference was observed in coliform populations ($P = 0.69$) or *E. coli* positive samples ($P = 0.52$). Prior to processing, 53% ($n = 215$) of samples tested positive for coliforms and after processing 79% ($n = 688$) of samples tested positive for coliforms. The increase in coliform populations was fairly large, from 1.16 ± 0.14 log CFU/g to 2.37 ± 0.08 log CFU/g. For samples that were paired before and after testing, 27% had no difference in counts, 53% had an increase in counts, and only 20% of samples had a decrease in coliform counts (Ilic and others 2008).

Ilic and others (2008) also found that of all samples tested 8.9% where contaminated with *E. coli* and only 2.8% (38 of 122) and 2.4% (33 of 122) of the positive samples had higher than 10^2 and 10^3 CFU/g, respectively. Of the samples, 1.2% (5 of 409) tested positive for *L. monocytogenes* and 0.4% (5 of 1,311) tested positive for *Salmonella* spp. None of the samples tested positive for *Shigella* spp. or *E. coli* O157:H7.

Chlorinated Washes

The most commonly post-harvest processing practice in the fresh produce industry is the use of chlorinated water to wash produce. Chlorinated water serves as a sanitizer in wash, spray and flume waters and assists in removing soil on the surface of fruits and vegetables. Soil may harbor microorganisms such as *L. monocytogenes* and *E. coli* O157:H7 (Beuchat 1997). Chlorine washes are more effective when the pH of water is increased or when a surfactant is used, but these can have an adverse effect on produce quality (Beuchat and Ryu 1997). Increasing the time of chlorinated washes does not increase the effectiveness of the wash at eliminating pathogens (Beuchat and Ryu 19997). Hypochlorous acid is the most effective form

of chlorine. Hypochlorous acid is believed to be effective because of high oxidative activity damages the cell membrane, thus causing bacterial inactivation (Inatsu and others 2010).

Most commercial systems use free chlorine concentrations in the single digit range, but FDA allows up to 100 mg/ liter total chlorine (Lou and others 2011). Chlorinated washes are cheaper to use than alternative methods, such as irradiation or organic acids, and it is fairly safe to use in a commercial setting. As noted by Barmore (1995), no chlorine alternatives are very effective for washing fruits and vegetables. Most alternative treatments studied only have minimal effects, resulting in less than 2 log CFU/g reduction of bacterial numbers. Hypochlorous acid and other chlorine washes can react with natural organic matter and it can result in the formation of carcinogenic halogenated disinfection by-products (DBP), like trihalomethanes (THMs) and haloacetic acids (HAAs) (Hua and Reckhow 2007; Singer 1994). The use of chlorine is also associated with the production of high amounts of wastewater with very high levels of biological oxygen demand (Ölmez and Kretzxchmar 2009).

Chlorine washes can possibly even increase the likelihood of cross-contamination of leafy green produce, especially if wash water is reused and recirculated. Ilic and others (2008) concluded that the increase in populations of coliforms and other microorganisms were due to cross-contamination from more heavily contaminated product and from the washing step. Despite washing, it is possible to increase pathogen numbers on produce if heavily contaminated product is introduced, if chlorine levels are not closely monitored, or if the oxidation-reduction potential is not closely controlled.

Lou and others (2011) looked at the effect of free chlorine levels in wash water on *E. coli* O157:H7 reduction, survival, and transference during the washing of fresh-cut lettuce. Using free chlorine concentrations ranging from 0 to 100 mg/liter (0 to 100 ppm), four strains of *E. coli* O157:H7 were added to wash solutions to determine survival. When inoculum was added to chlorinated water, all four strains decreased significantly after 30 s of exposure to 0.125 mg/liter free chlorine and no *E. coli* O157:H7 cells survived when free chlorine concentrations were increased to 0.5 mg/liter or above (Lou and others 2011). One of the major problems with chlorine washes is maintaining a high level of hypochlorous acid because organic material rapidly depletes the amount of chlorine. Also, pH has an effect on the amount of free chlorine available in wash water. Lettuce inoculated with *E. coli* O157:H7 at 4.0 logs MPN/g before washing was washed with water to achieve a reduction of 1.0 log MPN/g (Lou and others 2011).

Washing with solutions having free chlorine at 1, 5, 25, and 100 mg/liter resulted in a significant reduction of 0.2, 0.6, 0.9, and 1.2 logs MPN/g in *E. coli* O157:H7, respectively. When concentration of free chlorine was 5 mg/liter or higher, *E. coli* O157:H7 was below the detection limit of 0.36 MPN/g and addition of cut lettuce did not deplete the level of free chlorine (Lou and others 2011).

Chlorine washes have the potential to cross-contaminate produce if levels of free chlorine are below certain levels. Lou and others (2011) found that transference of *E. coli* O157:H7 happened between inoculated and non-inoculated lettuce when levels of free chlorine were less than 1 mg/liter. Even in the presence of 1 to 2 mg/liter of free chlorine, transference still occurred and when concentrations increased to 5 mg/liter *E. coli* O157:H7 was found on non-inoculated leaves post washing even though the wash solution did not contain *E. coli* O157:H7 (Lou and others 2011). When produce is washed in water containing insufficient chlorine, it is a common industry practice to rewash produce. Lou and others (2011) found that rewashing freshly contaminated lettuce in 50 mg/liter free chlorine for 30 s did not eliminate *E. coli* O157:H7 populations. It was concluded that 5 mg/liter free chlorine was needed to prevent pathogen survival in wash solutions, but 10 mg/liter was necessary to prevent cross-contamination of produce. Lou and others (2011) also concluded that rewashing is not an effective intervention method for eliminating *E. coli* O157:H7 once cross-contamination occurs.

One concern of the food and medical industries is the emergence and spread of microorganisms with reduced susceptibility to antimicrobials. Inatsu and others (2010) studied the possibility that *E. coli* O157:H7 can acquire chlorine resistance and subsequently be resistant to chlorine washes was evaluated. Using chemically induced mutant cultures of *E. coli* O157:H7, Inatsu and others (2010) noted that it is difficult for bacteria to acquire resistance to oxidative compounds, such as hypochlorous acid, that react readily with subcellular compounds, such as protein, lipids, and nucleic acid. No significant increases in chlorine resistance or in oxidative stress response genes occurred before or after exposure to chlorine water (Inatsu and others 2010).

Irradiation

While irradiation is not commonly used in the food industry, is a very effective method for eliminating or reducing pathogens in foods (Satin 1993; Gomes and others 2008; Neal and others 2008; Lester and Hallman 2010). Each of the major ionizing irradiation technologies

(electron beam, X-ray, and gamma ray) has the ability to penetrate tissue and effectively eliminate any bacterial cells that may be internalized. The use of irradiation on leafy green produce was approved by the FDA in August 2008 (CFR 21 Part 3 Section 179.26). Gamma rays, X-rays, and electron beam irradiation are the most commonly used processes to cold sterilize food and all three work in similar manners (Satin 1993). These three techniques are ionizing radiation because they move electrons out of their normal orbits in atoms and molecules and thus produce free radicals (Satin 1993). Each of the three techniques has advantages and disadvantages.

X-rays are beneficial because they are able to be turned on when needed and are therefore safer. Gamma radiation is most effective due to its ability to deeply penetrate materials uniformly, but more safety systems are required in addition to a supply of cobalt 60 (Co^{60}), the common irradiation source of the food industry (Satin 1993). Electron beam (E-beam) radiation is advantageous because this system is easy to control and is not related to the nuclear industry (Satin 1993). Electron beam radiation uses higher energy than gamma and X-rays, but has limited penetrating power of up to 2-4 inches (5-10 cm) (Neal and others 2008 and Satin 1993). E-beam radiation is produced using linear accelerators, that use electricity to accelerate electrons up to 99% of the speed light and these accelerated electrons then collide with chemical bonds causing them to break (Neal and others 2008). Gomes and others (2008) found that dose distribution of electron beam radiation depended on the arrangement of leafy green leaves inside the bag. By using these different ionizing radiation technologies, the produce industry could greatly reduce pathogens associated with leafy greens and, therefore, help prevent future foodborne illnesses associated with spinach, lettuce, cabbage, and other leafy green vegetables.

As electron beam radiation advances, the effectiveness of this technology against pathogens needs to be evaluated. Neal and others (2008) studied the effect of low-dose electron beam (e-beam) radiation on the reduction of *E. coli* O157:H7 and *Salmonella* in spinach. Radiation doses above 1.16 kGy reduced *E. coli* O157:H7 to levels near or below the 0.8 log CFU/g detection limit. When spinach was inoculated with 7.0 logs CFU/ml of *E. coli* O157:H7, radiation doses of 0.4, 0.79, 1.07, and 1.16 kGy resulted in estimated reductions of 3.7, 4.1, 6.3, and 6.3 logs CFU/g, respectively. When irradiated leaves were stored under refrigeration, leaves treated with 0.4 kGy had populations remain constant for the first two days of storage, but then declined to 2.5 CFU/g by day eight. Leaves inoculated with *E. coli* O157:H7 that were treated

with 0.79 kGy were reduced 3.0 CFU/g on day 0 and decreased to 2.3 CFU/g on day 8. *Escherichia coli* O157:H7 populations decreased from 1.0 CFU/g on day 0 but were below the detection limit by day 2 when treated with 1.07 kGy.

Neal and others (2008) found that e-beam radiation at 1.16 kGy was successful in reducing *E. coli* O157:H7 on baby spinach from 7.3 log CFU/g to levels at or below the detection limit (0.8 log CFU/g). The D-value for *E. coli* O157:H7 in baby spinach was determined to be 0.2 kGy (±0.01). D-values are the time required to kill 90% of the organisms being studied, and they usually differ based on the moisture content and the matrix of a particular food item. Neal and others (2008) concluded that the use of electron beam radiation can reduce the risk of pathogenic bacteria in fresh baby spinach and that low doses (1.16 kGy) will effectively reduce *E. coli* O157:H7 and *Salmonella* by at least 6 logs.

The use of ionizing radiation may have an effect on the organoleptic and nutritional quality of spinach and other leafy green produce. Neal and others (2008) found that e-beam radiation doses of 1.2 and 2.1 kGy had little impact on the respiration rate of spinach leaves. These results are similar to those from Gomes and others (2008), who found that treating leaves with up to 1.0 kGy did not cause significant changes in the produce quality. A study done by Lester and Hallman (2010) looked at the effect of various doses of irradiation on Vitamin C, E, K, and B_9 and carotenoid (lutein/zeaxanthin, neoxanthin, violoxanthin, and β-carotene) concentrations in flat leaf and crinkled leaf spinach. Four of the phytonutrients (vitamins B_9, E, K, and neoxanthin) did not exhibit a change in concentration with increasing doses of irradiation. Ascorbic acid (Vitamin C) declined with irradiation doses as low as 0.5 kGy (Lester and Hallman 2010). Lutein/zeaxanthin, violaxanthin, and β-carotene were all significantly ($P < 0.05$) reduced at affected at lower doses of irradiation (0.5 and 1.5 kGy) depending on the cultivar of spinach (Lester and Hallman 2010). Of the compounds studied, the one with the greatest loss in concentration was ascorbic acid, which was reduced 42% (Lester and Hallman 2010).

The effectiveness of irradiation compared to chlorine washes has been studied several times (Niemira 2007; Niemira and Cooke 2010). Niemira (2007) compared the efficacy of sodium hypochlorite and ionizing radiation for internalized *E. coli* O157:H7 when treating Romaine lettuce and baby spinach. For the Romaine lettuce, washing in water had no significant difference compared to the untreated control, but the washes with 300 and 600 ppm sodium hypochlorite had a significant effect on the survival of internalized bacterial cells (Niemira

2007). For the irradiated Romaine lettuce samples, all of the doses, except the 0.25 kGy dose, were significantly ($P < 0.05$) different than the sodium hypochlorite washes. For spinach, neither the water wash nor both sodium hypochlorite solutions (300 and 600 ppm) had significant effect on survival of internalized *E. coli* O157:H7 (Niemira 2007). The concentration of surviving bacteria for all of the irradiated samples were significantly different than the water wash and both sodium hypochlorite washes. Niemira (2007) noted concentrations of chlorine commonly used in the processing of leafy produce are inadequate to address internalized bacterial cells. Niemira (2007) concluded that sodium hypochlorite solutions were not effective against internalized *E. coli* O157:H7 from Romaine lettuce or baby spinach and that ionizing radiation effectively reduced the viable population of the pathogen.

Niemira and Cooke (2010) determined the efficacy of chlorine-based sanitizing washes and irradiation on surface biofilms on Romaine lettuce and baby spinach. Washing spinach leaves in water gave a reduction of bacterial populations ranging from 0.45 to 0.78 log CFU/g. Treating with 300 ppm sodium hypochlorite gave a reduction ranging from 0.95 to 1.26 log CFU/g while the 600 ppm solution reduced the bacterial population between 1.03 and 1.34 log CFU/g. Niemira and Cooke (2010) noted that time in storage and associated changes in biofilm development reduced the efficacy of irradiation on spinach leaves inoculated with *E. coli* O157:H7. Immediately following inoculation, treating leaves with 1.0 kGy reduced bacterial populations by 5.07 logs CFU/g, but the same dose gave reductions of 3.20, 1.93, and 2.19 log CFU/g after 24, 48, and 72 h, respectively. Niemira and Cooke (2010) concluded that washing treatments with 0, 300, or 600 ppm chlorine were only moderately effective whereas, gamma radiation was effective in reducing viable cells found in biofilms on spinach leaves. Niemira and Cooke (2010) noted that irradiation would be best applied as a post-packaging intervention because it can effectively reduce populations of *E. coli* O157:H7 on and in leaves and state that irradiation on a commercial scale needs to include efforts to minimize the time between final packaging and irradiation.

Alternative Methods

Besides using chlorinated wash water and irradiation, many other studies have been done on possible methods of preventing *E. coli* O157:H7 on spinach and other leafy green vegetables. Some of the alternative methods used to prevent cross-contamination could help reduce the bacterial load on spinach and other leafy green vegetables. These alternative processing methods

need to undergo further research to ensure they will be applicable to the produce industry and will be effective when large quantities of produce are being treated. Some alternative methods studied include the use of modified atmosphere packaging, lactic acid washes, UV light, and ozonated water.

Research has also been conducted to determine the effects of packaging environment on *E. coli* O157:H7. The packaging film for prewashed spinach leaves is typically selected to maintain 1-3% O_2 and 8-10% CO_2 but atmospheres of 7-10% O_2 and 5-10% CO_2 offer a moderate benefit by delaying yellowing in spinach (Suslow and Cantwell, 1999). Sharma and others (2011) looked at modified atmosphere packaging (MAP) on the persistence and expression of virulence factors of *E. coli* O157:H7 on shredded Iceberg lettuce. Using three different packaging environments, near-ambient air atmospheric conditions, high CO_2 and low O_2, or MAP, bags of shredded lettuce was stored at 4 and 15°C for up to 10 days. The largest population reduction (1.70 logs CFU/g) of *E. coli* O157:H7 was observed for the near-ambient air atmospheric condition after 10 days at 4°C. The MAP and high CO_2 and low O_2 packages had reductions of 0.85 and 1.10 log CFU/g, respectively, after storage at 4°C for 10 days (Sharma and others 2011). Under atmospheric conditions, the expression of virulence factors increased when stored at 15°C for 10 days when specific genes were tested using RT-PCR, while the MAP and high CO_2 and low O_2 packages did not have the same trend. Sharma and others (2011) noted that 15°C could be an abusive temperature that products could reach during commercial, retail, or home storage. In this study, MAP most closely resembles that of commercial packaging and the near-ambient air atmospheric conditions would be similar to those achieved once packages of lettuce have been opened by consumers. Sharma and others (2011) found that the conditions that had the greatest reduction in the population of *E. coli* O157:H7 also promoted the highest levels of virulence factors when bags were stored at 15°C.

Brown and others (2011) investigated the use of modified atmosphere packaging (MAP) after treatments with chlorine and lactic acid bacteria (LAB) to control *E. coli* O157:H7 and *Clostridium sporogenes* in fresh spinach. Using a treatment of sterile tap water, chlorine wash (200±10 ppm), and LAB rinse (Lactiguard®), a 1.43 log reduction in *E. coli* O157:H7 populations was achieved. When washed with sterile tap water, a 0.6 log reduction was obtained. When inoculated spinach was packaged in air, 80% O_2/20% CO_2 (high-oxygen), or 80% N_2/20% CO_2 (high-nitrogen) MAP, the most effective treatment was the air and the high-

oxygen packaging which achieved 0.26 log and 0.15 log reductions, respectively. Using LAB in the produce industry might prove to be beneficial, especially when combined with other treatments. The use of MAP for the produce industry needs to be furthered studied to look at the effect on the organoleptic characteristics of the spinach as well as the effect on the native flora and pathogens.

The use of organic acids, such as lactic acid, citric acid, or ascorbic acid, has the potential to reduce the risk of foodborne illnesses while still being generally regarded as safe (GRAS) compounds to be used with produce. Huang and Chen (2011) studied the effects of hydrogen peroxide and various organic acids alone or in binary combinations on the inactivation of *E. coli* O157:H7 on spinach. At 22°C, washing spinach with 200 ppm chlorinated water only decreased pathogen counts 1.2 to 1.6 logs CFU/g and was not significantly different from the deionized water wash. All the organic acid washes, except for the 1% acetic acid solution, resulted in higher reductions ($P < 0.05$) than washing with chlorine or deionized water when used at 22°C. When mild heat (40°C for 5 min) was added with the organic acid and hydrogen peroxide washes, the effectiveness was enhanced. For example, washing spinach in 1% lactic acid at 40°C for 5 min reduced the *E. coli* O157:H7 population by 2.7 logs CFU/g. Huang and Chen (2011) noted that the maximum reduction achieved by any of the treatments was 2.7 logs CFU/g by washing with 1% lactic acid at 40°C for 5 min.

Ölmez (2010) found that chlorine gave a higher reduction (2.57±0.27 log CFU/g) than a combination of citric acid (0.25%) and ascorbic acid (0.50%) (l.49±0.15 log CFU/g). Another lactic acid treatment was used in a study done by Ho and others (2011). In this pilot plant study, *Escherichia coli* K-12 was used as nonpathogenic surrogates to compare the microbial efficacy of lactic acid and peroxyacetic acid (LA-PAA) to chlorinated water, lactic acid alone, and peroxyacetic acid alone. A reduction of more than 4.88 logs was seen when spinach inoculated with K-12 was washed with 6,000 ppm of lactic acid and 80 ppm peroxyacetic acid. When inoculated spinach was washed with lactic acid or peroxyacetic acid alone, they achieved reductions of 0.83 and 2.47 logs, respectively. When the pH of the washing solution was increased from 3.0 to 3.9, the efficacy of the LA-PAA wash decreased from 4.07 to 2.83, respectively (Ho and others 2011).

The use of ozonated water and ozone gas has the potential to help reduce the bacterial load on various commodities, including leafy green produce. Klockow and Keener (2009)

looked at the use of a novel in-package ozone gas treatment in eliminating *E. coli* O157:H7 and the effects of treatment and storage of spinach leaves. When spinach leaves were treated with gaseous ozone for 5 min using a novel system and then stored in oxygen for 0.5 h at 5°C, leaves had a 1.3 and 0.5 log CFU/leaf reduction, respectively. When the storage time was increased to 2.0 h, reductions increased to 2.0 and 1.7 logs CFU/leaf, respectively. Leaf samples stored in oxygen and air for 24 h had reductions of 5.2 and 4.0 log CFU/leaf, respectively. When treated leaves were stored at room temperature (22°C) in oxygen and air for 0.5 h, both samples achieved a 0.6 log CFU/leaf reduction. When leaves were stored in oxygen for 2 h or 24 h, greater reductions of 3.5 and 5.6 log CFU/leaf were achieved, respectively. When leaves were stored in air for 2 h or 24 h, reductions of 1.7 and 3.2 logs CFU/leaf were reached, correspondingly (Klockow and Keener 2009). Klockow and Keener (2009) concluded that while the novel PK-1 system used to treat spinach leaves in package was effective at reducing *E. coli* O157:H7 up to 5 logs, however, notable color degeneration occurred.

Another alternative processing method that may help reduce the risk of pathogens is the use of Ultra Violet (UV) radiation with titanium dioxide (TiO_2) photocatalytic reactions which produce hydroxyl radicals through reduction and/or oxidation. Kim and others (2009) examined the effect of TiO_2- UV photocatalytic reaction on the natural microflora (total aerobic bacteria, coliforms, psychrotrophic bacteria, yeasts and molds) and inoculated pathogens (*Escherichia coli, Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella* Typhimurium) on Iceberg lettuce after treatment and during storage at both 4 and 25°C. The TiO_2-UV photocatalytic reactor reduced the populations of total aerobic bacteria, coliforms, psychrotrophic bacteria, and yeasts and molds by 2.5, 1.8, 1.8, and 1.9 logs CFU/g when treated for 20 min, respectively (Kim and others 2009). Treatment of inoculated Iceberg lettuce with a 150 ppm sodium hypochlorite solution, UV only, and TiO_2-UV for 20 min resulted in a reduction of 0.9, 1.4, and 2.6 log CFU/g for *E. coli*, 0.8, 1.0, and 2.5 log CFU/g for *L. monocytogenes*, 0.9, 1.4, and 2.3 log CFU/g for *S. aureus,* and 1.1, 1.4, and 2.8 log CFU/g for *S.* Typhimurium, respectively.

Kim and others (2009) concluded that the TiO_2-UV photocatalytic reaction resulted in large reductions in the populations of the natural microflora and inoculated pathogens on Iceberg lettuce and therefore, is an effective method of disinfecting lettuce. A comparison of the TiO_2-UV treatment and other non-thermal disinfection methods (UV radiation, ozonated water, electrolyzed water, and sanitizer solutions including sodium hypochlorite, citric acid and lactic

acid) was also done by Kim and others (2009) and the titanium dioxide-UV photocatalytic method was proved to provide better microbial reduction than other methods for various produce commodities.

Advanced Oxidation Technology

Advanced oxidation technologies (AOTs) or advanced oxidation processes (AOPs) have the potential to reduce pathogens on leafy green produce. Advanced oxidation technology is broadly defined as aqueous or gaseous phase oxidation methods based on highly reactive species, such as primarily hydroxyl radicals that are capable of damaging organic contaminants (Comninellis and others 2008; Vogelpohl and Kim 2004). Hydroxyl radicals are powerful, non-selective chemical oxidants (Table 1) that react approximately a million to a billon times faster than ozone and hydrogen peroxide (Vogelpohl and Kim 2004). AOPs use a variety of processes including, but not limited to, ozone, hydrogen peroxide, ultraviolet light, or any combination and AOPs often achieve oxidative inactivation of contaminants faster than conventional ozone, UV, or hydrogen peroxide, individually.

Table 3 Relative oxidation power of select oxidizing species (Oppenländer 2003).

Oxidizing Species	Relative Oxidation Power (Chlorine = 1)
Hydroxyl Radical	2.05
Atomic Oxygen	1.78
Ozone	1.52
Hydrogen Peroxide	1.31
Permanganate	1.24
Chlorine	1.00

One type of advanced oxidation technology is Photohydroionization (PHI), a patented technology from RGF Environmental Group Inc. (West Palm Beach, FL). Photohydroionization is particularly effective because of its method of operation. This technology uses a broad spectrum of wavelengths (185 to 366 nm), between ultra violet light and X-radiation, to produce ozone and vapor hydrogen peroxide at low levels. Photohydroionization has the ability to penetrate through packaging material and through the food surface (Fink and others 2011).

Advanced oxidation technology produces hydroperoxides, which are created whenever unstable oxygen molecules, water vapor, and electromagnetic energy are present (Fink 2012). Photohydroionization utilizes oxidizers, such as ozone, that revert back to oxygen and hydrogen after interacting with organic materials, such as foodborne pathogens. Hydroperoxides are very effective at eliminating harmful bacteria and do so by cell lysis or by altering the molecular structure of cellular components and thus rendering them harmless (Fink 2012). Ozone is more effective at killing bacteria than chlorine and some sources say that it is 3,000 times faster and 150% more powerful (Fink and others 2011). Wavelengths of 240 to 280 nm inactivate microorganisms by causing irreparable damage to their nucleic acid (Wolfe 1990). Photohydroionization is capable of damaging microorganisms because it utilizes wavelengths of 366, 310, 287, and 185 nm.

Photohydroionization technology has been utilized to reduce 90% of the bacterial loads on corn, peas, carrots, and celery (Fink and others 2011). Fink and others (2011) claim that PHI is a technology that is low-cost and provides an extra level of food safety. Another report by RGF environmental group states that advanced oxidation technology, such as PHI, has the ability to reduce *Pseudomonas* spp., *Listeria* spp., *Escherichia coli,* and *Bacillus* spp. by >99% (Fink 2012). Photohydroionization has been used to treat water, air, sewage, grease, and numerous food commodities, such as chicken, grains, and beef carcasses.

Photohydroionization has the potential to be used in the leafy produce industry to help reduce surface bacteria, including *E. coli* O157:H7. When combined with other washing methods, such as chlorine washes or lactic acid washes, the risk of foodborne pathogens could be greatly reduced.

Chapter 3 - Efficacy of Advanced Oxidation Technology and Lactic Acid Wash for Controlling *Escherichia coli* O157:H7 in Bagged Baby Spinach

3.1 Abstract

Escherichia coli O157:H7 outbreaks have been linked to leafy green produce and bagged spinach. The objective of this study was to evaluate Photohydroionization panel (novel advanced oxidation technology) and varying concentrations of lactic acid washes for controlling *E. coli* O157:H7 in baby spinach. Leaves were dip inoculated with a five-strain cocktail of *E. coli* O157:H7 inoculum having a concentration between 5-6 log CFU/ml. Leaves were submerged in inoculum for 30 s and then dried for 1 h. Non-inoculated and inoculated leaves were washed for 30 s in food grade lactic acid diluted to concentrations of 0.5, 1.0, or 2.0% and then allowed to dry for 10 min. For the PHI treatment, leaves were treated for 1, 2, or 5 min on both sides for total treatment times of 2, 4 or 10 min. Following treatments, samples were either sealed in low-density polyethylene bags or enumerated on 0, 3, 7, 10, and 14 days post inoculation. Ten gram samples were diluted with sterile peptone and stomached for one min, then 0.1 ml was plated onto sorbitol MacConkey agar with cefixime and tellurite plates that were incubated at 37°C for 24 h. For lactic acid treatments, *E. coli* O157:H7 populations were different ($P < 0.05$) compared to the control. There was no difference ($P > 0.05$) due to sampling time so sampling times where pooled together for each lactic acid concentration of 0.5, 1.0, and 2.0% and resulted in 2.01, 2.78, and 3.67 log CFU/g reductions, respectively. Lactic acid concentrations of 0.5, 1.0, and 2.0% resulted in 2.01, 2.78, and 3.67 log CFU/g reductions, respectively. Leaves treated with 1.0 and 2.0% lactic acid had color degradation and were organoleptically unacceptable by day 14. When leaves were treated with PHI for 1, 2, or 5 min per side, *E. coli* O157:H7 populations were reduced 1.6, 1.49, or 1.95 log CFU/g, respectively. Leaves treated with PHI were not different from one another, but were different ($P < 0.05$) from the positive control. The PHI panel and lactic acid washes of 0.5% or higher are effective in reducing *E. coli* O157:H7 in baby spinach.

3.2 Introduction

Bagged spinach is one of the fastest growing segments of the produce industry and this value added product accounted for a tenth of total supermarket sales in the $2 billion fresh-cut salad industry (Lucier and others 2004). Such high demand for fresh-cut produce has caused an increase in the production of spinach and other leafy green produce, such as Iceberg lettuce, Romaine lettuce, and cabbage. The production of these leafy greens includes many possible routes of contamination such as irrigation water, soil amendments, processing equipment, wild animals, and people involved with the growing and harvesting of the produce. One of the largest foodborne pathogen outbreaks occurred in 2006 when bagged spinach became contaminated with *E. coli* O157:H7. This outbreak caused 205 illnesses, 104 hospitalizations, 31 cases of Hemolytic Uremic Syndrome, and three deaths in 26 states and one Canadian province (Arnade and others 2009). Following extensive environmental sampling in the fields used for production, in the processing plant, and in the watershed used for spinach production, samples taken from river water, cattle feces, and wild pig feces, which were located within one mile of the spinach field, matched the PFGE pattern from the outbreak strain (CalFERT 2007).

The minimally processed vegetable industry uses aqueous chlorine at concentrations between 50 and 200 ppm to help control the risk of foodborne pathogens. This traditionally used disinfectant only causes minimal pathogen reduction, however. Studies have shown that the most commonly used chlorine treatment, sodium hypochlorite, results in a reduction of approximately 2 logs CFU (Huang and Chen 2011 and Ölmez 2010). Based on the limited bacterial reduction achieved by chlorine washes, new alternative methods of decontaminating leafy greens need to be researched.

The use of organic acids, such as lactic acid, citric acid, or ascorbic acid, has been shown to reduce the risk of *E. coli* O157:H7 by as much as 4.88 log CFU/g while still being generally regarded as safe (GRAS) compounds (Huang and Chen 2011; Ölmez 2010; Ho and others 2011). Lactic acid has the potential to be used an alternative method because of its GRAS status and its effectiveness at reducing bacterial populations. Advanced oxidation technology, such as Photohydroionization (PHI), is being utilized in the food industry and has the potential to greatly reduce microbial populations due to numerous antimicrobial compounds produced, including vapor hydrogen peroxide and gaseous ozone. One type of advanced oxidation technology is PHI, a patented technology from RGF Environmental Group Inc. (West Palm Beach, FL).

Photohydroionization is particularly effective because of its use of a broad spectrum of wavelengths (185 to 366 nm), between ultra violet light and X-radiation, to produce ozone and vapor hydrogen peroxide at low levels. RGF environmental group states that their advanced oxidation technology, such as PHI, has the ability to reduce *Pseudomonas* spp., *Listeria* spp., *Escherichia coli*, and *Bacillus* spp. by >99% (Fink 2012).

Photohydroionization has the potential to be used in the leafy produce industry to reduce surface bacteria, including *E. coli* O157:H7. When combined with other washing methods, such as chlorine washes or lactic acid washes, the risk of foodborne pathogens could be greatly reduced. The objectives of this study were to evaluate the efficacy of a PHI panel and lactic acid wash at reducing *E. coli* O157:H7 on the surface of baby spinach throughout a 14 day storage period after treatment. The funding for this project was solely by Kansas State University and there was no affiliation with RGF Environmental.

3.3 Materials and Methods

Experimental Design

This study consisted of two different phases; phase one was treating baby spinach leaves with lactic acid at three concentrations (0.5%, 1.0, and 2.0%) and phase two consisted of treating baby spinach leaves with a Photohydroionization (PHI) panel for three treatment times (1, 2, and 5 min). Inoculated controls were inoculated but did not undergo any treatment and non-inoculated samples were not inoculated or treated. Each treatment and control consisted of two samples taken from different bags of spinach. Inoculated controls, non-inoculated controls, and treated spinach leaves were sampled on days 0, 3, 7, 10, and 14 following inoculation. The experiment consisted of three replications and two new, unopened bags of spinach were used for each replication.

Preparation of Spinach

Bags containing 1.134 kg of baby spinach (*Spinacea oleracea)* were obtained from a commercial food service distributor. Prior to use, bags were stored at refrigeration (1-3°C) for approximately 2 days. Distributor bags were opened with sterile scissors and sorted to remove leaves that were bruised, torn, or had decay. A sample consisting of 10 g of leaves was weighed out. Average weight of leaves used in all experiments was 0.948±0.492g and average surface area of leaves was 23.688±8.252 cm^2.

Inoculum Preparation and Inoculation

A five-strain cocktail of *Escherichia coli* O157:H7 (RM 6069, RM 5280, ATCC 35150, ATCC 43895, and ATCC 43888) was used. Strain RM6069 was a clinical isolate from a person in Pennsylvania during the 2006 spinach outbreak and strain RM5280 was also from the 2006 spinach outbreak and were kindly provided by Dr. Robert Mandrel; USDA ARS, Albany, CA (Parker and others 2011). Strains ATCC 35150, ATCC 43895, and ATCC 43888 were isolated from either human feces (ATCC 35150 and ATCC 43888) or from raw hamburger implicated in hemorrhagic colitis outbreak (American Type Culture Collection (ATCC); Manassas, VA). Cultures were maintained in tryptic soy broth (TSB) (Difco; Franklin Lakes, NJ) at 4°C.

For inoculum preparation, one loopful of culture was transferred to 9.0 ml of TSB and incubated at 37°C for 24 h without shaking. For each strain, 1.0 ml was transferred into a sterile 800 ml beaker containing 495 ml of sterile 0.1% peptone water (Difco; Franklin Lakes, N.J.) for a total inoculum of 500 ml with a final *E. coli* O157:H7 cell density of 5-6 logs CFU/ml. The spinach leaves were aseptically inoculated by dipping leaves in inoculum for 30 s. Using sterile forceps, leaves were transferred to dry on sterile test tube racks in a laminar flood hood for 1 h to allow for attachment of cells.

Lactic Acid Preparation and Photohydroionization Treatment Method

Food-grade lactic acid (Sigma-Aldrich; St. Louis, MO) was used to prepare the lactic acid washes. Solutions were made mixed at 5,000 ppm (0.5%), 10,000 ppm (1.0%), and 20,000 ppm (2.0%) using deionized water. When used for treatments, each lactic acid solution was transferred to a sterile washing container. Two 10 g samples of leaves underwent treatment following inoculation. Leaves undergoing lactic acid wash were dipped in lactic acid for 30 s and stirred using a sterile inoculating loop and submerged using a sterile inoculating loop before removing them with sterile forceps and allowing them to dry on sterile test tube racks for 10 min.

For the PHI panel, as per the manufacturer, the 6 lamp ultra-violet advanced oxidations (UVAO) panel, at a distance of 15.24 cm above the samples, delivers an average 16.65 mJ/cm^2 germicidal 254 nm UV energy, and requires lamp replacement after 8,000 h or annually. The Photohydroionization unit was allowed to stabilize for 30 min in an enclosed chamber prior to treatment of samples. Leaves undergoing Photohydroionization treatment were placed on sterile trays and both the abaxial and adaxial sides of the leaf were treated for 0 s, 1 min, 2 min or 5 min per side, for a total treatment time of 0 min, 2 min, 4 min, or 10 min. Leaves were turned over

using sterile forceps. Ozone and vapor hydrogen peroxide measurements in the chamber were taken using Draeger Tube system (Draeger Safety Inc. PA, USA, Figure 5 in Appendix A). Following treatment leaves were placed in low density polyethylene (LDPE) bags (Hubert; Harrison, OH) and heat sealed (Multivac; Kansas City, MO). Bags were kept at 5°C in a dark refrigerator and stored for 0, 3, 7, 10, and 14 days until they were sampled for enumeration.

Recovery and Enumeration

On days 0, 3, 7, 10, and 14, bags were opened with sterile scissors and leaves were transferred to sterile stomacher bags (Seward Limited; Worthing, Great Britain). To each stomacher bag , 90 ml sterile 0.1% peptone water (Difco; Franklin Lakes, N.J.) was added to the bags and then stomached on medium speed for 1 min (Seward 400 Stomacher, Seward Limited; Worthing, Great Britain). Serial dilutions were then made using 9.0 ml of 0.1% peptone water and 0.1 ml of dilutions was spread plated in duplicate onto sorbitol MacConkey agar (Difco; Franklin Lakes, NJ) with cefixime and tellurite (Sigma-Aldrich; St. Louis, MO) (CTSMAC) for *E. coli* O157:H7 enumeration. Plates were incubated at 37°C for 24 h before enumeration. Typical colonies on CTSMAC were placed in TSB and were incubated at 37°C for 10 to 12 h before undergoing confirmation with VIP Gold for EHEC (BioControl; Bellevue, WA). Atypical colonies were streaked on tryptic soy agar (Difco; Franklin Lakes, NJ) and incubated at 37°C for 24 h. Colonies from TSA were then analyzed using API 20E (BioMerieux; Durham, NC). API 20E test strips were incubated at 37°C for 24 h prior to reading.

For non-inoculated samples, 10 g samples of leaves were weighed out and stored at 5°C in a dark refrigerator for 0, 3, 7, 10, and 14 days. Each of the bags used in the shelf-life study had the oxygen content monitored using Checkpoint Handheld Gas Analyzer (PBI Dansensor; Glen Rock, NJ) throughout storage. Non-inoculated samples on day 0 had 150 ml of *E. coli* enrichment broth (Remel; Lenexa, KS) added and were incubated for 18 h at 37°C. After enrichment, 0.1 ml was plated on CTSMAC to verify no *E. coli* O157:H7 was present in the sample. On each of the sampling days, another non-inoculated 10 g sample had 90 ml of 0.1% sterile peptone water added and was then stomached for 1 min on medium speed. Then, 0.1 ml of the serial dilutions was plated in duplicate on CTSMAC and 1.0 ml was plated in duplicate on Aerobic Plate Count Petrifilm (3M; St. Paul, MN) on days 0, 3, 7, 10, and 14.

Statistical Analysis

The experimental design for this study was a completely randomized design with sampling time as the repeated measure. *Escherichia coli* 157:H7 data was analyzed using PROC MIXED in SAS version 9.0 (SAS Institute, Cary, N.C., U.S.A). Fixed effects for statistical analysis were treatment and storage time and the random effect was replication. Least squares means differences ($P < 0.05$) were determined using the Tukey test to compare interactions.

3.4 Results and Discussion

The ozone and vapor hydrogen peroxide readings taken during treatment using the Dreager Tube System reached up to 0.7 ppm for ozone and ranged from 2.5 to 3.0 ppm for vapor hydrogen peroxide. The level of atmospheric oxygen in the sealed LDPE bags remained similar to that of the ambient air. The levels in the LDPE bags ranged from 20.5 to 21% O_2 and the ambient oxygen levels ranged from 20.6 to 21.2% O_2.

Following 24 h of enrichment, none of the non-inoculated controls had *E. coli* O157:H7 populations present. For non-inoculated controls, the average aerobic populations slowly increased throughout the 14 day period sampled. On day 0, the average aerobic population for the four non-inoculated samples was 5.73 log CFU/g (Table 4). The population initially decreased on day 3 to 5.51 logs CFU/g, but by day 7 the population increased to 5.84 logs CFU/g, day 10 was 5.91 logs CFU/g, and finally on day 14 the population reached 5.83 logs CFU/g. These results showed that throughout the 14 day sampling period the aerobic population on the spinach leaves was able to withstand refrigeration temperatures (5°C).

Table 4 Mean aerobic populations (log CFU/g) and standard error through the 14 day sampling period for non-inoculated controls of spinach (*n*=12).

Day	Average Count	St. Error
0	5.73	0.16
3	5.51	0.17
7	5.84	0.23
10	5.91	0.20
14	5.83	0.22

Atypical colonies on CTSMAC, were confirmed using API 20E test strips. Other Enterobacteriaceae found on spinach included *Psuedomonas luteola*, *Stenotrophomonas maltophila, Klebsiella pneumonia* spp. *ozaenae, Serratia ficaria, Salmonella* cholerasuis spp. *arizonae, Pantoea* spp., and *Psuedomonas aeruginosa,*. Other bacterial populations present on spinach leaves indicate that neither process was entirely effective at eliminating pathogens. Some of these *Enterbacteriaceae* species are pathogens and more research is needed to see how they are affected by either lactic acid or PHI.

For inoculated samples, *Escherichia coli* O157:H7 populations on the spinach leaves declined over time, but the only significance difference was seen on day 14 (Figure 1, Table 5 in Appendix A). There was no interaction seen between the sampling time and the treatment (P = 0.3402).

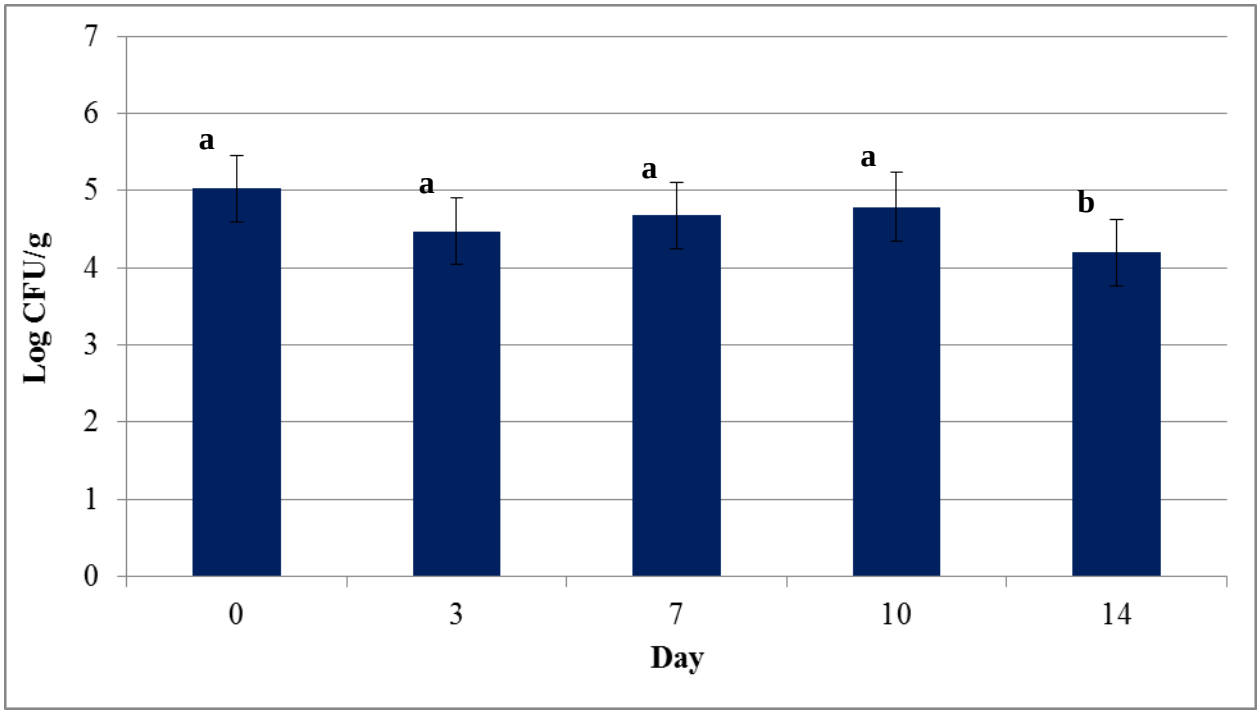

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Figure 1 Mean *E. coli* O157:H7 populations (log CFU/g) and standard error on inoculated spinach leaves throughout the 14 day sampling period. Pooled by treatment (0, 0.5, 1, and 2% lactic acid, 0 s UV, 1, 2, and 5 min UV. Superscripts indicate differences (P <0.05) across day sampled.

Lactic Acid Wash

The initial population of *Escherichia coli* O157:H7 on the positive control (0% lactic acid) was 5.91±0.45 log CFU/g (Table 6 in Appendix A). When leaves were treated with lactic acid solutions, *E. coli* O157:H7 populations decreased ($P < 0.05$) with all three treatments (0.5% lactic acid, 1% lactic acid, and 2% lactic acid). Each of the treatments was significantly different from one another. The 0.5% lactic acid treatment provided a 2.01 log CFU/g reduction. The 1% lactic acid provided a greater reduction of 2.78 log CFU/g and the 2% lactic acid treatment provided the greatest reduction of 3.67 log CFU/g.

These results differ from those achieved by Ho and others (2011), whose highest reduction observed with 6,000 ppm (0.6%) was 0.83±0.13 log CFU/g. The differences in these results are due to concentration of lactic acid, but some difference might be due to pH or temperature of the lactic acid solutions. Treating leaves with 1 and 2% lactic acid caused the leaves to undergo a visually notable color change and by the end of the 14 day sampling period, the leaves were organoleptically unacceptable (Figure 6 in Appendix A).

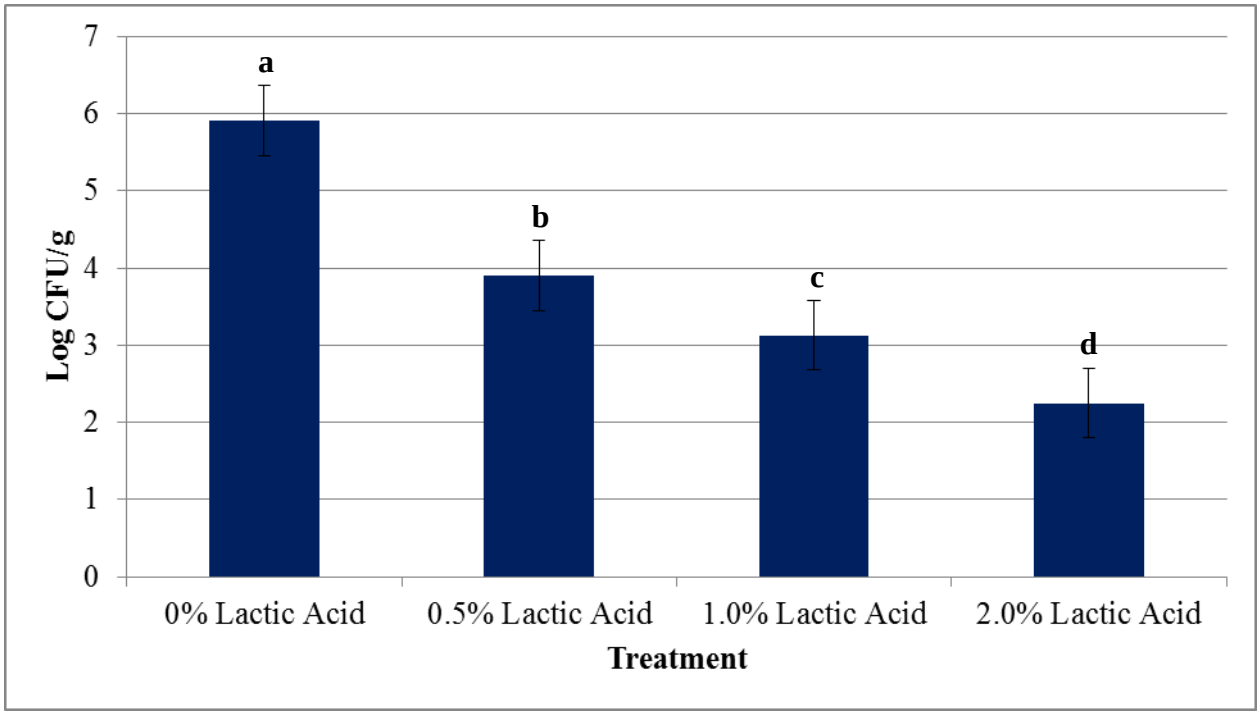

Figure 2 Mean *E. coli* O157:H7 populations (log CFU/g) and standard error on inoculated spinach leaves treated with lactic acid pooled on day (0, 3, 7, 10, and 14). Superscripts indicate differences ($P < 0.05$).

Photohydroionization

Initial *Escherichia coli* O157: H7 population for the positive controls (0 s PHI) was 6.83±0.45 log CFU/g (Table 7 in Appendix A). Leaves treated with the Photohydroionization panel had decreased ($P < 0.05$) *E. coli* O157:H7 populations when compared to positive control (0 s PHI). There was no significance between the treatments with the PHI panel. The 60 s (per side) treatment with the PHI panel reduced the population of *E. coli* O157:H7 1.6 log CFU/g and the 120 s (per side) Photohydroionization treatment produced a 1.49 log CFU/g reduction. The 5 min (per side) Photohydroionization treatment provided the greatest reduction of 1.95 log CFU/g. Leaves treated with Photohydroionization panel did not undergo wilting and by the end of the 14 day sampling period, they appeared more organoleptically acceptable than the positive control (0 s PHI) and non-inoculated samples.

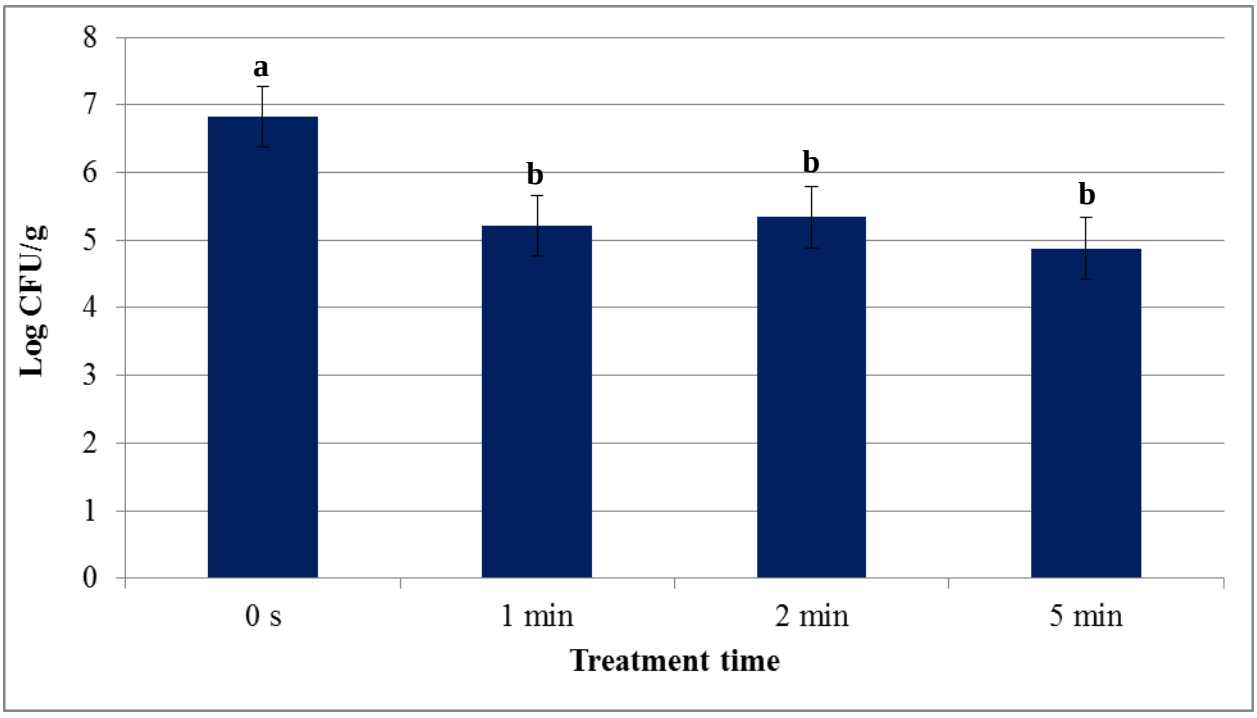

Figure 3 Mean *E. coli* O157:H7 populations (log CFU/g) and standard error on inoculated spinach leaves treated with Photohydroionization panel pooled on day (0, 3, 7, 10, and 14). Treatment time is per side. Superscripts indicate differences ($P < 0.05$).

The Photohydroionization technology has the potential to be used in the produce industry to help reduce surface bacteria. This technology is effective against numerous types of

microorganisms including *L. monocytogenes*, severe acute respiratory syndrome (SARS), *Bacillus cereus,* and Norwalk virus (Fink 2012). Some of the negative ascpects of the PHI technology is the formation of free radicals and the risk of ozone exposure (Fink 2012). These risks are minimal however, because the free radicals produced quickly react to produce water and oxygen and the ozone produced is within the federal limit of 0.04 ppm that is allowed (Fink 2012). The effects of treating leafy greens would have to be further evaluated for additional risks or side effects. One of the challenges with using the Photohydroionization panel is the necessity for leaves to lay flat and be treated on both the abaxial and adaxial sides, otherwise there may still be niches for bacteria to still be present. In a commercial application though, several PHI panels would be used at once to ensure that leaves were being treated on both sides simultaneously. Additional studies would need to be conducted to see if the Photohydroionization panel causes any additional sensory or nutritional changes in the spinach leaves.

3.5 Conclusions

The results show that treating leaves with 0.5, 1, and 2% lactic acid greatly reduces the population of *E. coli* O157:H7 on inoculated leaves by more than 2 logs; however, notable color degradation occurs in leaves treated with 1 and 2% lactic acid. Treating leaves with an advanced oxidation system, such as Photohydroionization technology, significantly reduces the population of *E. coli* O157:H7 but the treatment time is not significant. In general, treating spinach leaves with lactic acid produced greater than 2 logs of reduction and treating with the Photohydroionization panel produced >1.5 log reductions. Treating leaves with lactic acid has been done in previous studies (Ho and others 2001) and provided up to 0.83±0.13 log reduction when *E. coli* K-12 was treated with 6,000 ppm (0.6% lactic acid). Treating leafy green produce, such as spinach or Romaine lettuce, with low levels of lactic acid may prove to be a safer alternative to chlorinated water wash flumes. Since lactic acid is a GRAS substance, it would be safe to use on produce. More studies would need to be conducted to see if any sensory or nutritional changes are made to spinach treated with lactic acid.

The use of Photohydroionization as a processing method in the food industry is a rather novel technology. The development of new technology, such as Photohydroionization™, has the potential to aid in reducing bacterial loads and reduce the risk of pathogens, such as *E. coli* O157:H7. In this study, PHI showed a reduction of up to 1.95 log CFU on inoculated spinach

leaves. When added to the washing processes already used in the fresh produce industry, this technology might have the potential to further reduce the risk of pathogens.

While both lactic acid and PHI may be novel technologies in the fresh produce industry, both have the potential to be used as alternatives to or in addition to current processing practices. Combining either or both of these methods with current practices has the potential to create a hurdle effect to eliminating pathogens. Reducing the risk of foodborne pathogens on fresh cut produce is vital to providing safe food to consumers. This study proves that alternative methods may be more effective than chlorine washes and need to be researched further.

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Appendix A - Additional Figures and Tables

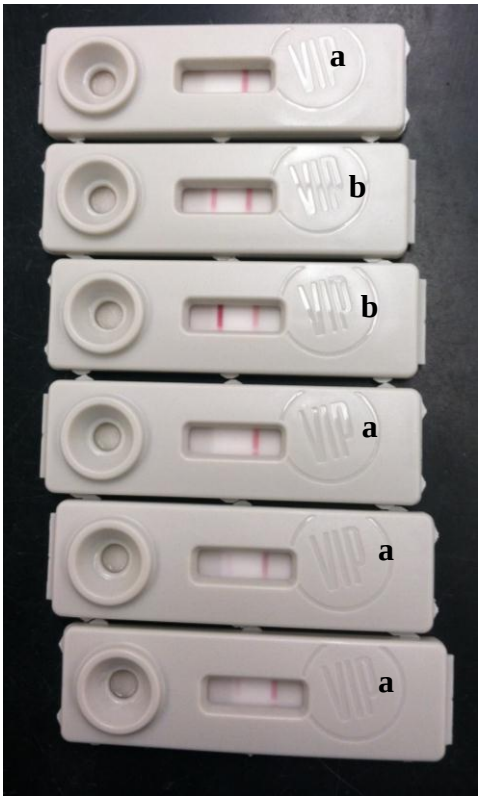

Figure 4 VIP Gold for EHEC (BioControl; Bellevue, WA) test strips showing negative (a) and positive (b) test results.

Figure 5 Draeger-Tube System with Dreager Accuro pump and Dreager-Tubes.

Figure 6 Leaves treated with 1% lactic acid (left) and 2% lactic acid (right).

Table 5 Average *E. coli* O157:H7 population (log CFU/g) through the 14 day sampling period for inoculated samples pooled on treatment.**

Day*	Average Count	St. Error
0	5.03[a]	0.43
3	4.47[a]	0.43
7	4.68[a]	0.43
10	4.79[a]	0.45
14	4.20[b]	0.43

* Denotes day sampled following inoculation
** 0% lactic acid, 0.5% lactic acid, 1% lactic acid, 2% lactic acid, 0s UV, 60s UV, 120s UV, and 5min UV.
[ab] Superscripts indicate significant differences ($P < 0.05$).

Table 6 Average *E. coli* O157:H7 populations (log CFU/g) on inoculated spinach leaves treated with lactic acid pooled on day*.

Treatment	Average Count	St. Error
0% Lactic Acid	5.91[a]	0.45
0.5% Lactic Acid	3.90[b]	0.45
1.0% Lactic Acid	3.13[c]	0.45
2.0% Lactic Acid	2.25[d]	0.45

* Day 0, 3, 7, 10, and 14.
[ab] Superscripts indicate significant differences ($P < 0.05$).

Table 7 Average *E. coli* O157:H7 population (log CFU/g) on inoculated spinach leaves treated with PHI light pooled on day*.

Treatment (per side)	Average Count	St. Error
0s PHI	6.83[a]	0.45
60s PHI	5.22[b]	0.45
120s PHI	5.34[b]	0.45
5min PHI	4.88[b]	0.45

* Day 0, 3, 7, 10, and 14.
[ab] Superscripts indicate significant differences ($P < 0.05$).

Appendix B - SAS Code for *Escherichia coli* O157:H7 for Inoculate Spinach Leaves

```
options noncenter 1s = 120;
data;
input Rep Sample Trt $ Day Population
datalines;
 1   1    0LA    0   6.736
 1   1    0LA    3   6.932
 1   1    0LA    7   7.470
 1   1    0LA   10     .
 1   1    0LA   14   4.699
 1   2    0LA    0   6.562
 1   2    0LA    3   7.648
 1   2    0LA    7   6.398
 1   2    0LA   10     .
 1   2    0LA   14   4.699
 1   1  0.5LA    0   6.968
 1   1  0.5LA    3   4.398
 1   1  0.5LA    7   6.763
 1   1  0.5LA   10     .
 1   1  0.5LA   14   4.301
 1   2  0.5LA    0   4.484
 1   2  0.5LA    3   4.161
 1   2  0.5LA    7   4.954
 1   2  0.5LA   10     .
 1   2  0.5LA   14   3.699
 1   1    1LA    0   5.166
 1   1    1LA    3   2.699
 1   1    1LA    7   6.940
 1   1    1LA   10   5.667
 1   1    1LA   14   3.602
 1   2    1LA    0   4.114
 1   2    1LA    3   3.398
 1   2    1LA    7   5.398
 1   2    1LA   10   5.667
 1   2    1LA   14   4.597
 1   1    2LA    0   3.602
 1   1    2LA    3   2.699
 1   1    2LA    7   4.851
 1   1    2LA   10   6.509
 1   1    2LA   14   2.699
 1   2    2LA    0   4.484
 1   2    2LA    3   2.699
 1   2    2LA    7   2.949
 1   2    2LA   10   5.122
```

1	2	2LA	14	2.699
2	1	0LA	0	5.863
2	1	0LA	3	6.736
2	1	0LA	7	5.677
2	1	0LA	10	6.892
2	1	0LA	14	5.114
2	2	0LA	0	6.161
2	2	0LA	3	6.172
2	2	0LA	7	5.455
2	2	0LA	10	7.031
2	2	0LA	14	5.312
2	1	0.5LA	0	4.484
2	1	0.5LA	3	2.699
2	1	0.5LA	7	3.292
2	1	0.5LA	10	5.648
2	1	0.5LA	14	2.462
2	2	0.5LA	0	3.978
2	2	0.5LA	3	4.470
2	2	0.5LA	7	2.690
2	2	0.5LA	10	2.863
2	2	0.5LA	14	2.643
2	1	1LA	0	2.699
2	1	1LA	3	3.903
2	1	1LA	7	1.000
2	1	1LA	10	1.000
2	1	1LA	14	2.477
2	2	1LA	0	2.699
2	2	1LA	3	4.712
2	2	1LA	7	1.954
2	2	1LA	10	2.580
2	2	1LA	14	3.049
2	1	2LA	0	3.740
2	1	2LA	3	3.000
2	1	2LA	7	2.653
2	1	2LA	10	1.000
2	1	2LA	14	1.000
2	2	2LA	0	3.699
2	2	2LA	3	3.566
2	2	2LA	7	1.000
2	2	2LA	10	1.000
2	2	2LA	14	1.000
3	1	0LA	0	6.447
3	1	0LA	3	4.778
3	1	0LA	7	4.778
3	1	0LA	10	4.720
3	1	0LA	14	4.544
3	2	0LA	0	6.242
3	2	0LA	3	5.477
3	2	0LA	7	4.813
3	2	0LA	10	5.267

3	2	0LA	14	4.752
3	1	0.5LA	0	3.929
3	1	0.5LA	3	2.255
3	1	0.5LA	7	2.146
3	1	0.5LA	10	4.352
3	1	0.5LA	14	3.201
3	2	0.5LA	0	4.531
3	2	0.5LA	3	3.262
3	2	0.5LA	7	2.792
3	2	0.5LA	10	2.415
3	2	0.5LA	14	3.542
3	1	1LA	0	4.491
3	1	1LA	3	2.602
3	1	1LA	7	2.519
3	1	1LA	10	2.556
3	1	1LA	14	1.301
3	2	1LA	0	3.041
3	2	1LA	3	2.633
3	2	1LA	7	1.903
3	2	1LA	10	3.093
3	2	1LA	14	1.000
3	1	2LA	0	2.699
3	1	2LA	3	1.000
3	1	2LA	7	1.000
3	1	2LA	10	1.000
3	1	2LA	14	1.000
3	2	2LA	0	2.699
3	2	2LA	3	2.681
3	2	2LA	7	1.000
3	2	2LA	10	1.000
3	2	2LA	14	1.000
1	1	0UV	0	7.447
1	1	0UV	3	7.491
1	1	0UV	7	7.903
1	1	0UV	10	.
1	1	0UV	14	6.826
1	2	0UV	0	7.498
1	2	0UV	3	7.244
1	2	0UV	7	7.906
1	2	0UV	10	.
1	2	0UV	14	6.597
1	1	60UV	0	5.160
1	1	60UV	3	3.699
1	1	60UV	7	5.633
1	1	60UV	10	.
1	1	60UV	14	4.699
1	2	60UV	0	4.806
1	2	60UV	3	5.973
1	2	60UV	7	7.117
1	2	60UV	10	.

1	2	60UV	14	4.699
1	1	120UV	0	5.228
1	1	120UV	3	4.724
1	1	120UV	7	6.987
1	1	120UV	10	8.041
1	1	120UV	14	8.342
1	2	120UV	0	4.498
1	2	120UV	3	4.653
1	2	120UV	7	7.398
1	2	120UV	10	7.470
1	2	120UV	14	5.602
1	1	5mUV	0	5.816
1	1	5mUV	3	4.681
1	1	5mUV	7	7.092
1	1	5mUV	10	6.900
1	1	5mUV	14	3.699
1	2	5mUV	0	5.574
1	2	5mUV	3	5.927
1	2	5mUV	7	6.491
1	2	5mUV	10	7.378
1	2	5mUV	14	3.699
2	1	0UV	0	6.736
2	1	0UV	3	6.866
2	1	0UV	7	6.638
2	1	0UV	10	6.190
2	1	0UV	14	4.301
2	2	0UV	0	8.000
2	2	0UV	3	6.332
2	2	0UV	7	5.550
2	2	0UV	10	6.006
2	2	0UV	14	5.878
2	1	60UV	0	6.312
2	1	60UV	3	5.816
2	1	60UV	7	2.699
2	1	60UV	10	6.860
2	1	60UV	14	6.389
2	2	60UV	0	5.638
2	2	60UV	3	4.243
2	2	60UV	7	6.427
2	2	60UV	10	5.842
2	2	60UV	14	4.740
2	1	120UV	0	5.937
2	1	120UV	3	6.210
2	1	120UV	7	5.908
2	1	120UV	10	5.748
2	1	120UV	14	6.371
2	2	120UV	0	3.477
2	2	120UV	3	4.658
2	2	120UV	7	2.699
2	2	120UV	10	6.663

2	2	120UV	14	6.568
2	1	5mUV	0	3.301
2	1	5mUV	3	5.155
2	1	5mUV	7	6.538
2	1	5mUV	10	7.109
2	1	5mUV	14	6.352
2	2	5mUV	0	4.720
2	2	5mUV	3	5.027
2	2	5mUV	7	3.954
2	2	5mUV	10	7.130
2	2	5mUV	14	6.585
3	1	0UV	0	6.980
3	1	0UV	3	6.884
3	1	0UV	7	6.934
3	1	0UV	10	6.906
3	1	0UV	14	6.648
3	2	0UV	0	7.019
3	2	0UV	3	7.011
3	2	0UV	7	6.477
3	2	0UV	10	6.944
3	2	0UV	14	6.447
3	1	60UV	0	5.462
3	1	60UV	3	5.041
3	1	60UV	7	4.498
3	1	60UV	10	3.176
3	1	60UV	14	5.613
3	2	60UV	0	4.889
3	2	60UV	3	.
3	2	60UV	7	3.875
3	2	60UV	10	5.799
3	2	60UV	14	4.470
3	1	120UV	0	3.602
3	1	120UV	3	4.672
3	1	120UV	7	4.161
3	1	120UV	10	4.342
3	1	120UV	14	5.447
3	2	120UV	0	5.961
3	2	120UV	3	5.462
3	2	120UV	7	5.389
3	2	120UV	10	4.556
3	2	120UV	14	2.279
3	1	5mUV	0	4.146
3	1	5mUV	3	6.132
3	1	5mUV	7	3.241
3	1	5mUV	10	2.041
3	1	5mUV	14	1.000
3	2	5mUV	0	3.778
3	2	5mUV	3	3.407
3	2	5mUV	7	2.813
3	2	5mUV	10	4.439

```
  3     2     5mUV     14     3.978
;
proc mixed;
class rep sample trt day;
model pop = trt|day/ddfm = satterth;
random rep;
1smeans trt day trt*day/pdiff adjust=tukey;
1smeans trt*day/ slice = day;
run;
```