VALIDATION OF WASHING TREATMENTS TO REDUCE *ESCHERICHIA COLI* O157:H7 AND *ESCHERICHIA COLI* SURROGATES, *SALMONELLA* SPP., AND *LISTERIA MONOCYTOGENES* POPULATIONS ON THE SURFACE OF GREEN LEAF LETTUCE, TOMATOES, AND CANTALOUPES

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Produce such as tomatoes, lettuce, and cantaloupes have been associated repeatedly with food outbreaks connected to various *Salmonella* serovars, *Listeria monocytogenes*, and *Escherichia coli* O157:H7. The aim of this research was to validate washing solutions and techniques in reducing pathogens on produce surfaces. Lettuce (25 ± 0.3g) and tomatoes were inoculated with *E. coli* O157:H7 and *Salmonella* spp., respectively. Samples were treated with tap water (TW) or a chemical wash treatment (CWT; containing citric acid) for 30, 60, or 120 s. Reduction of *E. coli* O157:H7 and *Salmonella* spp. populations on the surface of leaf lettuce and tomatoes, respectively, were greater (P<0.05) for CWT (ca. 3.0 logs) than for TW (ca. 2.3- 2.5 logs). Cantaloupes were washed with TW, 9% vinegar solution, or a commercial antimicrobial for fruit and vegetables treatment (CAFVT; containing lactic acid) for 2 min using a washing system. Cantaloupes were cut into wedges or cubes and stored at 4ºC for aerobic plate counts (APC) on days 0, 1, 3, and 6. APC populations of cubed and wedged cantaloupes were different over time (P=0.00052); cantaloupes washed with 9% vinegar solution showed the lowest APC populations after day 1 and 3 of storage. *Salmonella* spp. or *L. monocytogenes* inoculated cantaloupes were washed with CPW for 30, 60 or 120 s. Washing cantaloupes for 120 s with CPW showed greater (P<0.05) reductions of *Salmonella* spp. and *L. monocytogenes* populations (1.26 and 1.12 $\log_{10}$ CFU/cm^2) than TW (ca. 0.63 $\log_{10}$ CFU/cm^2) on cantaloupe surface. Lettuce leaves were inoculated with rifampicin-resistant *E. coli* surrogates and then washed with CAFVT, 5% vinegar solution or TW for 2 min with agitation (washing system) or without. Log reductions of CAFVT (2.25 $\log_{10}$ CFU/g) were greater (P=0.0145) than those by tap water (1.34 $\log_{10}$ CFU/g), but similar to 5% vinegar solution (2.09 $\log_{10}$ CFU/g). Washing lettuce with continuous agitation achieved higher (P=0.0072) *E. coli* reductions (2.26 $\log_{10}$ CFU/g) than

without agitation (1.53 $\log_{10}$ CFU/g). Overall, incorporation of wash solutions or agitation (washing system) in the washing process compared to TW alone reduced greater (P<0.05) APC, pathogens, or surrogates populations from lettuce, tomato, and cantaloupe surfaces.

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Dedication

I would like to dedicate this doctoral dissertation to my parents, Hernan R. López and Gladys A. Girón, and my brother, Edwin R. López Girón. I would not be the person I am today without your immense love and continued support.

Chapter 1
Introduction

Consumption of raw produce has been associated with foodborne-disease outbreaks due to contamination with pathogenic microorganisms. In the United States (U.S.), foodborne-disease outbreaks are estimated to cause 48 million foodborne illnesses, 128,000 hospitalizations, and over 3,000 deaths each year (Scallan and others 2011a, b). Callejón and others (2015) examined foodborne outbreaks due to produce in the U.S. during the period of 2004 to 2012, in which a total of 377 outbreaks were reported and approximately 54% of produce-associated outbreaks took place in foodservice establishments, especially restaurants, followed by private homes.

Currently, fresh produce is regularly considered to be a possible source of foodborne outbreaks caused by a variety of pathogens. The etiological agents linked with fresh produce outbreaks are many, bacteria agents such as *Salmonella* spp., *Shigella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Yersinia enterocolitica*, viruses such as Norovirus, Hepatitis A, and Calicivirus, and parasites such as *Cyclospora cayetanensis*, *Crytosporidium parvum*, and *Giardia lamblia* (Matthews 2006). Documented reviews of outbreaks noted that the bacterial etiological agents *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* are the most frequently linked to large multistate outbreaks associated with fresh produce. Generally, produce commonly associated in foodborne-disease outbreaks are "salads," leafy vegetables, sprouts, tomatoes, and melons (Sivapalasingam and others 2004; Callejón and others 2015).

The first outbreak of *E. coli* O157:H7 associated with lettuce occurred during July 1995 in the state of Montana. Since 1995, several outbreaks of Shiga toxin-producing *E. coli* illnesses have been associated with leafy greens (Olaimat and Holley 2012). Epidemiological

investigations indicated that between 1995 and 2006 there have been 22 produce outbreaks documented in the U.S., and 9 of these outbreaks were traced to lettuce or spinach grown in, or near the Salinas Valley region on the Central Coast of California, which is the major producer of leafy vegetables in the U.S. (Cooley and others 2007).

Multiple outbreaks of *Salmonella* illnesses associated with tomatoes and cantaloupes have occurred in the past 15 years. For example, during 2005 and 2006, 4 multistate outbreaks of *Salmonella* infections linked to the consumption of raw tomatoes in restaurants resulted in 459 laboratory confirmed cases in 21 states (Bidol and others 2007). Melons, especially cantaloupes, have been associated with foodborne illness outbreaks (Sivapalasingam and others 2004). In 2008, a multistate outbreak of *S.* Litchfield infections associated with contaminated cantaloupes involved 51 ill persons in 16 states in the U.S. and 9 illnesses in Canada (CDC, 2008). Moreover, in 2011, a multistate outbreak of listeriosis was linked to consumption of cantaloupes. This outbreak involved 147 illnesses, 33 deaths, and 1 miscarriage in 28 states. Collaborative investigations indicated implicated cantaloupes came from Jensen Farms in Colorado (CDC 2011).

Fresh produce can become contaminated at any point along the supply chain including contamination of seed stocks, during production, harvesting, post-harvest handling and processing, storage, distribution, retail display, and home/foodservice preparation (Gorny 2006). Washing plays an important role on fresh produce quality and safety. Washing during post-harvest processing is used mainly to improve produce' quality (e.g. remove soil, chemical residues, and other debris from produce surfaces) and safety. Chlorine is regularly used as a sanitizer in wash and flume waters in the fresh fruit and vegetable industry. Commonly added to wash water as sodium and calcium chloride (NaOCl and $CaCl_2O_2$), or as chlorine gas (Cl_2),

aqueous chlorine is frequently used at 50 to 200 ppm free chlorine concentrations and exposure times from 1 to 2 min in washing operations to control the risk of foodborne pathogens.

Studies have indicated that use of chlorinated water on fresh produce decreases microbial load by values ranging from <1 to <3 log CFU/g (Beuchat and others 2004; Bari and others 2005; Akbas and Ölmez 2007; Allende 2009). Several researchers have also explored the antimicrobial potential of organic acids such as citric, lactic, and acetic acids, which have also been classified as Generally Recognized as Safe (GRAS; 21 CFR184.1005, 1033, 1061). For example, dipping inoculated fresh-cut iceberg lettuce in 0.5% citric acid or 0.5% lactic acid solutions for 2 min showed reductions of about 2 log CFU/g units for *E. coli* populations (Akbas and Ölmez 2007). Washing procedures and sanitizing agents are of concern because inadequate handling can result in produce damage, cross-contamination, and chemical and/or microbial contaminants internalization (Park and others 2008; Pao and others 2012).

With the increased demand for fresh produce year round and a more globalized food trade structure, challenges exist in developing and implementing measures to control foodborne illnesses linked to pathogenic contaminated produce. Therefore, the aim of this research was to validate washing treatments in reducing bacterial pathogens on fresh produce surfaces (leaf lettuce, tomatoes, and cantaloupes). The research consisted of three phases: 1) validation of washing treatments to reduce *Escherichia coli* O157:H7 and *Salmonella* spp. on the surface of green leaf lettuce and tomatoes; 2) investigation of handling practices for fresh produce and the efficacy of commercially available produce washes on removal of *Salmonella* spp. and *Listeria monocytogenes* and natural microflora from whole cantaloupes surfaces; and 3) effectiveness of produce washes and a washing system in reducing and inactivating *Escherichia coli* surrogates from lettuce leaves at refrigeration temperature.

The first phase was on the use of a chemical produce wash (antimicrobial in powder form containing citric acid, sodium lauryl sulfate, sodium carbonate, magnesium carbonate, and grapefruit oil extract) to reduce pathogenic contamination. The objective of this phase was to determine the efficacy of the antimicrobial in reducing *E. coli* O157:H7 and *Salmonella* spp. on the surface of green leaf lettuce and tomatoes, respectively.

The second phase was on the use of a produce washer (continuous water motion washing system), water, vinegar, or commercially available produce washes to reduce natural microflora or pathogenic contamination on whole cantaloupes. The objective of this phase was to collect descriptive data of handling and washing practices for fresh produce used at foodservice facilities and to evaluate the efficacy of commercially available washes (Vinegar, CAFVT, and CPW) for reducing natural microflora and pathogens on whole cantaloupes. This phase was conducted in three sections: 1) a survey that was directed to foodservice employees, exploring four different variables related to produce washing: i) identification of personnel responsibilities, ii) equipment in facilities for washing produce, iii) produce washing practices, and iv) produce storage practices. The results obtained were used to expand research on the following sections; 2) an experiment in which non-inoculated cantaloupes were washed with water, a solution of vinegar, or a Commercial Antimicrobial Fruit and Vegetable Treatment (CAFVT, in liquid form containing lactic acid, sodium hydrogensulfate, docecylbezesulfonic acid) by using a continuous water motion washing system to determine natural microbial reductions; and 3) an experiment in which *Salmonella* spp. or *Listeria monocytogenes* inoculated cantaloupes were washed with a commercial produce wash (CPW, in powder form containing citric acid, sodium lauryl sulfate, sodium carbonate, magnesium carbonate, and grapefruit oil extract) to reduce pathogenic contamination on whole cantaloupes.

The third phase was on the use of the produce washer (continuous water motion washing system), water, a solution of 5% vinegar, and CAFVT to reduce contamination on the surface of green leaf lettuce. The main objective of this phase was to test the efficacy of the continuous motion washing system and produce washes in reducing *E. coli* surrogates on the surface of green leaf lettuce. A secondary objective was to study the shelf life of green leaf lettuce throughout a 6-day storage period after washing treatment application.

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Chapter 2
Literature Review

Overview of outbreaks associated with fresh produce

According to the Centers for Disease Control and Prevention (CDC), a foodborne-disease outbreak is defined as the occurrence of two or more cases experiencing similar illness after ingestion of a common food. The recognized foodborne pathogens include parasites, protozoa, fungi, bacteria, viruses, and prions (Jay and others 2005a). Despite advances in food safety, foodborne-disease outbreaks remain a constant concern to public health. In the United States (U.S.), foodborne-disease outbreaks are estimated to cause 48 million foodborne illnesses, 128,000 hospitalizations, and over 3,000 deaths each year (Scallan and others 2011a, b).

In a review of outbreaks in the U.S. from 1973 to 1997, it was reported that the proportion of illnesses attributed to produce increased by eightfold (Sivapalasingam and others 2004). A total of 32 states reported 190 produce-associated outbreaks, which caused over 16,058 illnesses, 598 hospitalizations, and 8 deaths. Painter and others (2013) analyzed documented outbreaks between 1998 and 2008, estimating that approximately 46% of annual U.S. foodborne illnesses are attributed to produce, with a large number of illnesses (2.2 million (22%)) linked to leafy vegetables. Additionally, it was estimated that illnesses associated with leafy vegetables were the second most frequent cause of hospitalizations (14%) and the fifth most frequent cause of death (6%) during the period of time under study.

Callejón and others (2015) examined foodborne outbreaks due to produce in the U.S. during the period of 2004 to 2012, in which a total of 377 outbreaks were reported. Findings of this study showed that 49 (13%) of all produce-associated outbreaks were multistate outbreaks. *Salmonella* spp. was the leading cause of multistate outbreaks in the U.S. and also the predominant pathogen in sprout-associated outbreaks. Additionally, 54% of produce-associated

outbreaks took place in foodservice establishments, particularly restaurants, followed by private homes.

Currently, fresh produce is regularly considered to be a possible source of foodborne outbreaks caused by a variety of pathogens. Generally, produce most commonly associated in outbreaks are "salads," leafy vegetables, sprouts, tomatoes, and melons (Sivapalasingam and others 2004; Callejón and others 2015). Documented reviews of outbreaks by previous authors note that *Salmonella* spp. and *Escherichia coli* O157:H7 regularly cause large foodborne outbreaks associated with produce. In the U.S. since 2006, there have been over 80 foodborne outbreaks associated with leafy vegetables, including salads, due to contamination of *E. coli* O157:H7 and *Salmonella* spp., resulting in 1,430 illnesses, 392 hospitalizations, and 7 deaths (Table 2.1,Table 2.2). Since 1998, at least 29 (over 2,500 illnesses, 4 deaths) outbreaks have been linked to *Salmonella* spp. contaminated tomatoes and at least 3 major outbreaks associated with *Salmonella* spp. contaminated cantaloupes (334 cases, 6 deaths). Additionally, there have been multiple foodborne outbreaks of *Salmonella* spp. and *E. coli* O157:H7 associated with sprouts (e.g. alfalfa, clover, and mung bean sprouts).

Fresh produce can become contaminated at any point along the supply chain including contamination of seed stocks, during production, harvesting, post-harvest handling and processing, storage, distribution, retail display, and home/food service preparation (Gorny 2006). Furthermore, according to Lynch and others 2009, the likelihood of contamination is considerably higher during three periods: while growing in the field, during initial processing, and during final preparation in the kitchen. Field contamination may come from contaminated manure compost, sewage sludge, irrigation water, and runoff water from nearby livestock operations (Doyle and Erickson 2008; Erickson 2012). Additionally, indirect sources of

contamination may include interaction of birds, mammals, and insects with crop (Doyle and Erickson 2008; Lynch and others 2009). It is important to mention that contamination in the field may also come from farm workers without access to lavatories and/or hand washing stations. Moreover, the risk of contamination in the field is also influenced by the contamination introduced in the production environment and the nature of the plant. For example, melons are grown in direct contact with the ground, which may facilitate the adsorption of microorganisms present in the soil (Bach and Delaquis 2009). During processing, produce may be prone to contamination if contaminated water is used for washing and cooling (e.g. vacuum, chilling tanks, spray; Li and others 2008; Zhuang and others 1995; Takeuchi and Frank 2000). During food handling and preparation, hands of foodservice employees and food contact surfaces play an important role in the risk of cross-contamination with microorganisms (Yuhuan and others 2001). For example, during the preparation of a meal, microorganisms present on the surface of raw foods can be transferred to various food contact surfaces and utensils (e.g. knives, cutting boards, counter space) and vice versa (Yuhuan and others 2001). Additionally, contamination may be present on other surfaces in the kitchen such as water faucets, sponges, and dishcloths (Scott and Bloomfield 1993). Researchers have reported that pathogenic bacteria, including *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus* are capable of surviving for a few hours to days on hands, cloths, and utensils after initial contact with microorganisms (Scott and Bloomfield 1993). Therefore, it is imperative for the prevention of contamination through workers to implement constant personnel training, maintenance of good health, and proper hygiene (Bihn and Gravani 2006).

With the increased demand for produce year round and a more globalized food trade structure, foodborne illnesses caused by bacterial pathogens can be expected to continue as a

major public health concern. Therefore, food safety efforts will need to continually evolve in order to have a better understanding of risk factors associated with produce, so that more effective intervention strategies may be developed.

Table 2.1 Summary of confirmed outbreaks associated with fresh fruits and vegetables in United States, 1998 to 2013[*]

Type of pathogen	[1]Food vehicle								*Total outbreaks*
	Vegetables					Fruits			
	Salad	*Leafy*	*Tomato*	*Sprout*	*Other*	*Berries*	*Melon*	*Other*	
[2]*Escherichia coli*	26	26	1	7	2	3	0	5	70
Salmonella spp.	15	14	29	32	25	3	28	15	161
Listeria monocytogenes	0	0	0	1	1	0	1	0	3

[1]Food vehicle categories:
Salad: all produce items related to salad (i.e. Cesar salad, prepackaged salad, mixed salad, lettuce-based salad, house salad, green salad)
Leafy vegetables: all produce items related to leaves (i.e. iceberg lettuce, romaine lettuce, spinach, kale, ambrosia lettuce, lettuce, cilantro)
Sprouts: all produce items related to sprout (i.e. alfalfa sprouts, bean sprouts, clover sprouts)
Other vegetables: remaining vegetables (i.e. carrots, green beans, cucumber, avocado, vegetable snacks, peppers, onion, green onions, mushrooms, guacamole, celery)
Berries: strawberries, blueberries
Melon: melon, watermelon, cantaloupe, honeydew melon
[2]*Escherichia coli* O157:H7 and other *Escherichia coli* strains
Other fruits: fruit cocktail, fruit salad, grapes, mamey, mango, mixed fruits, papaya, pear,
[*]*Adapted from: Callejón and others 2015 and CDC-Foodborne Outbreak Online Database(FOOD), Available at (http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx)*

Outbreaks of Shiga toxin-producing Escherichia coli infections linked to fresh, and fresh-cut lettuce and leafy greens

Escherichia coli O157:H7 have been extensively studied and recognized as an important foodborne pathogen. The first documented outbreaks of *E. coli* gastroenteritis in the U.S. occurred in 1971. However, the microorganism was not recognized as a human pathogen until 1982, after two hemorrhagic colitis outbreaks in the states of Oregon (26 cases) and Michigan (21 cases) (Riley and others 1983). It was not until 1993, after a large multistate *E. coli* O157:H7 outbreak associated with undercooked ground beef patties from a fast-food chain restaurant, that the microorganism was recognized as a health threat (Rangel and others 2005). Although undercooked beef and dairy products have been identified as the leading source of foodborne *E. coli* O157:H7 illnesses (Harris and others 2003), other food commodities of non-bovine origin (e.g. lettuce, apple cider, salad, and sprouts) have also been associated with *E. coli* O157:H7 outbreak illnesses (Rangel and others 2005).

Produce-associated outbreaks of *E. coli* O157:H7 (38 of 183) reported during the period of 1982 to 2002 accounted for 34% of 5,269 foodborne outbreak-related cases. Approximately 34% (13) of the produce related outbreaks were linked with lettuce, 16% (6) with salad, and 11% (4) with coleslaw (Rangel and others 2005). The first outbreaks of *E. coli* O157:H7 associated with lettuce occurred during July 1995 in the state of Montana. Over 70 persons in western Montana developed bloody diarrhea and abdominal cramps. An epidemiological study of the outbreaks identified 40 laboratory-confirmed cases of *E. coli* O157:H7 infection in the area. Another 52 cases of possible *E. coli* O157:H7, without laboratory confirmation, were identified. However, only 13 hospitalizations were reported, with only one person developing Hemolytic-Uremic Syndrome (HUS). No deaths were reported. Stool cultures from 29 patients yielded *E.*

coli O157:H7, and 23 of the isolates were confirmed as *E. coli* O157:H7. These isolates were then subtyped by pulsed-field gel electrophoresis (PFGE). A common strain pattern was identified in 22 of the 23 PFGE subtyped isolates. In the case-controlled study, 19 of 27 patients indicated eating purchased leaf lettuce before illness and 15 of the patients were able to identify 6 names of grocery stores where they bought the lettuce. These stores received lettuce from 3 distributors, whom obtained the lettuce from 2 shippers. One of the shippers received leaf lettuce from 6 farms located near each other in Washington State, while the second shipper was a small local produce grower. None of the environmental and leaf lettuce samples from the local produce grower tested positive for *E. coli* O157:H7.

However, additional investigation at the 6 grocery stores identified that 4 of 6 stores followed the leaf lettuce handling practice "crisping." This practice was considered a possible source of cross-contamination, since various batches of leaf lettuce were submerged in fairly warm water, which was changed infrequently, and then followed by refrigeration. Although it was not determined how contamination of leaf lettuce occurred, four possible ways of contamination were discussed. First, fertilization of leaf lettuce with improperly aged compost contained contaminated bovine manure. Second, contamination of irrigation water and surface water runoff by feces of infected cattle that were present in the adjacent pasture areas to the leaf lettuce farms. Third, cattle with access to the streams above the pond used for irrigation water may have contaminated water. Lastly, feces of other animal reservoirs of *E. coli* O157:H7, such as sheep kept on the local producer farm or deer, may have contaminated irrigation water (Ackers and others 1998).

Since 1995, several outbreaks of Shiga toxin-producing *E. coli* illnesses have been associated with leafy greens (Olaimat and Holley 2012). Epidemiological investigations

indicated that between 1995 and 2006 there have been 22 produce outbreaks documented in the U.S., and 9 of these outbreaks were traced to lettuce or spinach grown in, or near the Salinas Valley region on the Central Coast of California, which is the major producer of leafy vegetables in U.S. (Cooley and others 2007). Moreover, baby spinach linked to a large multistate outbreak of *E. coli* O157:H7 in 2006 was traced to this area. The spinach outbreak involved a total of 205 cases, 51% hospitalizations, 16% developed HUS, and 3 deaths (Table 2.2; CDC 2006; Jay and others 2007) in 26 states and Canada. After case patients were identified, spinach from opened bags that had been consumed by case patients were analyzed. The contaminated spinach was traced to a specific brand, and eventually the investigation was narrowed to a ranch located in San Benito County, California. The ranch was used for cattle grazing and a leased portion of the ranch was used for spinach and other leafy green vegetables production (Jay and others 2007).

Epidemiological and environmental investigations indicated that most abundant wildlife observed in the ranch were wild swine, followed by birds, black-tailed and cotton-tailed deer, rabbits, coyotes, and ground squirrels (Jay and others 2007; Cooley and others 2007). Isolates of *E. coli* O157:H7, with a PFGE pattern indistinguishable from the outbreak pattern, were positive for samples of river water, cattle manure, and wild swine feces in and near the field used to grow spinach. Additionally, it was observed that wild swine had access to the crop field through gaps formed at the base of the fence caused by erosion and rooting. Moreover, cattle and wild swine had access to surface water on the ranch (Jay and others 2007).

Between 2006 and 2015, other outbreaks of *E. coli* O157:H7 linked with leafy green vegetables have been reported. In 2011, a multistate outbreak associated with romaine lettuce resulted in 58 persons infected with the outbreak strain in 9 states. Outbreak investigations indicated illnesses began from October 9, 2011 to November 7, 2011. Among the 49 illnesses

reported, there were 33 hospitalizations, and 3 developed HUS. No deaths were reported (CDC, 2012a). Epidemiological investigations focused on ill persons that reported eating at salads bars, which were located at different grocery stores from the same chain (Chain A) and at university campuses in Minnesota and Missouri. Results from the investigation indicated that a single common lot of romaine lettuce harvested from one farm was used to supply the grocery stores of Chain A as well as the university campus in Minnesota.

Another multistate outbreak of *E. coli* O157:H7 infections linked to organic produced spinach and spring mix blend was reported in 2012. Collaborative investigations linked the outbreak to pre-packaged leafy greens, produced by State Garden of Chelsea, Massachusetts. However, the source of contamination was not identified. A total of 33 persons infected with the outbreak strain were reported from 5 states. Among 28 cases with available information, 13 case patients were hospitalized, 2 developed HUS, and no deaths were reported (CDC 2012b).

Furthermore, between October and November 2013, a multistate outbreak of *E. coli* O157:H7 infections associated with ready-to-eat salads resulted in 33 ill persons from 4 states. Two ill persons developed HUS, and no deaths were reported. Results from epidemiological investigations linked this outbreak to 2 ready-to-eat salads: field fresh chopped salad with grilled chicken and Mexicali salad with chili lime chicken. These salads were produced by Glass Onion catering and sold at Trader Joe's grocery store locations (CDC 2013a). Between 2006 and 2015, there have been numerous other recalls involving leafy greens, a summary of these outbreaks is listed in (Table 2.2).

Table 2.2 *Escherichia coli* confirmed outbreaks associated with leafy vegetables from 2006 to 2013*

Year	Month	Serotype	Total ill (deaths)	Vehicle
2006	August	O157:H7	238 (5)	Spinach
2006	July	O121	3	Lettuce-based salads unspecified
2006	November	O157:H7	77	Lettuce, unspecified
2006	November	O157:H7	80	Lettuce, unspecified
2006	November	O157:H7	3	Vegetable-based salads unspecified
2007	January	O157:H7	2	Caesar salad
2007	June	O157:H7	26 (1)	Lettuce-based salads unspecified
2008	August	O157:H7	13	Spinach
2008	May	O157:H7	10	Lettuce, prepackaged
2008	May	O157:H7	6	Pre-packaged salad
2008	September	O157:H7	74	Iceberg lettuce, unspecified
2009	April	O157:H7	16	Ambrosia; lettuce
2009	September	O157:H7	22	Lettuce, unspecified
2009	September	O157:H7	10	Romaine lettuce, unspecified
2010	April	O145	31	Romaine lettuce, unspecified
2011	December	O157:H7	22	Pizza, tostada; sandwich, submarine
2011	May	O6:H16	19	Spinach struedel; tabouleh salad
2011	October	O157:H7	26	Lettuce
2011	October	O157:H7	60	Romaine lettuce, unspecified
2012	April	O157:H7	12	Vegetable-based salads unspecified
2012	June	O157:H7	52	Romaine lettuce, unspecified
2012	March	O157:H7	24	Leaf lettuce
2012	November	O157:H7	8	Leaf lettuce, unspecified
2012	October	O145	16	Lettuce
2012	October	O157:H7	33	Prepackaged leafy greens
2012	September	O157:H7	9	Salads
2013	April	O26	26	Lettuce
2013	April	O157:H7	14 (1)	Prepackaged leafy greens
2013	December	O157:H7	9	Lettuce
2013	July	O157:H7	5	Green leaf lettuce
2013	July	O157:H7	94	Lettuce
2013	June	O157:H7	6	Lettuce-based salads unspecified
2013	October	O157:H7	33	Romaine lettuce, unspecified
2013	September	O157:H7	7	Kale

**Data collected from: CDC-Foodborne Outbreak Online Database (FOOD), Available at: (http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx).*

Outbreaks of salmonellosis and listeriosis infections linked to produce

United States (U.S.) is a leading world producer of tomatoes, with commercial-scale production in 20 states. Moreover, California is the primary producer of all tomatoes in the U.S. with a share in the market between 25 and 37% since the 1980's (USDA-ERS, 2012). According to a United States Department of Agriculture Economic Research Service (USDA- ERS), annual per capita use of fresh tomatoes increased by 14% during the 1990's, which averaged to be approximately 17 pounds per person (Lucier and others 2000).

Multiple outbreaks of *Salmonella* illnesses associated with tomatoes and cantaloupes have occurred in the past 15 years (Table 2.3 and Table 2.4). During 2000 and 2004, the number of outbreaks associated with tomatoes increased in frequency and magnitude (Table 2.3). Three major outbreaks of *Salmonella* infections were linked to consumption of Roma tomatoes in the summer of 2004, two outbreaks in the U.S. and one outbreak in Canada (Corby and others 2005). In one of the multistate tomato outbreaks in the U.S., a total of 429 laboratory-confirmed cases were identified in 9 states. The cases occurred among persons eating at delicatessen chain A locations, where 30% of case patients were hospitalized and no deaths were reported. Multiple *Salmonella* serotypes were isolated, including: Javiana, Typhimurium, Anatum, Thompson, Muenchen, and Group D untypable. In the second U.S. multistate tomato outbreak, a total of 125 confirmed cases of *S*. Braenderup infections were reported in 16 states. Twenty percent of case patients were hospitalized, but no deaths were reported. Moreover, in Ontario, Canada, seven confirmed cases of *S*. Javiana infections were identified, with illness onset occurring between July 4 and 8, 2004. All case patients ate at the same restaurant, and only Roma tomatoes were the common food among all patients. Collaborative investigations of the multiple serotype outbreak identified one field-packing operation and three packinghouses located in three different states as

possible sources for contamination. One of the packinghouses, located in Florida, was also identified as a possible source of contamination for the other two single serotype outbreaks (Corby and others 2005).

Similarly, during 2005 and 2006, four multistate outbreaks of *Salmonella* infections linked to the consumption of raw tomatoes in restaurants resulted in 459 laboratory confirmed cases in 21 states (Bidol and others 2007). The outbreaks occurred between July and December of both years. In one outbreak during 2005 outbreaks, a total of 72 laboratory-confirmed *S.* Newport infections were identified from stool specimens collected in 16 states. Investigations determined the implicated tomatoes were grown on two farms located on the eastern shore of Virginia. Environmental samples tested positive for the implicated outbreak (i.e. pond water near to the tomato fields in the region). Interestingly, tomatoes from the same region had been the source of contamination for other *S.* Newport infections outbreaks in 2002 (Greene and others 2008). In a second outbreak during 2005, a total of 82 laboratory confirmed cases of *S.* Braenderup infections were identified in 8 states. A control study was conducted with 38 case patients. Twenty of the 38 patients had eaten at Chain Restaurant A and illness was associated with eating food items containing raw pre-diced Roma tomatoes. Implicated tomatoes were grown in Florida, pre-diced and packaged by a firm in Kentucky, and then shipped to Chain Restaurant A.

Outbreaks associated with tomatoes contaminated with *S.* Newport and *S.* Typhimurium during 2006 involved a total of 305 cases in 21 states. In one of the outbreaks, the source of implicated tomatoes was traced to a single packinghouse in Ohio. The packinghouse was supplied by 3 tomato growers from 25 fields in 3 counties in Ohio (Bidol and others 2007).

Table 2.3 *Salmonella* spp. confirmed outbreaks associated with tomatoes from 2000 to 2013[*]

Year	Month	Genus species	Serotype	Total ill (deaths)	Vehicle
2000	November	Enterica	Thompson	43	Tomato, unspecified
2002	February	Enterica	Newport	8	Grape tomato
2002	July	Enterica	Newport	510	Tomato, unspecified
2002	June	Enterica	Javiana	3	Tomato, unspecified
2002	June	Enterica	Javiana	159	Tomato, unspecified
2003	March	Enterica	Virchow	11	Tomato, unspecified
2004	July	Enterica	Javiana; Typhimurium; Thompson; Muenchen; unknown; Anatum	429	Roma tomato
2004	June	Enterica	Braenderup	137	Roma tomato
2005	July	Enterica	Newport	72	Tomato, beefsteak
2005	November	Enterica	Braenderup	84	Roma tomato
2006	January	Enterica	Berta	16	Tomatoes
2006	June	Enterica	Newport	115	Tomato, unspecified
2006	September	Enterica	Typhimurium	8	Tomato (see fruit)
2006	September	Enterica	Typhimurium	192	Tomato, unspecified
2007	July	Enterica	Newport	10 (1)	Tomato, unspecified
2007	October	Enterica	Typhimurium	23	Tomato, unspecified
2009	May	Enterica	Saintpaul	21	Tomatoes
2010	July	Enterica	Javiana	30	Tomatoes
2010	July	Enterica	Newport	24	Tomatoes
2010	May	Enterica	Newport	64	Tomatoes
2011	April	Enterica	Newport	166	Tomatoes
2011	July	Enterica	Newport	10	Tomatoes
2012	June	Enterica	Newport	102	Tomato, unspecified
2013	January	Enterica	Newport	14	Salad, unspecified; sandwich, unspecified
2013	January	Enterica	Newport	14	Salad,
2013	March	Enterica	Saintpaul	131	Cherry & grape tomato
2013	May	Enterica	Enteriditis	27	Tomatoes, raw
2013	May	Enterica	Enteriditis	27	Tomatoes, raw

[*]*Data collected from: CDC-Foodborne Outbreak Online Database (FOOD), Available at: (http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx).*

Melons, especially cantaloupes, have been associated with foodborne illness outbreaks (Sivapalasingam and others 2004; Table 2.4). Walsh and others (2014) reviewed outbreaks reported to CDC, literature published, and records obtained from investigating agencies during the time period between 1973 and 2011. Their findings indicated that a single melon type (e.g. cantaloupe, honeydew, or watermelon) caused a total of 34 outbreaks, involving 3,602 infection cases, 322 hospitalizations, 46 deaths, and 3 fetal deaths. Fifty-six percent (19) of the 34 single melon type outbreaks were caused by cantaloupes, followed by watermelons and honeydews responsible for 38% and 6%, respectively.

Among outbreaks with known etiology (34), *Salmonella* was the most common etiology reported (56%). Among 20 outbreaks with available contamination information, 13 (65%) were contaminated during production, while 7 (35%) were contaminated at the point of service. Precutting of melons was reported as a main factor for contamination. Outbreaks associated with contaminated cantaloupes at production level (13) involved imported (9) and domestic (4) cantaloupes as well. For example, in 2008, a multistate outbreak of *S.* Litchfield infections involved 51 ill persons in 16 states in the U.S. and 9 illnesses in Canada, with no reported deaths. Investigations indicated cantaloupes imported from Honduras (Agropecuria Montelibano) were the source of illnesses (CDC, 2008). Once again, in 2011, a multistate outbreak of *S.* Panama infections were linked to cantaloupe consumption (CDC 2011a). A total of 20 cases were reported. Investigation indicated that among ill persons, 11 ate purchased cantaloupes sourced from a single farm in Guatemala.

Later in 2011, a multistate outbreak of listeriosis infections were linked to consumption of cantaloupes. This outbreak involved 147 illnesses, 33 deaths, and 1 miscarriage in 28 states. Reported illnesses onset ranged from July 31 through October 27, and ages ranged from <1 to 96

years old. Collaborative investigations indicated implicated cantaloupes came from Jensen Farms in Colorado (CDC 2011b).

Table 2.4 *Salmonella* spp. and *Listeria monocytogenes* confirmed outbreaks associated with melons and cantaloupes from 2000 to 2013*

Year	Month	Genus species	Serotype	Total ill (deaths)	Vehicle
2000	April	Enterica	Poona	47	Cantaloupe
2000	June	Enterica	Heidelberg	4	Melon, unspecified
2001	April	Enterica	Poona	50 (2)	Cantaloupe
2001	April	Enterica		2	Cantaloupe
2001	June	Enterica	Poona	23	Honeydew melon; musk melon; watermelon
2002	April	Enterica	Poona	48	Cantaloupe
2002	September	Enterica	Berta	29	Cantaloupe; grapes, unspecified; watermelon
2003	January	Enterica	Newport	68 (2)	Honeydew melon
2003	May	Enterica	Muenchen	58	Cantaloupe; honeydew melon
2005	July	Enterica	Newport	24 (1)	Cantaloupe; ground beef, unspecified
2006	August	Enterica	Newport	20	Watermelon
2007	December	Enterica	Litchfield	53	Cantaloupe
2007	May	Enterica	Litchfield	30	Cantaloupe; fruit salad; grapes, unspecified
2008	August	Enterica	Newport	3	Cantaloupe; watermelon
2008	November	Enterica	Javiana	10	Cantaloupe
2008	October	Enterica	Javiana	594	Watermelon
2009	February	Enterica	Carrau	53 (1)	Melon
2010	July	Enterica	Saintpaul	17	Watermelon
2011	August	Enterica	Typhimurium	15	Watermelon
2011	February	Enterica	Panama	20	Cantaloupe
2011	June	Enterica	Uganda	25	Cantaloupe
2011	June	Enterica	Typhimurium	6	Cantaloupe & Strawberry mix
2012	July	Enterica	Newport	33 (1)	Cantaloupe
2012	July	Enterica	Typhimurium	14	Cantaloupe
2012	July	Enterica	Typhimurium; Newport	261 (3)	Cantaloupe
2012	July	Enterica	Newport	24	Cantaloupe
2013	April	Enterica	Typhimurium; Typhimurium	14	Cantaloupe
2011	July	*Listeria monocytogenes*		147 (33)	Cantaloupe

** Data collected from: CDC-Foodborne Outbreak Online Database (FOOD), Available at: (http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx)*

Escherichia coli and *Escherichia coli* O157:H7

Description of the organism

In 1885, Theodor Escherich in his studies of the neonatal and infant fecal flora isolated and described some microorganisms, which were initially named *Bacterium coli commune* (the common colon bacteria). It wasn't until the 1960s, after numerous works on the phenotyping characteristics, that the genus *Escherichia* was described as a gram-negative, non-spore-forming, straight rod, facultative anaerobe, often motile by means of peritrichous flagella (Bell and Kyriakides 2002a; Frataminco and Smith 2006).

Escherichia coli is one of the predominant enteric species in human and warm-blooded animal gastrointestinal tracts. Although most *E. coli* strains are harmless, certain strains, particularly Shiga toxin-producing *E. coli* (STEC) strains, can cause illness in humans. Serological typing of *E. coli* allows identifying three major surface antigens: O (somatic lipopolysaccharide), K (capsular), and H (flagellar). There are approximately 173 O antigens, 56 H antigens, and 80 K antigens recognized (Ørskov and Ørskov 1992). Serotyping and serogrouping of *E. coli* strains facilitates distinction between pathogenic and non-pathogenic strains. Currently, there are six recognized pathogenic groups, based on serological grouping, characteristics, and disease syndromes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC).

Enterotoxigenic E. coli *(ETEC)*

These strains are among the leading cause of traveler's diarrhea. In both developing and developed countries, ETEC infection is transmitted via ingestion of contaminated food or water. Moreover, ETEC-contaminated food handlers with poor hygiene can contaminate food and

water. In human volunteer challenge studies, infective dose was estimated as 10^8 to 10^{10} ETEC cells (Levine and others 1979). ETEC disease syndrome is rarely accompanied by fever or vomiting, but diarrhea is sudden. However, blood, mucus, and leukocytes are not present in stools. Diarrhea may be prolonged in childen causing severe dehydration. Fimbrial structures, known as colonization factors, are significant for ETEC cells attachment and colonization of the small intestine (Jay and others 2005b; Fratamico and Smith 2006; Fleckenstein 2013) that subsequently causes diarrheal illness. Once attached, the cells synthesize and release either one or two toxins. One of the *E. coli* enterotoxins is heat-labile (LT) and the other is heat-stable (STa and STb). The LT strain can be destroyed at 60°C in approximately 30 min, whereas ST toxins require over 100°C for 15 min to be destroyed (Jay and others 2005b).

Enteropathogenic* E. coli *(EPEC)

Generally, these strains do not produce enterotoxins, however they can cause diarrhea. The main symptom of EPEC infections is diarrhea, and it may contain mucus, or blood on rare occasions. Other symptoms may include abdominal pain, fever, myalgia, vomiting, and nausea. EPEC is a frequent cause of infantile diarrhea in developing countries, where water quality and hygiene are poor. Infant dehydration due to EPEC infections is common, which may lead to weight loss, malnutrition, and death (Nisa and others 2013). EPEC strains are characterized by their ability for localized adherence. EPEC cells possess adherence factor plasmids, which encode the bundle-forming pilus (BFP) that facilitates adherence to the intestinal epithelium cells. Upon colonization of the mucosa, attachment-effacement (A/E) lesions occur (Fratamico and Smith 2006). The genetic factor responsible for the A/E lesions is the pathogenicity island known as Locus of Enterocyte Effacement (LEE), which encodes multiple virulence factors (Jay and others 2005b; Nisa and others 2013).

Enteroaggregative E. coli *(EAEC)*

Enteroaggregative strains are characterized as *E. coli* strains that do not produce SL and Stx enterotoxins, and adhere to HEp-2 (epithelial) cells in a localized and aggregative arrangement (Nataro and others 1998). EAEC is a common cause for persistent diarrhea (> 14 days) in infants and children in developing countries. Common infection indicators include diarrhea with or without blood and mucus, abdominal pain, nausea, vomiting, borborygmi, and fever (Nataro and others 1998). Infections of EAEC may lead to pathogen-induced malnutrition, caused by increased metabolic demand due to intestinal inflammation. Thus, the persistent diarrhea in EAEC infected infants and child patients may be due to inability to repair the damage done in the intestinal mucosa (Nataro and others 1998). The ability of *E. coli* strains to evolve and acquire virulence is a major concern, since novel and unusual STEC strains emerge. For example, a highly virulent Shiga toxin-producing enteroaggregative *E. coli* strain (O104:H4) was the cause of an outbreak in Germany in 2011 (Bielaszewska and others 2011; Alexander and others 2012).

Enteroinvasive E. coli (EIEC)

Enteroinvasive E. *coli* strains cause bacillary dysentery, which causes frequent small volume of stools with mucus and blood. Thus, EIEC strains resemble *Shigella* in their pathogenic potential (Nataro and others 1995). The infective dose of EIEC ranges from 200 to 5,000 cells (Feng 2012). The bacterial cells attach to the epithelial cells of the colon and then spread from cell to cell, penetrating as far as the lamina propia (Maurelli 2013). Infections occur via ingestion of contaminated food and water. In most patients, EIEC infection results in watery diarrhea with traces of mucus and blood. Other symptoms include fever, severe abdominal cramps, and tenesmus (Maurelli 2013).

Diffusely adherent E. coli (DAEC)

These strains are characterized by their ability to attach to the surface of epithelial cells. This adherence is termed diffuse. The genetic factor responsible for the diffused pattern of attachment was characterized by Bilge and others (1989). Their findings indicated that a fimbrial adhesin, F1845, was found responsible for the diffuse epithelial cell adherence (Bilge and others 1989). Although not all DAEC strains cause diarrhea, strains in this group vary in their level of pathogenicity. However, diarrhea induced by some DAEC strains is watery and with mucus, followed by with fever and vomiting.

Enterohemorrhagic E. coli (EHEC)

Escherichia coli strains that synthesize Shiga toxins are referred as Shiga toxin-producing *E. coli* (STEC). A STEC strain may produce Stx1, Stx2, or a combination of both toxins. Virulence factors of EHEC strains, including *E. coli* O157:H7, is the production of one or more types of Shiga toxins, intestinal colonization, and A/E lesions (Fratamico and Smith 2006). Enterohemorrhagic *E. coli* strains, similarly to EPEC, have the ability to induce A/E lesions in the intestinal mucosa. EHEC strains also possess the LEE locus, which encodes various surface factors, such as frimbrial and non-fimbrial adhesins (Vanaja and others 2013). One of the most studied adhesins of EHEC is the outer-membrane intimin, encoded by the *eae* gene. Intimin participates in formation of A/E lesions by binding to the intimin receptor *Tir*, subsequently translocated to the host by a type III-secretor effector, which acts as an export apparatus that connects inner and outer membranes and facilitates injection of bacterial effectors from bacteria cytoplasm into the host (Garmendia and others 2005). Tir, intimin, and the entire type III secretor system (T3SS) are encoded in the LEE.

Shiga toxin-producing *E. coli* strains, particularly *E. coli* O157:H7, have a very low infectious dose. It has been estimated that *E. coli* O157:H7 has an infectious dose of 10 to 100 cells (Feng 2012). The estimated number of *E. coli* O157:H7 organisms in beef patties, implicated in an outbreak between November 1992 and February 1993, was 1.5 cells per gram or 67.5 organisms per patty (Tuttle and others 1999).

Shiga toxin-producing *E. coli* infections display various manifestations. They may cause asymptomatic infections or may cause mild non-bloody diarrhea to acute grossly bloody diarrhea also known as Hemorrhagic Colitis (HC). Incubation periods for HC range from 3 to 8 days, but can be as short as 1 to 2 days. HC is accompanied by severe abdominal cramps, and in some cases vomiting. Some cases, particularly infants, children, and the elderly, develop severe complications, being the most lethal HUS and Thrombotic Thrombocytopenic Purpura (TTP). This disorder is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and failure of affected organs (Noris and others 2012); central nervous system complications may also occur (Su and Brandt 1995). Researchers have reported that Shiga toxin-producing *E. coli* strains, particularly *E. coli* O157:H7 that produces Stx2 but not Stx1, were more likely than other genotypes to be isolated from patients with HUS or TTP (Griffin and others 1988; Panos and others 2006). HUS and TTP are pathologically indistinguishable and have been classified as similar disorders, due to common incidence of thrombosis. Moreover, HUS can involve manifestations other than renal disease and patients with TTP often present renal disease. Therefore, the two conditions can be difficult to differentiate based only in clinical presentation (Tarr and others 2005; Noris and others 2012). According to Noris and others (2012), however, advances of molecular pathology have helped to recognize three different diseases: HUS caused by Shiga toxin-producing *E. coli*, atypical HUS associated with genetic or acquired disorders of

regulatory components, and TTP as resulting from a deficiency of ADAMTS 13, a plasma methalloprotease that cleaves von Willebrand factor. All these diseases have a pathogenic effector in common (known as complement system) that leads to endothelial damage and microvascular thrombosis (Noris and others 2012).

Escherichia coli can grow in temperatures ranging from 7 to 46°C, with optimum temperature conditions ranging from 35 to 40°C. Although *E. coli* do not grow in refrigeration or frozen temperature conditions, the microorganism can survive for weeks at 4°C or -20°C (Fratamico and Smith 2006; Strawn and Danyluk 2010). The minimum water activity (a_w) required for growth of *E. coli* is 0.95. Heat resistance of *E. coli* O157:H7 is affected by composition, pH, and a_w of the food. For example, Ahmed and others (1995) found differences in D-values between different meat and poultry products. Higher fat levels in all products resulted in higher D-values, and the D_{60}-values (minutes) ranged from 0.45 to 0.47 in beef, 0.37 to 0.55 in pork, 0.38 to 0.55 chicken, and 0.55 to 0.58 in turkey.

Heat sensitivity can also be affected by a microorganism's exposure to prior stress (Yuk and Marshalll 2003). *Escherichia coli* O157:H7 grows at pH levels ranging from 4.4 to 9.0, with an optimum pH of 6 to 7. However, studies have shown that *E. coli* O157: H7 can survive for extended periods of time in foods at pH values of 3.5 to 5 (Zhao and others 1994; Fisher and Golden 1998). In a study by Zhao and others (1994), *E. coli* O157:H7 survived for 5 to 7 weeks in mayonnaise with pH levels ranging from 3.6 to 3.9, when stored at 5°C and 20°C, respectively. Fisher and Golden (1998) studied the fate of *E. coli* O157:H7 in four varieties (Golden Delicious, Red Delicious, Rome, and Winesap apples) of ground apples. *Escherichia coli* O157:H7 survived for 18 days at 4°C in the four variety groups with pH values ranging from 3.91 to 5.11.

Enterohemorrhagic *E. coli* was recognized as a human pathogen in 1982 after two hemorrhagic colitis outbreaks in U.S. However, it was not until 1993, after a large multistate *E. coli* O157:H7 outbreak associated with undercooked ground beef patties from a fast-food chain restaurant, that *E. coli* O157:H7 was recognized as a major health threat. Although *E. coli* O157:H7 is the strain accountable for the greatest proportion of illnesses worldwide (Tarr and others 2005), other non-O157 serotypes of STEC (O26, O45, O103, O111, O121, O145) are of major concern in the U.S. Between 1983 and 2002, non-O157 serotypes isolated from submitted cases of sporadic illnesses included O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145 (5%). Moreover, O111 accounted for most HUS cases and 3 of 7 non-*E. coli* O157 outbreaks reported in U.S. (Brooks and others 2005). Table 2.5 summarizes information regarding the pathogenicity of different *E. coli* groups and characteristics of illnesses associated with it.

Table 2.5 Pathogenicity and characteristics of foodborne illness caused by pathogenic *E. coli*

Pathogenic group	Selected serogroup	*E. coli*/ host interaction	Time to onset of illness	Duration of illness	Symptoms
ETEC (enterotoxigenic)	O6, O15, O25, O27, O63, O78, O115, O148, O153, O159	Adhere to the small intestinal mucosa and produce toxins that act on the mucosal cells.	8 to 44 h, average 26 h	3 to 19 d	Watery diarrhea, low fever, abdominal cramps, malaise, and nausea. When severe, causes cholera-like extreme diarrhea with rice water like stools, dehydration.
EPEC (enteropathogenic)	O18ab, O18ac, O26, O44, O55, O86, O114, O119, O125, O126, O127, O128, O142, O158	Attach to intestinal mucosal cells causing cell structure alterations (attaching and effacing).	17 to 72 h, average 36 h	6 h to 3 d, average 24 h	Severe diarrhea in infants, which may persist for more than 14 d. In adults, sever watery diarrhea with prominent amount of mucus without blood (main symptom), nausea, headache, fever, and chills.
EHEC (enterohemorrhagic)	O2, O4, O5, O6, O15, O18, O22, O23, O26, O55, O75, O91, O103, O104, O105, O111, O113, O114, O117, O118, O121, O128ab, O145, O153, O157, O163, O168	Attach and efface mucosal cells and produce toxin.	3 to 9 d, average 4 d	2 to 9 d, average 4 d	Hemorrhagic colitis: sudden onset with severe cramps and abdominal pain, bloody diarrhea, vomiting, no fever. Hemolytic uremic syndrome (HUS): bloody diarrhea, acute renal failure in children, thrombocytopenia, acute nephropathy, seizures, coma, and death. Thrombotic thrombocytopenia purpura (TTP): similar to HUS, nervous system disorder, abdominal pain, gastrointestinal hemorrhage, blood cloths in brain, and death.
EIEC (enteroinvasive)	O28ac, O29, O112ac, O121, O124, O135, O144, O152, O167, O173	Invade cells in the colon and spread laterally, cell to cell.	8 to 24h, average 11 h	Days to weeks	Profuse diarrhea or dysentery, chills, fever, headache, muscular pain, abdominal cramps.
EAEC (Enteroaggregative)	O3, O44, O51, O77, O86, O99, O111, O126	Bind in clumps to cells of the small intestine and produce toxins.	7 to 22 h	Days to weeks	Persistent diarrhea in children. Occasionally bloody diarrhea or secretory diarrhea, vomiting, dehydration.
DAEC (diffusely adherent)	O1, O2, O21, O75	Fimbrial and non-fimbrial adhesion	Not yet established	Not yet established	Childhood diarrhea

Adapted from: Blackburn and McClure 2002; Bad Bug Book 2nd edition

Salmonella species (spp.)

Description of the organism

The genus *Salmonella* is part of the Enterobacteriaceae family. *Salmonella* spp. are facultative anaerobes, Gram negative, oxidase negative, straight rod-shaped which are usually motile by means of peritrichous flagella (Bell and Kyriakides 2002b). The genus consists of two main species, which can cause illnesses in humans: *S. enterica* and *S. bongori*. *Salmonella enterica*, which is of major concern for public health, is diverse and consists of six subspecies: *S. enterica* subsp. *enterica, S. enterica* subsp. *salamae, S. enterica* subsp. *arizonae, S. enterica* subsp. *diarizonae, S. enterica* subsp. *houtenae, S. enterica* subsp. *indica* (Hammack 2012). According to the WHO Collaborating Centre for Reference and Research on *Salmonella*, over 2,500 serotypes of *Salmonella* have been recognized by using the White-Kauffmann Scheme (Molbak and others 2006; Grimont and Weill 2007). Table 2.6 summarizes information regarding the present number of serovars in each specie and subspecies.

Table 2.6 Present number of serovars in each *Salmonella* species and sub-species

Specie	Sub-specie	No. serovars
S. enterica		2,557
	enterica	1,531
	salamae	505
	arizonae	99
	diarizonae	336
	houtenae	73
	indica	13
S. bongori	-	22
Total (genus *Salmonella*)		2,579

Adapted from: Grimont and Weill 2007.

Salmonella can grow in temperatures ranging from 5.2 to 46.2°C, with optimal growth in temperatures of 35 to 37°C (Jay and others 2005c). Optimum pH and a_w values for *Salmonella*

growth are between 6.6 to 8.2 and 0.94 to 0.99, respectively (Bell and Kyriakides 2002b; Jay and others 2005c). However, survival of *Salmonella* in low a_w (<0.7) foods for long periods of time have been reported (Juven and others 1984; Farakos and others 2014). Moreover, *Salmonella* strains exposed to temperatures ≥ 70°C at low a_w were more heat resistant than strains at higher a_w and exposed to temperatures below 65°C (Mattick and others 2001).

Salmonella infections in humans are usually caused by ingestion of contaminated food or water. Certain groups of people are more susceptible to *Salmonella* infections; young children are especially at risk since their immune system is still developing. The elderly, and those with chronic illness or immunocompromised individuals are also vulnerable to salmonellosis. Incubation period ranges from 6 to 48 hours after ingestion, but with an ingestion of a high dose, the incubation may be as short as few hours (Molbak and others 2006). Most patients develop acute diarrhea as the main symptom. Other common symptoms are abdominal pain, nausea, mild fever and chills. Diarrhea varies in volume and frequency, and blood in stools may occur. Illness usually lasts from 2 to 7 days. However, in some cases sequela or late-onset complications have been observed; arthritis, Reiter's syndrome, and erythema nodosum are some of the reactive consequences.

Some *Salmonella* serovars are specifically host related, for example *S.* Gallinarum (poultry), *S.* Abortusequi (horse), and *S.* Abortusovis (sheep), among others. Moreover, some serovars are more infectious to some animals, for example *S.* Dublin in cattle and *S*. Choleraesuis in pigs, but may still cause illnesses in humans (Bell and Kyriakides 2002b; Forshell and Wierup 2006). Despite various research efforts, the precise mechanism of how *Salmonella* causes illness is not fully understood. However, research highlights that both plasmid and chromosomal genes are involved in *Salmonella* virulence (Marcus and others 2000; Forshell and Wierup 2006).

Many of these genes are found in pathogenicity islands in the chromosomes. *Salmonella* pathogenicity islands (SPIs) encode specific determinants responsible for *Salmonella/*host interactions (Marcus and others 2000). A total of five SPIs have been studied. The SPI1 primarily participates in the bacterial penetration of epithelial cells of the intestine, while SPI2, 3, and 4 are required for growth and survival of *Salmonella* within the host, which characterizes the systemic phase of disease. The SPI1 and 2 both encode type 3 secretion systems, whose main role is to deliver bacterial effector proteins from bacteria cytoplasm into the host cell. Virulence factors encoded by SPI5 mediate the inflammation and chloride secretion that characterize the enteric phase of disease (Marcus and others 2000). It is noteworthy to clarify that the five SPIs are not present in all *Salmonella* species, which may explain the varying host specificity.

Historically, foodborne *Salmonella* infection outbreaks have been associated with raw or undercook eggs, meat and poultry, unpasteurized milk, and also other cross-contaminated food during preparation. However, an increasing number of *Salmonella* outbreaks have been linked with fresh fruits and vegetables. Moreover, processed products such as chocolate, peanut butter, powdered milk, and bakery products have been implicated in *Salmonella* infections outbreaks (Podolak and others 2010; Beuchat and others 2013). Dose-response modeling research has indicated that infective dose of *Salmonella* is reduced (10 to 100 cells) when food matrixes contain high fat and protein levels, such as chocolate, salami, cheese (Teunis and others 2010). For example, chocolate's low a_w (0.4 – 0.5) and high content of sugar and fat do not support the growth of microorganisms, however estimates of *S.* Oranienburg per gram (1.1 – 2.8) recovered from samples of chocolate implicated in a *Salmonella* outbreak suggest a low infectious dose (Werber and others 2005). Generally, it is thought that high numbers (between 10^5-10^6 cells) of

Salmonella cells need to be consumed to cause illness (Jay and others 2005c; Molbak and others 2006).

Listeria monocytogenes

Description of the microorganism

Six species of *Listeria* are recognized: *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii,* and *L. grayi*. *Listeria monocytogenes* is a Gram-positive, non-spore forming, facultative anaerobic rod motil by means of peritrichous flagella (Jay and others 2005d). *Listeria monocytogenes* is the main pathogenic specie of the *Listeria* genus and is comprised of 13 serovars including 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. However, most foodborne outbreaks of *Listeria* infections have been attributed to serogroups 1/2a, 1/2b, and 4b (Chen 2012). Among the other species in the genus *Listeria*, only *L. ivanovii* is recognized as pathogenic, and mainly in ruminants rather than in humans (Chen 2012).

Nutritional requirements for *Listeria* growth in common media requires B-vitamins including: biotin, riboflavin, thiamine, and thiotic acid. Additionally, the amino acids cysteine, glutamine, isoleucine, leucine, and valine are required for *Listeria* spp. growth. *Listeria* spp. can grow at temperatures ranging from < 0°C to 45°C, pH values between 6 to 8, a_w values as low as 0.90, and salt concentrations as high as 10% (Jay and others 2005d; Pagotto and others 2006). Temperature plays an important role for the microorganism motility, which is exhibited at 20°C to 25°C, but absent at 37°C (Pagotto and others 2006). Peel and others (1988) reported that transcription of the flagellin-encoding gene in *L. monocytogenes* is more prominent at 22°C, but undetectable at 37°C. Moreover, *L. innocua* produces flagellin at 37 °C, suggesting that differences in motility exists among species (Kathariou and others 1995).

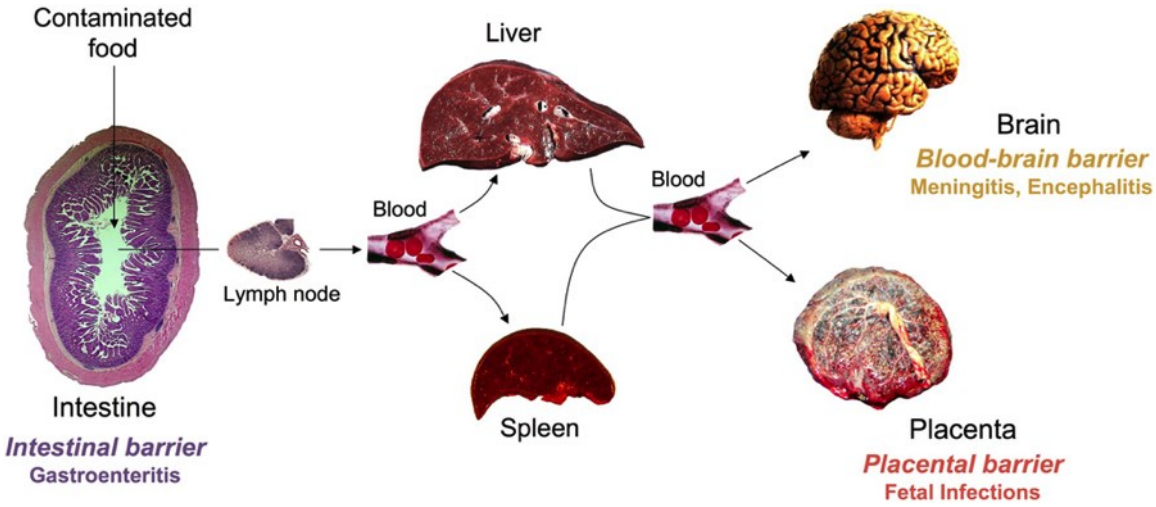
Listeria monocytogenes ability to proliferate at low temperatures, broad pH range, and low a_w, facilitates its prevalence in the environment and in a variety of food items. In nature, for example, *Listeria* can be found in decaying vegetation and soil, animal feces, sewage, silage, and water (Jay and others 2005d). Foods associated with *L. monocytogenes* include raw milk, inadequately pasteurized milk, cheeses, ice cream, raw vegetables, liquid whole eggs, raw meat and poultry, fermented raw meats (sausages), hot dogs and deli meats, raw and smoked fish and seafood (Chen 2012). Infective dose of *L. monocytogenes* varies among the strains, vulnerability of the host, and nature of the food matrix involved. According to Chen (2012) the infectious dose for vulnerable individual consuming raw or inadequately pasteurized milk is less than 1,000 cells.

Individuals principally at risk from *Listeria* infections (Listeriosis) include: organ transplant patients, pregnant women/fetuses/neonates, immunocompromised patients by the use of anticancer drugs, corticosteroids, and graft suppression therapy and AIDS, patients with cancer, and the elderly (Bell and Kyriakides 2002c; Chen 2012). Additionally, reports suggest that use of antacids or cimetidine may predispose healthy persons to *Listeria* infections. Moreover, if ingested food has been contaminated with high levels of *L. monocytogenes,* healthy uncompromised persons could develop illness (Chen 2012). Three main routes transmit *L. monocytogenes*: contact with animals, cross-infection of new-born babies, and foodborne infection (Bell and Kyriakides 2002c). The incubation period for Listeriosis is between 10 to 70 days. Symptoms may include influenza like symptoms such as fever, myalgias, arthralgias, headache and backache, sometimes preceded by diarrhea or other gastrointestinal symptoms, but might also be clinically silent (Lecuit 2007; CDC 2013b). Additionally, symptoms vary with the infected person for example, pregnant women present fever, fatigue, and aches. Infections during

pregnancy can lead to miscarriage, stillbirth, premature delivery, or severe infection of the newborn. In elderly or immunocompromised patients, septicemia and meningitis are the most common clinical manifestations (CDC 2013b). *Listeria monocytogenes* is able to cross the gastrointestinal, materno-fetal and blood-brain protective barriers (Pagotto and others 2006; Lecuit 2007).

After ingestion of contaminated food, the organism invades the intestinal epithelial cells. Subsequently, the bacteria can enter the mesenteric lymph nodes and translocate via bloodstream to the liver and spleen. If not properly controlled by the immune system in the liver and spleen, asymptomatic bacteremia may occur and then reach brain or placenta, resulting in meningitis, encephalitis, abortions in pregnant women, and/or generalized infections in neonates (Figure 2.1; Lecuit 2007).

Figure 2.1 Steps involved in the development of human listeriosis (Lecuit 2007)



There are many virulence factors involved in *L. monocytogenes* intracellular invasion of host's cells. *Listeria monocytogenes* can invade phagocytes and non-phagocyte cells (Jay and

others 2005d). In phagocyte cells, internalization occurs when bacteria become immersed within the phagocytic vacuole through disruption of the vacuole membrane. This step is critical for *Listeria* survival and proliferation, and is facilitated by the Listeriolysin O (LLO), a pore-forming hemolysin, encoded by *hly* locus (Marquis and others 1995; Vasquez-Boland and others 2001). In non-phagocyte cells, InlA and InlB, encoded by the *inlAB* operon, mediate *listeria* internalization.

Other surface proteins associated with *Listeria* invasion are: p60 (encoded in the invasion associated protein gene, *iap*), ActA (actin polymerizing protein), and Ami (an autolysin with a C-terminal cell wall-anchoring domain similar to InlB) (Jay and others 2005d; Vasquez-Boland and others 2001). The virulence factors of *Listeria* spp. are organized in genetic units (pathogenicity islands or PAIs). Virulence factors (*prfA, plcA, hly, mpl, actA, and plcB*) involved in *L. monocytogenes* intracellular parasitism are physically linked in a chromosomal island known as *hly* or PrfA (dependent virulence gene cluster), which is currently referred as LIPI-1 (*Listeria* pathogenicity island 1) (Vasquez-Boland and others 2001).

Sanitizer washes and safety practices used to minimize microbial contamination of fresh produce

Fresh and ready-to-eat produce have repeatedly been associated with foodborne outbreaks. Pathogenic microorganisms such as *Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes,* and lately non-O157 STEC strains have been the cause of foodborne outbreaks. Fresh produce can become contaminated with these pathogens at any point along the farm-to-table food chain. Washing plays an important role on fresh produce quality and safety. Washing procedures are intended to remove soil, chemical residues (i.e. pesticides), and other

debris on produce surfaces. Washing procedures are of imperative concern since inadequate handling can result in produce damage, cross contamination, and internalization of chemical and/or microbial contaminants (Park and others 2008; Pao and others 2012). Methods to reduce microbial contamination on produce surfaces usually involve the use of sanitizers and mechanical action. The use of different sanitizing agents and application methods to reduce microbial populations on the surfaces of whole and fresh cut produce has been documented. Brief summaries of the antimicrobial characteristics of common sanitizer agents currently used in the produce industry are discussed below in this document.

Chlorine

Chlorine is a broadly used sanitizing agent for fresh produce (Sapers 2001; Rico and others 2007; Ölmez and Kretzschmar 2009). The most common form of chlorine includes liquid chlorine and hypochlorite (sodium and calcium hypochlorite), which are commonly utilized at concentrations of 50 to 200 ppm of free chlorine, with exposure times of less than 5 min (Beuchat and others 2004; Bari and others 2005; Akbas and Ölmez 2007; Allende and others 2009). The antimicrobial activity of chlorine is attributed to hypochlorous acid (HOCl), which forms when chlorine dissolves in water (CDC 2009). In aqueous solutions, chlorine reactivity is pH dependent, with the concentration of HOCl increasing as pH decreases (FDA 2013). Although, chlorine solutions are more effective at low pH levels (4 to 5), in the fresh produce industry chlorine solutions are commonly utilized at pH values ranging from 6.0 to 7.5 to minimize corrosion of processing equipment (Beuchat 2000). HOCl concentration is also affected by temperature, presence of organic matter, light, air, and metal (FDA 2013). The use of chlorine-based sanitizers on leafy greens has shown reductions of pathogenic microorganisms between 90 and 99.99% (1 to

4 log units; Beuchat and others 2004; Bari and others 2005; Akbas and Ölmez 2007; Allende and others 2009).

Yuk and others (2006a) reported that application of chlorinated water (200 ppm) on the smooth surface of bell peppers achieved 2 log reductions of *Salmonella* spp. populations while application of acidified sodium chlorite (1,200 ppm) showed undetectable populations of *Salmonella* spp. Rinsing *E. coli* O157:H7 or *Salmonella* spp. inoculated mung bean seed for 15 min with aqueous solutions of calcium hypochlorite (1,900 to 18,000 ppm) resulted in reductions of approximately 4 to 5 logs CFU/g (Fett 2002a; Fett 2002b). At foodservice facilities, chlorine is a convenient and inexpensive method used against pathogenic microorganisms (FDA 2013). However, improper use of chlorine can affect the quality and shelf life of fresh and fresh-cut produce. Moreover, overuse of chlorine can produce undesired toxic by-products known as chlorination by-products (CBPs) (Lopez-Galvez and others 2010). Chlorine dioxide also is used to reduce pathogenic microorganisms from fresh produce surface. Unlike chlorine, chlorine dioxide is more stable at a wide range of pH levels. A maximum of 200 ppm of chlorine dioxide is allowed to sanitize processing equipment and a maximum of 3 ppm is allowed for contact with whole produce (CFR 2014). This method is more commonly used in commercial facilities than at foodservice facilities. However, reactions with chlorine can lead to the formation of harmful CBPs, such as chloroform (Lopez-Galvez and others 2010).

Organic acids

Citric acid, lactic acid, ascorbic acid, and acetic acid are GRAS (Generally Recognized as Safe) status and are frequently used antimicrobial agents. The antimicrobial action of organic acids is due to pH reduction in the internal cell pH (FDA 2013). Organic acids are stable in the presence of organic matter. Washing treatments of citric acid and lactic acid at 0.5, 1, and 5%

concentrations with exposure times less or equal to 5 min have been studied (Torriani and others 1997; Francis and O'Beirne 2002; Akbas and Ölmez 2007; Chang and Fang 2007). Washing ready-to-use mixed salad with 1% lactic acid resulted in total and fecal coliform population reductions of approximately 99 and 90% (2 and 1 logs), respectively, while 0.5% lactic acid did not affect vegetable indigenous microbial flora (Torriani and others 1997). Dipping fresh-cut iceberg lettuce in 0.5% citric acid or 0.5% lactic acid solutions for 2 min showed reductions of 90 and 99% (1 and 2 log units) for *L. monocytogenes* and *E. coli* populations, respectively (Akbas and Ölmez 2007). Washing solutions of 5% acetic acid resulted in 99.9% reduction (3 logs) of *E. coli* O157:H7 populations on iceberg lettuce; however this concentration of acetic acid may detriment sensory characteristics of produce (Chang and Fang 2007). Leaf lettuce dipped in a solution containing 0.25% citric acid plus 0.5% ascorbic acid for 2 min showed approximately 1.5 log reduction of *E. coli* populations (Ölmez 2010). The antimicrobial activity of organic acids varies among the type of organic acid and its concentration, contact time, and microbial load (Rico and others 2007; Ölmez and Kretzschmar 2009; FDA 2013).

Ozone

Ozone is a strong antimicrobial agent. After gaining GRAS status in 1997, it has been commonly used in aqueous form as a method to reduce microbial contamination in fresh produce. There are mixed findings reported on the efficacy of ozone against pathogenic microorganisms on artificially contaminated lettuce. Spray washing lettuce with cold water (2°C) at 5 ppm ozone concentration for 1 min showed about 99% (2 logs) reduction of *Staphylococcus aureus, Escherichia coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* populations (Kim and others 2006). Populations of *E. coli* O157:H7 and *L. monocytogenes* were reduced 99.999% (5 logs) when whole green leaf lettuce leaves were immersed in ozonated water (3 ppm ozone

concentration) for 5 min or when shredded lettuce leaves were spray treated with ozonated water for 3 min (Rodgers and others 2004).

Other experiments have reported only 90% (1 log) reduction in *E. coli* populations and no effect on *L. monocytogenes* populations on shredded lettuce after application of aqueous ozone (5 ppm concentration) for 5 min (Yuk and others 2006b). Advantages of ozone include greater antimicrobial effect against certain microorganisms and the absence of potentially toxic reactions, since decomposition in water occurs very rapidly. Nevertheless, ozone's strong oxidizing activity may cause physiological damage to produce above certain levels (Kim and others 2006) and can cause corrosion to metals and other materials in processing equipment. In addition, ozone has the potential to produce toxic vapor, so adequate ventilation is necessary for employee safety (FDA 2013).

Peroxyacetic acid or peracetic acid (PAA)

Peroxyacetic acid is a mixture of acetic acid and hydrogen peroxide in a water solution. Benefits of PAA include lack of harmful decomposition material (i.e., acetic acid, water, oxygen, and hydrogen peroxide), effective removal of organic material, and lack of remaining residues (CDC 2009). The U.S. Food and Drug Administration (FDA) allows the addition of PAA prepared by reacting acetic acid with hydrogen peroxide to water at concentrations that do not exceed 80 ppm in wash water for fruits and vegetables (CFR 2014). However, under Environmental Protection Agency (EPA) regulations, the established level is up to 100 ppm of PAA as a direct application to fruits, vegetables, tree nuts, cereal grains, herbs and spices because of an exemption (CFR 2015). Whole iceberg lettuce washed for 2 min with a water solution of 80 ppm PAA in a continuous-flow tank showed about 99% (2 logs) reductions of *E. coli* O157:H7 populations (Palma-Salgado and others 2014). In another study, effectiveness of

PAA against the natural microbiota of lettuce was assessed after keeping lettuce in contact with a water solution of 80 ppm PAA for 15 min. Results of the study indicated that mold, yeast, and total coliform populations were reduced by about 99% (2 logs) (Silva and others 2003). Bell peppers and cucumbers contaminated with *Salmonella* species were wash-floated in a recirculating water bath containing PAA (75 ppm concentration). After washing treatment, *Salmonella* spp. populations were not detected on the intact surface of bell peppers and cucumbers. However the washing treatment was able to reduce only 99% (2 logs) and 90% (1 log) of *Salmonella* spp. populations on the stem scar and punctured wounds on the surface of bell peppers and cucumbers, respectively (Yuk and others 2006a).

It is important to emphasize that the efficacy of the washing techniques used for reducing bacterial pathogens are influenced by: sanitizer agent, method used for application, exposure time and concentration of sanitizer, pH, temperature, and characteristics of produce surface (i.e., texture, crack, complex surface), and type and physiology of the target microorganism (FDA 2013). Although, washing methods can reduce the microbial contamination of fresh and fresh-cut produce, the lack of safe handling practices after washing procedures can result in microbial cross-contamination. Therefore, besides the use of sanitizers described above, safe handling practices (Table 2.7) are recommended to minimize or reduce microbial contamination on fresh produce in foodservice operations.

Table 2.7 Recommendations for handling fresh produce in foodservice operations

Activity	Safe handling practice
Purchasing and receiving	• Purchase produce from reliable sources (licensed, reputable suppliers). • Check storage and handling practices of vendor/suppliers (i.e., maintaining produce at proper temperature below 41°F (5 °C) during transportation. • Check the condition of produce at receiving and reject produce with visible signs of damage or partially decay.
Storage	• Store raw produce separately from other refrigerated foods by using a separate set of storage racks, or separate cooler if possible, so that it does not contaminate other food with soil, debris, etc. • Store all produce off the floor (at least 6" off the floor). • Maintain whole or cut produce at temperature recommended below 41 °F (5 °C) to prevent growth of pathogenic microorganisms. • Store washed cut produce in properly sanitized covered containers above other food products that might cause cross contamination. • Keep prepare produce (i.e. shredded lettuce, washed lettuce) in its original package until needed and follow manufacture's instruction for the produce ("keep refrigerated", "best used by", "ready-to-use", etc.)
Food manipulation	• Wash hands thoroughly for 20 s with soap and warm water before and after handling produce. Also, rewash hands as necessary. • Use a barrier such as clean, intact gloves or sanitized utensils to handle ready-to-eat produce. • Do not reuse disposable gloves and change if they are damaged. • Make sure that food service employees who are ill do not work while sick.
Washing and preparation	• Inspect produce for signs of soil and damage. • Carefully read labels to determine if product is a raw produce (i.e., hearts of romaine) that should be washed before consumption or a ready-to-eat product (i.e. pre-washed romaine lettuce). Do not rewash products that are "washed"," triple washed" or "ready-to-eat." • Remove outer leaves, stems, and hulls from produce like head lettuce, cabbage, berries and tomatoes • Always wash produce before serving under running, potable water. • Use registered (EPA, FDA, state and local jurisdiction) antimicrobial washes according to the manufacture's label instructions for recommended concentrations and contact time. • Use a sanitize produce brush to scrub firm fruits and vegetables (i.e. cantaloupes, carrots, potatoes) under running, potable water
Service	• Keep fresh produce at or below 41°F or surrounded by ice. Fresh produce should not be held in direct contact with ice • Dispose cut, pealed, or prepared produce that have not been refrigerated within 4 h of preparation. • Provide appropriate sanitize utensils for self-service of fresh produce • Do not add freshly prepared produce in containers that previously held produce.

Adapted from: Albretch 2008; NFSMI-USDA 2010 and Palumbo 2007.

Attachment of pathogenic microorganism to fresh produce

Fresh produce contamination can occur at any point along the supply chain; there are many scenarios that can favor potential contamination including seed stocks, production, harvesting, post-harvest, retail display, and home/food service preparation (Gorny 2006). Generally, there are two very different environments within a plant, the rhizosphere (roots below the ground environment) and phyllosphere (leaves above ground and the surrounding environment in contact with leaves). Factors such as temperature, humidity, UV solar radiation, nutrient availability, and presence of bacteria, insects, and wild animals may change or influence the microbial communities of the rhyzosphere and phyllosphere (Mandrell and others 2006). For example, in one study various bacteria and viruses were found to survive on the surface of cantaloupes, lettuce, and bell peppers for approximately 14 d under controlled environmental conditions. However, microorganisms survived significantly longer on cantaloupes than on lettuce and bell peppers. This suggests that surface characteristics of produce play an important role in the attachment and survival of microorganisms (Stine and others 2005).

It is noteworthy to clarify that human pathogens are not considered to be part of the phylloshere microbial communities. However, many bacterial pathogens can survive in both soil and water and can also persist in the spermosphere (germinated seed), rhizosphere (roots), and phyllosphere (leaves) of plants, which subsequently allows them to interact with plant tissues through various adhesins and surface proteins that can facilitate attachment, colonization, and biofilm formation (Morris and Monier 2003; Danhorn and Fuqua 2007). Research on human pathogens has indicated that genes required for virulence also are required for attachment and colonization of plant tissue (Barak and others 2005; Barak and others 2007).

Escherichia coli

Several mechanisms of attachment used by *E. coli* O157 have been studied. Jeter and Matthysse (2005) studied the attachment characteristics of pathogenic and non-pathogenic *E. coli* strains. Their findings indicated that conversely to non-pathogenic strains, *E. coli* O157:H7 strongly attached to plant surfaces, including alfalfa sprouts and their open coat seeds, tomatoes, and *Arabidopsis thaliana* (an edible flowering plant) seedlings. The ability of *E. coli* O157:H7 attachment to these plant surfaces was facilitated by curli (Jeter and Matthysse 2005). However, insertion of plasmids that encode or regulate synthesis of curli on non-pathogenic strains (*E. coli* K12) have been shown to be sufficient to enable bacterial attachment to alfalfa sprouts (Jeter and Matthysse 2005). Interestingly, deletion of these genes in *E. coli* O157:H7 did not restrict their ability to bind to plant surfaces. The production of curli is sufficient to allow attachment of non-pathogenic strains to plant surfaces. However curli is not the only factor required for the attachment of pathogenic strains to plant surfaces (Jeter and Matthysse 2005).

The ability of Shiga toxin-producing *E. coli* to cause A/E lesions is encoded mainly in the LEE pathogenicity island, which employs transcriptional regulators, the adhesin intimin, the T3SS, chaperons, translocators (EspA, EspB, and EspD), and effector proteins. Additionally, the ATPase (EscN) plays a key role providing energy to the system in order to accomplish the translocation of proteins into the host cell (Garmendia and others 2005). Ultimately, the T3SS - EspA filament is utilized by the microorganism to directly translocate effector proteins into host cells through a translocation pore formed in the plasma membrane of host cell by the EspB and EspD.

Research by Shaw and others (2008) indicated that attachment of *E. coli* O157:H7 and non-O157 EHEC strains to phyllosphere (lettuce, spinach, and arugula leaves) is mediated by the

filamentous T3SS, which is composed of EspA filaments (Garmendia and others 2005). The results by Shaw and others (2008) illustrated that O157 and non-O157 EHEC strains implement the same molecular mechanism (EspA filaments) used to colonize mammalian epithelial cells to bind to plant phyllosphere. Moreover, the adhesion of EHEC to phyllosphere is independent of effector protein translocation (EspB; Shaw and others 2008). Additionally, Xicohtencatl-Cortes and others (2009) reported that *E. coli* O157:H7 colonizes spinach and lettuce phyllosphere via flagella and T3SS. Their observations indicated that mutation of the *fliC* gene (which encodes flagellin) and the *escN* gene (ATPase; which provides the energy to T3SS for protein translocation into host cell) reduced the level of adhesion of bacteria. This research suggested participation of flagella and T3SS in the bacteria-leaf interaction. Berger and others (2009a) observed that an *aaf* mutant of EAEC O42 lost ability to bind to leaf epidermis and retained stomatal adherence, whereas an *fliC* mutant of the same strain retained the ability to bind to the epidermis and lost stomatal tropism.

Salmonella spp. and Listeria monocytogenes

Berger and others (2009b) used *S.* Senftenberg, a strain implicated in an outbreak associated with basil, and other serovars to study the mechanism used by *S. enterica* to attach to salad leaves. Their results indicated that flagella played a major role in adherence of *S.* Senftenberg to leaf epidermis, since deletion of *fliC* (encoding phase-1 flagella) resulted in reduced level of bacteria adhesion. Moreover *S.* Typhimurium and *S.* Enteritidis were able to adhere efficiently through flagella, whereas *S.* Arizona, *S.* Heidelberg, and *S.* Agona did not. Although flagella were observed as the major mean of attachment for *S.* Typhimurium, the deletion of *fliC* did not affect adherence ability of this serovar. Therefore, the mechanism of attachment differs among serovars. Barak and others (2005) also reported the importance of curli

(Tafi; regulated by *agfD*) for the attachment of *S*. Enteriditis and *S*. Newport to alfalfa sprouts. However, they also found that *S. enterica* uses other adhesins or mechanisms (*rpoS*) in addition to curli to attach to plants. The stationary-phase sigma factor*, RpoS,* plays a significant role by transcribing *agfD*, which is important for the synthesis of thin aggregative frimbriae (Tafi or curli). Additionally, *agfB* functions as a cell-bound fimbrial subunit that secures Tafi to the cell surface.

Further studies have indicated that surface polymers composing the cellular matrix, bacterial cellulose, and O-antigen capsule are key factors for *Salmonella enterica* attachment and colonization of plant tissue. Induced mutations to the O-antigen capsule assembly and translocation (encoded by *yihO*) and bacterial cellulose synthesis (encoded by *bcsA*) reduced the ability of *Salmonella* to attach and colonize alfalfa sprouts. Furthermore, curli, cellulose, and O-antigen capsule are all regulated by *agfD*, which suggests that *agfD* plays a major role for *Salmonella* survival outside the host (Barak and others 2007).

Gorski and others (2003) examined the ability of *L. monocytogenes* to attach to freshly sliced radish. Various *L. monocytogenes* strains were selected to test their ability to attach to radish tissue. The strains belong to the serotyope 1/2a, a/2b, and 4b, and it was observed that the ability to attach to radish tissue appeared to be similar among the strains and to be independent of the source of the strain. Moreover, increased levels of attachment were observed at 10, 20,and 30°C, with attachment at 37°C showing at least 1 log unit below the other three temperatures. Others researchers have reported that transcription of the flagellin-encoding gene in *L. monocytogenes* is more prominent at 22°C, but undetectable at 37°C (Peel and others 1988).

Additionally, differences in motility among species have been suggested (Kathariou and others 1995). In the study by Gorski and others (2003), three mutants with defects in attachment

were identified and characterized. Two of the identified mutants were located in genes with unknown functions, but both genes mapped to a region suspected to contain genes necessary for flagellar export. The third mutation was predicted to encode a sugar transport phosphoenol-pyruvate-sugar phosphotransferase system (PTS). The role of the sugar transport system for the attachment for *L. monocytogenes* to radish tissue remains unclear. All three mutations showed reduction in attachment when tested at 30°C, suggesting that temperature may play a significant role along with the attachment factor available in *L. monocytogenes*. Table 2.8 lists foodborne pathogens and identified mechanism used for attachment and colonization of phyllosphere (plant tissue).

Overall, pathogenic microorganisms such as *E. coli*, *Salmonella* spp. and *L. monocytogenes* employ different mechanisms to attach and colonize plant tissue, which ensures its survival under hostile environmental conditions and allows transmission to human host. Additionally, this interaction may be dependent upon plant and pathogen characteristics. Therefore, understanding the attachment mechanisms of human pathogens to produce surfaces will contribute to the development of novel intervention strategies to prevent produce outbreaks.

Table 2.8 Possible attachment factors and genes of pathogens linked to attachment and colonization of plant tissue

Pathogen	Attachment factor or gene(s)	Target plant tissue	Reference
ETEC *Escherichia coli*	*fliC* – flagella	Lettuce (Arugula)	Shaw and other 2011
EAEC *E. coli*	*fliC* – flagella *aaf* – adherence fimbriae	Lettuce (Arugula)	Berger and others 2009a
E. coli O157:H7	curli	Cabbage, lettuce	Patel and others 2011
E. coli O157:H7	*fliC* – flagella *escN* – ATPase gene associated with the function of the type III secretion system	Lettuce, spinach	Xicohtencatl-Cortes 2009
E. coli O157:H7 and O26	*EspA* filaments via fT3SS – filamentous type III secretion system	Arugula, lettuce, spinach	Shaw and others 2008
Salmonella enterica	*agfB* – surface -exposed aggregative fimbria (curli) or Tafi nucleator *agfD* – a transcriptional regulator of the LuxR superfamily *rpoS* – stationary-phase sigma factor	Alfalfa sprout	Barak and others 2005
Salmonella enterica	Tafi – thin aggregative frimbriae (encode by *agfB)* O-antigen capsule (encoded by *yihO*) Cellulose synthesis (encoded by *bcsA*)	Alfalfa sprouts	Barak and others 2007
Listeria monocytogenes	Flagellin	Raddish (freshly cut)	Gorski 2003

Adapted from: Erickson 2012

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Chapter 3
[1]Validation of Washing Treatments to Reduce *Escherichia coli* O157:H7 and *Salmonella* spp. on the Surface of Green Leaf Lettuce and Tomatoes

Abstract

Outbreaks associated with consumption of fresh produce have been linked to *Escherichia coli* O157:H7 and *Salmonella* contamination. The objective of this study was to determine the efficacy of a chemical wash treatment (citric acid, sodium lauryl sulfate, sodium carbonate, magnesium carbonate, and grapefruit oil extract) in reducing pathogens on the surface of leaf lettuce and tomatoes. Lettuce (25 ± 0.3g) and whole tomatoes were inoculated with *E. coli* O157:H7 (~7.8 $\log_{10}$ CFU/ml) and *Salmonella* spp. (9.39 $\log_{10}$ CFU/ml) cocktails, respectively. Samples were treated with cold tap water (negative control) or the chemical wash treatment with various exposure times (30, 60, and 120 s), and then rinsed with tap water. Samples then were plated on selective media. The chemical wash treatment was capable of reducing by ca. 3.0 $\log_{10}$ units of *E. coli* O157:H7 and *Salmonella* spp. populations on the surface of leaf lettuce and tomatoes, respectively. Even though there were no significant differences among results with different exposure times ($P > 0.05$), application of the chemical wash treatment for 120 s lowered the mean populations of recovered pathogens by 0.1 to 0.66 $\log_{10}$ CFU. Therefore, it is recommended that the chemical wash treatment be applied for 120 s to obtain optimal log reductions on the surface of leaf lettuce and tomatoes.

[1] Lopez, K., Getty, K. J. K., & Vahl, C.I. (2015). Validation of washing treatments to reduce *Escherichia coli* O157:H7 and *Salmonella* spp. on the surface of green leaf lettuce and tomatoes. *Food Protection Trends*, *35*(5), 377-384.

Introduction

Increasing demand for year-round availability of fresh produce, accessibility to ready-to-eat vegetables (pre-prepared or bagged produce), a changing ethnic composition of the population, and an emphasis on increasing consumption of fresh produce for a healthier lifestyle have contributed to increased per capita consumption of fresh produce in the United States (U.S.) *(5,12, 24)*. Concurrently with the increase in consumption, the U.S. Food and Drug Administration (FDA) has responded to several foodborne illness outbreaks linked to fresh produce. The increase in reported outbreaks associated with fresh produce is strongly linked to increased consumption of these commodities, and the improved epidemiological systems used to determine the source of a foodborne illnesses outbreak, such as PulseNet at the Centers for Disease Control and Prevention (1*3, 23)*, have enabled these associations to be made.

In a review of U.S. outbreaks from 1973 through 1997, Sivapalasingam et al. *(28)* reported an eightfold increase in the proportion of illness attributed to produce. In addition, the authors *(28)* found that 190 produce-associated outbreaks caused 16,058 illnesses, 598 hospitalizations, and 8 deaths in 32 states during that time. Painter et al. *(22)* recently analyzed data from documented outbreaks in1998 through 2008 and estimated the number of annual U.S. foodborne illnesses attributable to each of 17 commodities; their results attributed 46% of the illnesses to produce. Among the 17 commodities analyzed, more illnesses were associated with leafy vegetables (22%) than with any other commodity. According to the Centers for Disease Control and Prevention *(11),* the percentage of outbreaks associated with leafy vegetables increased, during 2006 through 2008, from 6 to 11%. Analysis of the settings of food preparation and consumption associated with recognized foodborne outbreaks in the U.S. showed that the largest outbreaks occur in institutional settings such as schools, prisons, and camps *(11)*.

Fresh produce such as tomatoes, lettuce, and cantaloupes, has been associated repeatedly with food outbreaks connected to various *Salmonella* serovars, *Listeria monocytogenes*, and *Escherichia coli* O157:H7. In 2005 and 2006, four multistate outbreaks of *Salmonella* infections that were linked to the consumption of raw tomatoes in restaurants resulted in 450 confirmed cases in 21 states *(9).* A multistate outbreak of *E. coli* O157:H7 infections linked to romaine lettuce affected 58 people from nine states in 2012 *(10).*

Contamination of fresh produce can occur at any point in the food chain (production, harvesting, transportation, processing, or preparation in food service or home kitchens) *(23).* To maintain organoleptic characteristics, fresh produce is usually exposed to minimal processing, which increases the potential risk of contamination *(2).* Washing produce with tap water is recommended to reduce potential microbial contamination on the produce surface, but this technique cannot be relied on to remove pathogenic contamination completely *(6).* Therefore, the aim of this study was to determine the efficacy of a chemical wash treatment in reducing pathogens on the surface of green leaf lettuce and tomatoes.

Materials and Methods

Bacterial strains

Mixtures of five strains of each pathogen, isolated from different sources, were used as inocula. *Escherichia coli* O157:H7 isolates used in this study included RM 6069 and RM 5280 (associated with a 2006 spinach outbreak; clinical isolations), both strains of which were kindly provided by Dr. Robert Mandrell (USDA ARS, Albany, CA). *Escherichia coli* O157:H7 mixture also included ATCC 35150 (human feces isolation; Manassas, VA), ATCC 43895 (hemorrhagic

colitis outbreak from raw hamburger meat; Manassas, VA), and ATCC 43888 (human feces isolation; Manassas, VA).

Salmonella spp. strains, also provided by Dr. Robert Mandrell, included RM 33363 (serovar Poona), RM 6832 (serovar Newport), RM 2247 (serovar Baildon), RM 6825 (serovar Gaminara), and ATCC 13311 (*Salmonella* Typhimurium); these strains have been associated with produce outbreaks. All culture strains were maintained in tryptic soy agar (TSA; Difco; Flankin Lakes, NJ) slants and then transferred to tryptic soy broth (TSB; Difco; Flankin Lakes, NJ) prior to preparation of inoculum.

Inoculum preparation

For green leaf lettuce *E. coli* O157:H7 inoculum preparation, one loopful of each culture strain was used to inoculate 9 ml of TSB and each broth was incubated at 37°C for 24 h. The cocktail was prepared by mixing the five strains in a sterile beaker to deliver a final volume of 50 ml of inoculum with a final *E. coli* O157:H7 cell density of 7.86 $\log_{10}$ CFU/ml. For tomato *Salmonella* spp. inoculum preparation, 100 µl of each strain was used to inoculate 100 ml of TSB and then incubated at 37°C for 24 h. A five-strain cocktail was prepared by transferring 20 ml of each inoculated broth into a sterile 800-ml beaker containing 400 ml of sterile 0.1% peptone water (Bacto; Flankin Lakes, NJ) for a total inoculum of 500 ml with a final *Salmonella* spp. cell density of 9.39 $\log_{10}$ CFU/ml. Inoculum suspensions were maintained at 22 ± 2°C and applied to produce within 1 h of preparation.

Inoculation procedure

Unwashed green leaf lettuce and unwaxed ripe tomatoes were obtained from the Kansas State University Dining Services and local retail stores (Manhattan, KS). Produce was stored at 4 ± 1°C for no more than 2 days prior to inoculation, and samples were tempered at room

temperature (22 ± 2°C) prior to inoculation. Inoculum suspensions containing *E. coli* O157:H7 and *Salmonella* spp. were used to inoculate green leaf lettuce and tomatoes, respectively. Lettuce samples (25 ± 0.3 g, 2 leaves) were placed on a sterile surface in a biosafety cabinet, and 1 ml of the five-strain *E. coli* O157:H7 cocktail was spot-inoculated with a micropipettor onto 10 sites on the adaxial side of lettuce leaves. Tomato surfaces were inoculated by submerging tomatoes in *Salmonella* spp. suspension for 30 s. After inoculation, produce was allowed to dry for 1 h at room temperature to permit attachment of cells.

Washing procedures

Green leaf lettuce and tomatoes were washed separately with a chemical wash sanitizer (antimicrobial powder containing citric acid, sodium lauryl sulfate, sodium carbonate, magnesium carbonate, and grapefruit oil extract, pH 3.6 (HealthPro Brands Inc., Cincinnati, OH) or with cold tap water (as negative control, 22.4 ± 2°C, 0 ppm free chlorine, and 50 mg/l of Chloride ions) for three exposure times (30, 60, and 120 s), using a procedure simulating the sequence of steps (washing, rinsing, and drying) followed for preparing produce for consumption in a food service operation. For green leaf lettuce, chemical wash treatment was prepared according to manufacturer's directions by mixing 14 g antimicrobial powder with 4 l of cold tap water to achieve an antimicrobial concentration of 0.35% (HealthPro Brands Inc., Cincinnati, OH). For tomatoes, chemical wash treatment was prepared by mixing 28 g of antimicrobial powder with 8 l of cold tap water (0.35% antimicrobial concentration).

Two inoculated lettuce samples (25 ± 0.3 g per sample; 2 leaves per sample) or two inoculated whole tomatoes per treatment combination were washed by submerging/dipping and gently stirring the produce item in the chemical wash treatment or cold tap water for 30, 60, or 120 s. A disinfected metal colander was used to hold produce during washing. After application

of washing procedures, lettuce or tomato samples were rinsed with tap water. During the rinsing step, each lettuce leaf was held with sterile tweezers and 50 ml of tap water was dispensed with a pipettor onto the adaxial and abaxial side of each lettuce leaf. Each tomato was held using a disinfected metal colander and 100 ml of tap water was dispensed with a pipettor onto the tomato surface (tomatoes were rotated to ensure coverage of the entire surface). Produce was allowed to air dry for at least 5 min after rinsing prior to enumeration.

Sampling, enumeration, and enrichment procedures

E. coli O157:H7 and *Salmonella* spp. populations on treated leaf lettuce and tomatoes were determined. Lettuce and tomatoes from all treatment combinations were sampled within 10 min after washing procedures. Lettuce samples (25 ± 0.3 per sample; 2 leaves per sample) were transferred to a sterile stomacher bag; 225 ml of sterile 0.1% peptone water (Bacto; Franklin Lakes, NJ) was added to the bag, which was then stomached on medium speed for 1 min (Seward 400 Stomacher, Seward Limited; Worthing, Great Britain). Samples were serial diluted using 9 ml of 0.1% peptone water, and dilutions were surface-plated (0.1 ml) onto sorbitol MacConkey agar (Difco; Franklin Lakes, NJ) with cefixime tellurite supplement (CTSMAC; Oxoid Limited; Remel Inc., Lenexa, KS) for *E. coli* O157:H7 enumeration. In addition, non-inoculated samples to which 225 ml of *E. coli* enrichment broth (Difco; Franklin Lakes, NJ) was added and were incubated for 18 to 24 h at 37°C. After enrichment, 0.1 ml aliquot was plated onto CTSMAC to verify absence of *E. coli* O157:H7 in background flora of the sample.

Surface tissue samples from two whole tomatoes were removed with a sterile scalpel. The procedure consisted of cutting around a core mark (11.34 cm^2) and excising a circular area of tissue to a depth of 1.5 ± 0.5 mm. Each sample was placed in a sterile stomacher bag to which 30 ml sterile 0.1% peptone water (Difco; Franklin Lakes, NJ) was added, then stomached on

medium speed for 1 min. Samples were subsequently surface-plated (0.1 ml aliquots in duplicate or 0.25 ml aliquots in quadruplicate (totaling 1 ml)) onto xylose-lisine deoxycholate (XLD; Difco; Franklin Lakes, NJ) agar for *Salmonella* spp. enumeration. An additional surface tissue sample from treated and non-inoculated tomatoes had 30 ml of universal preenrichment broth (UPB; Difco; Franklin Lakes, NJ) added and were incubated for 24 h at 37°C. After enrichment, a 0.1 ml aliquot was plated onto XLD to test for *Salmonella* spp. presence or absence in the sample.

After washing treatments were applied, the residual water from wash solutions was sampled to determine the bacterial load transferred from produce to water. Samples were surface-plated (0.1 ml in duplicate and 0.25 ml in quadruplicate) onto CTSMAC and XLD media for enumeration of *E. coli* O157:H7 and *Salmonella* spp., respectively. The detection limits for lettuce and tomato residual water were 1.95 and 0.95 $\log_{10}$ CFU/ml, respectively.

Inoculated samples (n = 6) were surface plated onto CTSMAC and XLD media for enumeration of *E. coli* O157:H7 and *Salmonella* spp. attached to lettuce and tomato samples, respectively. Additionally, non-inoculated lettuce and tomato samples (n = 6) were prepared and plated onto TSA to estimate aerobic plate counts.

Statistical analysis

A split-plot design (replication day as the whole-plot blocking factor) with three replications was used to test the effectiveness of washing treatments in combination with exposure time on *E. coli* O157:H7 and *Salmonella* spp. populations in lettuce and tomatoes, respectively. Two samples of lettuce and two whole tomatoes within each treatment combination [washing solution × exposure time] and replication were collected to determine the effectiveness of the washing procedure, resulting in n = 6 per treatment combination, or 2 samples per each of

3 replications. Washing treatment and exposure time were considered whole-plot factors, and washing order of the two samples was the subplot factor. Data were analyzed using PROC MIXED in SAS version 9.2 (SAS institute, Cary, NC; Appendix A), with washing treatment, exposure time, and sample order being treated as fixed effects and replicate day and replicate day × washing treatment × exposure time treated as random. The 3-way (washing treatment× exposure time × sample order) and 2-way (exposure time × sample order, washing treatment× sample order, or washing treatment × exposure time) interactions were tested first at a significance level of 0.05, followed by tests of main effects. The appropriate corresponding least squares means were determined, and pairwise comparisons were conducted using Fisher's protected LSD. Mean $\log_{10}$ reductions and associated standard errors were estimated by contrasts of the washing treatment combination minus the inoculated samples at each trial.

Results

Non-inoculated samples

Enrichment of non-inoculated samples was performed for detection of *E. coli* O157:H7 and *Salmonella* spp. on the background flora of lettuce and tomato surfaces, respectively. Following 24 h of enrichment, none of the non-inoculated lettuce and tomato samples had *E. coli* O157:H7 or *Salmonella* spp. populations present. Mean aerobic populations for non-inoculated lettuce samples (n = 6) were ca. 5.3 $\log_{10}$ CFU/g, whereas mean aerobic populations for non-inoculated tomatoes (n = 6) were ca. 1.2 $\log_{10}$ CFU/cm^2.

Green leaf lettuce

Inoculated samples not treated with the washing treatments (n = 6) showed an *E. coli* O157:H7 mean population of ca. 7.75 ± 0.2 $\log_{10}$ CFU/g, and this value was used to estimate

$\log_{10}$ reductions. *E. coli* O157:H7 populations were not affected by 3- or 2- way interactions, exposure time, and sample order; however, populations were significantly affected by the chemical washing treatment (Table 3.1).

Table 3.1 *P*-values of the main effects and interaction effects for viable *E. coli* O157:H7 and *Salmonella* spp. after application of washing treatments

Effect	*P*-values	
	E. coli O157:H7 on green leaf lettuce	*Salmonella* spp. on tomatoes
Washing treatment	0.0131[a]	0.2410
Exposure time	0.4594	0.6764
Sample order	0.3981	0.4767
Washing treatment × Exposure time	0.5295	0.7259
Washing treatment × Sample order	0.3502	0.8748
Exposure time × Sample order	0.8793	0.1404
Washing treatment × Exposure time × Sample order	0.6731	0.5180

[a]Main and/or interaction effect was significant ($P < 0.05$)

Overall, *E. coli* O157:H7 population reductions in green leaf lettuce were greater ($P < 0.05$) for chemical washing treatment (2.95 $\log_{10}$ CFU/g) than for cold tap water washing (2.25 $\log_{10}$ CFU/g). Mean $\log_{10}$ reductions in green leaf lettuce washed with the chemical wash treatment for various exposures times ranged from 2.53 to 3.21 $\log_{10}$ CFU/g, whereas mean $\log_{10}$ reductions with cold tap water applied for the same exposure times ranged from 2.16 to 2.34 $\log_{10}$ CFU/g (Table 3.2).

Sampling of residual water solutions indicated that *E. coli* O157:H7-contaminated lettuce transferred the pathogenic load to regular tap water by 4.92 $\log_{10}$ CFU/ml. However, *E. coli* O157:H7 recovery from the chemical wash treatment residual water was below the detection limit of 1.95 $\log_{10}$ CFU/ml (Table 3.3).

Tomatoes

Inoculated tomatoes not treated with the washing treatments (n = 6) showed *Salmonella* spp. populations of ca. 3.55 ± 0.57 $\log_{10}$ CFU/cm^2. *Salmonella* spp. populations on the surface of tomatoes were not significantly (P > 0.05) affected by 3- or 2- way interactions (exposure time, sample order, and washing treatments). *Salmonella* spp. reductions of 2.50 $\log_{10}$ CFU/cm^2 were achieved for cold tap water and 2.96 $\log_{10}$ CFU/cm^2 for the chemical wash treatment (P > 0.05; Table 3.2). However, 16 out of 18 tomatoes washed with the chemical wash treatment had contamination levels below the detection limit (0.42 $\log_{10}$ CFU/cm^2), whereas only 8 out of 18 tomatoes washed with cold tap water had *Salmonella* spp. populations below the detection limit.

Table 3.2 Mean $\log_{10}$ reductions ± standard error in populations of *E. coli* O157:H7 on green leaf lettuce and *Salmonella* spp. on tomatoes after chemical wash treatment or cold tap water wash

Effect	Treatment	Exposure time (s)	*E. coli* O157:H7 $\log_{10}$ Reduction (CFU/g) [b]	*Salmonella spp.* $\log_{10}$ Reduction (CFU/cm^2) [c]
Main effects [a]	Cold tap water	-	2.25 ± 0.34^x	2.50 ± 0.49
	Chemical wash treatment	-	2.95 ± 0.34^y	2.96 ± 0.49
Interaction effect	Cold tap water	30	2.16 ± 0.41	2.47 ± 0.60
	Cold tap water	60	2.24 ± 0.41	2.26 ± 0.60
	Cold tap water	120	2.34 ± 0.41	2.78 ± 0.60
	Chemical wash treatment	30	3.11 ± 0.41	2.62 ± 0.60
	Chemical wash treatment	60	2.53 ± 0.44	3.13 ± 0.60
	Chemical wash treatment	120	3.21 ± 0.41	3.13 ± 0.60

[a] Data pooled for exposure time (30, 60, 120); n= 18.
[b] *E. coli* O157:H7 inoculated samples mean population was 7.75 ± 0.37 (SD) $\log_{10}$ CFU/g.
[c] *Salmonella* spp. inoculated samples mean population was 3.55 ± 0.57 (SD) $\log_{10}$ CFU/cm^2.
[xy] Means ± standard error (SE) with different superscripts within a column are significantly different ($P < 0.05$)

Samples with *Salmonella* spp. populations below the detection limit were enriched in UPB to verify the presence or absence of *Salmonella* spp. remaining on the surface of tomatoes after application of washing treatments. After 24 h of incubation, 15 of 18 (83.3 %) tomatoes treated with the chemical wash treatment tested positive for *Salmonella* spp., while all tomatoes (n = 18) treated with cold tap water tested positive for *Salmonella* spp.

Sampling of residual wash solutions resulted in recovery of 2.73 $\log_{10}$ CFU/ml of *Salmonella* spp. from the cold tap water solution and populations below the detection limit (0.95 log10 CFU/ml) for the chemical wash treatment (Table 3.3). Overall, the chemical wash treatment was slightly more effective in reducing the potential transmission of pathogens from inoculated tomatoes than the cold tap water wash was.

Table 3.3 Mean ± standard error *Escherichia coli* O157:H7 and *Salmonella* spp. populations recovered from residual water after wash treatments (n=9)

Produce	Pathogen	Treatment	Log_{10} CFU/ml
Lettuce	*E. coli* O157:H7	Cold tap water	4.92 ± 0.23
		Chemical wash treatment	< 1.95 DL[a]
Tomatoes	*Salmonella* spp.	Cold tap water	2.73 ± 0.25
		Chemical wash treatment	< 0.95 DL

[a] Detection limits (DL) for lettuce and tomato samples were 1.95 and 0.95 $\log_{10}$ CFU/ml, respectively.

Discussion

Green leaf lettuce

Velaquez et al. *(29)* studied the efficacy of 0.1 mg/ml benzalkonium chloride and 0.2% lactic acid against *E. coli* O157:H7 on lettuce. Bezalkonium chloride reduced *E. coli* O157:H7 by 1.71 $\log_{10}$ CFU/g, while lactic acid reduced *E. coli* O157:H7 by 0.4 $\log_{10}$ CFU/g. Keeratipibul

et al. *(15)* reported that lettuce leaves dipped for 10 min in 75 ppm hypochlorous acid and 50 ppm peracetic acid reduced *E. coli* by 1.3 and 2.5 $\log_{10}$ CFU/g, respectively. Ölmez *(21)* found that treatment of lettuce with 1.5 ppm aqueous ozone and a mixture of organic acids (0.25 % citric acid + 0.50 % ascorbic acid) for 2 min reduced *E. coli* by 1.19 and 1.40 $\log_{10}$ CFU/g, respectively. Various studies have reported that chlorine solutions to reduce *E. coli* by < 1 to 3 $\log_{10}$ CFU/g on lettuce. These results are highly dependent on inoculation method, method of application, exposure time, and free chlorine concentrations *(1,4, 15,16, 21)*. In some cases, reductions achieved by chlorine solutions were the same as reductions achieved by water alone *(4)*.

Similar reductions of *E. coli* O157:H7 on leaf lettuce were obtained in our study. Although reductions using different exposure times were not significantly different, it is recommended that the chemical wash treatment be used for 120 s to reduce microbial load from the lettuce surface and to reduce possible cross-contamination in the washing tank.

Tomatoes

Beuchat et al. *(7)* reported reductions (> 6.83 $\log_{10}$) of *Salmonella* populations on tomatoes when a prototype wash (containing citric acid and distilled grapefruit oil, among other ingredients) was applied. In a scaled-up study using the same commercial prototype wash, reductions in *Salmonella* were greater than those achieved with sterile water or Dey and Engley (D/E) broth *(14)*. In both studies, *Salmonella* reductions achieved by the prototype wash were obtained by sampling the rinse and residual wash solutions used to wash tomatoes.

In our study, *Salmonella* spp. and *E. coli* O157:H7 reductions (ca. 3 $\log_{10}$) were obtained by sampling the tissue/skin of each treated tomato or lettuce leaf. Therefore, it is difficult to compare the reductions obtained in our study to those obtained in these studies, because of

differences in treatment application and methods used for recovery of *Salmonella.* However, in our study, *Salmonella* counts in the residual wash (Table 3.3) were consistent with those reported by Beuchat et al. *(7)* and Harris et al. *(14)*, who reported *Salmonella* reductions in rinse and residual water 2 to 4 $logs_{10}$ greater than for controls (water and D/E broth), respectively.

Various studies have reported the efficacy of different sanitizers in reducing populations of *Salmonella* on the surface of tomatoes. Sapers et al. *(27)* reported 2.59 log_{10} reductions of *Salmonella* in tomatoes treated with 5% hydrogen peroxide at 60°C for 2 min. Long et al. (*17)* who investigated the efficacy of ozone washing systems in reducing *Salmonella* and *E. coli* on tomatoes, reported that ozone systems did not significantly reduce the pathogenic load attached on tomato surfaces, but ozone application did significantly reduce *Salmonella* and *E. coli* (> 1 log_{10}) in wash water. Wei et al. *(30)* and Zhuang et al. *(31)* reported *Salmonella* Montevideo reductions between 1 to 2 log_{10} for tomato skin dipped for up to 2 min in 60 to 350 ppm free chlorine solutions; however, *Salmonella* populations were not eliminated. These results are similar to the results obtained in the current study, in which *Salmonella* reductions were between 2 to 3 log_{10}.

Multiple studies have investigated the microbiological quality of produce. In Canada, two surveys testing over 600 lettuce samples reported generic *E. coli* populations that ranged from <1 to 3 log_{10} CFU/g *(3, 8)*. Moreover, two surveys in United States (U.S.) reported coliform counts from 1.5 to 4.1 log_{10} MPN/g for lettuce and 1.8 to 2.3 log_{10} MPN/g for tomatoes *(19, 20)*. Additionally, Mukherjee et al. *(20)* reported lettuce samples with generic *E. coli* populations of 2.2 to 2.4 log_{10} MPN/g. Despite the prevalence of *E. coli*, the serotype O157:H7 was not detected on any lettuce samples *(3, 8, 19, 20)*. In various surveys of retail markets of United Kingdom (428 samples), Canada (120 samples) and the U.S. (108 samples), *Salmonella* was not isolated

from tomato samples *(8, 19, 25)*. However, in a survey in Canada that tested Roma tomatoes (148 samples), one sample tested positive for *Salmonella* spp; however, although *Salmonella* spp. was detected, the population recovered from the sample was not reported *(3)*.

If the initial population of *E. coli* O157:H7 and *Salmonella* spp. in a naturally contaminated fresh produce is ≤ 3 log, reduction levels (ca. 3 $\log_{10}$) obtained with the chemical wash treatment for both *Salmonella* and *E. coli* O157:H7 may reduce the risk of foodborne illnesses. This might be applicable for produce (lettuce and tomatoes) exposed to contamination prior to being washed with this product. However, it is important to note that this treatment might not be able to ensure produce safety if pathogens are present in populations > 3 logs on the surface or internalized in produce. Contamination can occur at numerous points along the farm-to-table food chain because produce is grown in open fields, handled by humans or automatized equipment prior, during, and post harvest, and eaten raw *(18)*. To reduce contamination of produce, multiple interventions (i.e., Good Agricultural Practices, GAP; Good Manufacturing Practices, GMPs; and Sanitation Standard Operating Procedures, SSOPs) at different points of the food chain (i.e., field production, harvesting, transportation, processing, or preparation in food service or home kitchens) need to be implemented.

Limitations of the effectiveness of the washing treatments used in our study may be the result of the specific surface characteristics of the produce (i.e., green leaf lettuce irregular surface, unwaxed or waxed tomatoes), time interval between inoculation and treatment, strong attachment of the pathogens to inaccessible sites, biofilm formation, and background microflora *(26)*. However, our observations indicate that using the chemical wash treatment during the washing procedure will reduce foodborne pathogens on the surface of produce and also reduce cross-contamination that occurs when new produce is introduced into a washing tank.

Conclusions and Recommendations

Data from this study expands knowledge of the chemical wash treatment as an alternative for produce decontamination and its potential value for preventing cross-contamination during produce washing. Overall, application of the chemical wash treatment was capable of reducing *E. coli* O157:H7 and *Salmonella* spp. by about 3 $\log_{10}$ units on the surface of green leaf lettuce and tomatoes, and post-treatment residual water with the chemical wash treatment contained populations below detection limits. Application of the chemical wash treatment (0.35%) by immersing the produce in the wash solution and gently stirring for 2 min, followed by rinsing with tap water, represents a potential intervention strategy for reducing pathogens on green leaf lettuce and tomato surfaces and in the wash water. However, further research exploring different microorganisms, levels of initial contamination, time intervals between produce inoculation and treatment application, application methods, and different antimicrobial concentrations are advisable to determine the effectiveness of the antimicrobial solution under different conditions.

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Chapter 4
Investigation of handling practices for fresh produce and the efficacy of commercially available produce washes on removal of pathogens and natural microflora from whole cantaloupe surfaces

Abstract

A survey (Phase I) of school foodservice employees was conducted between June and July 2014. The survey consisted of 23 questions, exploring four different variables related to produce washing: (1) identification of personnel responsibilities; (2) equipment in facilities for washing produce; (3) produce washing practices; and (4) produce storage practices. Based on results obtained from the survey, two more phases were conducted to evaluate the efficacy of commercial washes for reducing pathogens and natural microflora on whole cantaloupes.

In Phase II, cantaloupes were washed with water (control), 9% vinegar solution, or a commercial antimicrobial for fruit and vegetables (CAFVT) for 2 min by using a continuous water motion system. Surface of treated and untreated cantaloupes were tested on day 0 for initial aerobic plate counts (APC) and then cut into wedges or cubes and kept in refrigeration storage for enumeration of APC on days 1, 3, and 6. In Phase III, cantaloupes were inoculated with *Salmonella* spp. (8.54 $\log_{10}$ CFU/ml) or *Listeria monocytogenes* (8.52 $\log_{10}$ CFU/ml) cocktails, dried for 1 h, washed with cold tap water (control) or a commercial produce wash (CPW) at various exposure times (30, 60, and 120 s), and then rinsed with tap water. Samples were plated on selective media. The trials were replicated five times for *Salmonella* spp. inoculated cantaloupes and three times for *L. monocytogenes* inoculated cantaloupes.

For Phase I results, a significant number of respondents in this study were employees in school cafeterias (61%). Commonly used produce included: carrots, tomatoes, cantaloupes, romaine lettuce, green leaf lettuce, and other fruits. Respondents indicated using antimicrobial

washes (10%) and 31% indicated using washing sinks to wash fruits and vegetables. Over 31% of respondents indicated they store prepared produce in plastic containers with lids for 1 day or up to 7 days. Among all 51 respondents, 39% indicated to be "well trained" to correctly wash and prepare fruits and vegetables. Respondents highlighted the lack of sinks dedicated for produce washing and preparation.

In Phase II, day 0 APC populations for surface of untreated cantaloupes were 3.88 $\log_{10}$ CFU/cm^2, whereas populations for cantaloupes treated with water, 9% vinegar solution, or CAFVT were 3.39, 3.01, and 2.98 $\log_{10}$ CFU/cm^2, respectively. Wedges and cubes from cantaloupes washed with 9% vinegar solution showed the lowest APC populations after day 1, 3, and 6 of storage. Populations for wedges from all other treatments reached over 7 $\log_{10}$ CFU/g on day 6 of storage, while populations for cubes from untreated and CAFVT cantaloupes reached over 8 $\log_{10}$ CFU/g on day 6 of storage.

In Phase III, the CPW was capable of reducing ca. 1.26 and 1.12 $\log_{10}$ CFU/cm^2 of *Salmonella* spp. and *L. monocytogenes* populations, respectively, on the surface of cantaloupes. Pathogenic populations for residual wash water were reduced below the detection limit of 1.95 $\log_{10}$ CFU/ml.

Keywords: Produce, Handling, Washing, *Salmonella*, *Listeria*

Introduction

Changes in life style and the awareness of health benefits have markedly increased the demand and consumption of fruits and vegetables (Bruhn, 2009). However, along with this increase in consumption, produce-related outbreaks and illnesses have been recognized worldwide (Lynch, Tauxe, & Hedberg, 2009).

In the United States (U.S.), melons, especially cantaloupes, have been associated with foodborne illness outbreaks linked to *Salmonella* serovars and *Listeria monocytogenes*. In 2012, a multistate outbreak of salmonellosis resulted in a total of 261 ill persons with outbreak strains of S. Typhimuriun (228) and S. Newport (33), with 3 deaths reported in the state of Kentucky (CDC, 2012). During 2011, multistate outbreaks of listeriosis and salmonellosis (S. Panama) were linked to cantaloupe consumption. The listeriosis outbreak involved 147 illnesses, 33 deaths, and 1 miscarriage in 28 states, while salmonellosis outbreaks resulted in only 20 illnesses (CDC, 2011a,b). Moreover, in 2008 another salmonellosis outbreak (S. Litchfield) involved 51 ill persons in 16 states in the U.S. and 9 illnesses in Canada, no deaths were reported (CDC, 2008).

Fresh produce, including cantaloupes, can become contaminated with pathogenic microorganisms at any point along the farm-to-table food chain (e.g. production, harvesting, packing, processing, and foodservice handling). Washing plays an important role on fresh produce quality and safety. Washing procedures are used mainly to remove soil, chemical residues (i.e, pesticides), and other debris from the surface of produce. Washing procedures and sanitizing agents are of concern because inadequate handling can result in produce damage, cross-contamination, and chemical and/or microbial contaminant internalization (Park, Gray, Oh, Kronenberg, & Kang, 2008; Pao, Long, Kim, & Kelsey, 2012). Methods to reduce microbial

contamination on the surface of produce usually involve the use of sanitizers and mechanical action.

Studies exploring the efficacy of various washing treatments including antimicrobial chemicals such as hydrogen peroxide (Ukuku, 2006), peracetic acid (Rocha-Bastos, Ferreira-Soares, Andrade, Arruda, & Alves , 2005), nisin and its combination with EDTA, sodium lactate, and potassium sorbate (Ukuku & Fett, 2004), plant antimicrobial extracts (Upadhyay, Upadhyay, Mooyottu, & Kollanoor-Johny, 2014), or hot water surface pasteurization (Fan, Annous, Beaulieu, & Sites, 2008; Ukuku, 2006) in reducing pathogenic microorganisms on cantaloupes have yielded mixed results. However, washing treatments applied by immersion in the washing solutions with or without physical action (e.g. scrubbing or agitation) reduced attachment of pathogenic microorganisms on cantaloupes surface by 2 to 4.9 $\log_{10}$ CFU units. It is important to note that as the time interval between contamination and washing treatment application increases, washing treatment efficacy decreases (Gil, Selma, Lopez-Galvez, & Allende, 2009; Sapers, 2001).

The incidence of food-related outbreaks in school settings and the effort to improve availability of fruits and vegetables in meals offered to young children prompt the need to develop interventions and prevention strategies for handling produce at the school level. Reported foodborne disease outbreaks in schools have been analyzed to identify etiology, mode of transmission, number of affected children, morbidity and mortality, and strategies of prevention (Daniels et al., 2002; Venuto, Halbrook, Hinners, Lange, & Mickelson, 2010; Lee & Greig, 2010). Because limited information is known about fruit and vegetable handling practices in schools, and such information is imperative for the development and implementation of produce intervention strategies, there is a need to conduct research to examine produce-handling

practices in school foodservice facilities. Therefore, the objective of this study was to collect descriptive data of handling practices for fresh produce used in foodservice facilities. The secondary objective was to evaluate the efficacy of commercially available washes for reducing pathogens (*Salmonella* spp. and *Listeria monocytogenes*) and natural microflora on whole cantaloupes based on information provided for washing and storing of cantaloupes.

Materials and Methods

Investigation of handling practices for fresh produce (Phase I)

Questionnaire

The development of the questionnaire consisted of two phases. First, personnel of a large foodservice facility were interviewed to gather information for the procedures used to wash, prepare, and store produce (e.g. lettuce, tomatoes, and cantaloupes), then personnel were asked to demonstrate practices and observational data was collected. Utilizing this information, a draft of the questionnaire was prepared and then reviewed by researchers at the Center of Excellence for Food Safety Research in Child Nutrition Programs at Kansas State University, Manhattan, Kans., who work closely with school foodservice personnel. Appropriate modifications were made to assess food safety practices of interest. The final questionnaire was designed to ensure respondents were able to navigate the survey and respond quickly, containing multiple choice and ranking questions. The questionnaire comprised a total of 23 questions (Appendix B and Appendix C), which were grouped into four different categories/sections: (1) identification of personnel responsibilities; (2) equipment in facilities for washing produce; (3) produce washing practices; and (4) produce storage practices.

Sample selection and data collection

Through the collaboration of the Food Science Institute and the Center of Excellence for Food Safety Research in Child Nutrition Programs at Kansas Sate University and the Institute of Child Nutrition at the University of Mississippi, Oxford, Miss., the questionnaires were provided to foodservice employees in the states of Kansas and Mississippi attending a state workshop. Participation of the employees was voluntary and anonymous. Data were collected from June to July 2014.

Data analysis and further research

Descriptive statistics were used to assess participants' responses. Microsoft® Excel® (Excel:mac ^{2011},version 14.4.6) was used to arrange data, obtain frequencies, calculate medians and percentages, and to depict results in graphs and tables.

Results of Phase I provided information regarding the processing to which produce is subjected before it is served in school cafeterias. For example, the questionnaire results identified that 10% of the foodservice facilities, under the study, used antimicrobial products to wash produce (e.g. FIT, Eat Cleaner) while 53% used tap water. It was also identified that approximately 20% of respondents kept fresh-cut produce (e.g. prepared shredded lettuce, sliced tomatoes, and cubed or wedged cantaloupes) in refrigeration storage for 1 day while approximately 6% kept the prepared produce for 3 days; and produce was usually stored in plastic containers with lids. Remaining results from the survey are further discussed in the results and discussion section of this chapter.

Produce, such as cantaloupes, have been identified as the food vehicle for salmonellosis and listeriosis outbreaks (CDC, 2015). These outbreaks have stressed the need to investigate disinfectant agents for their effectiveness in reducing populations of microorganisms in produce. Thus, the information obtained in Phase I was used to develop research studies defined in Phase

II and Phase III with the purpose of determining efficacy of washing techniques for reducing pathogens (*Salmonella spp.* and *Listeria monocytogenes*) and natural microflora on whole cantaloupes surfaces.

Efficacy of washing treatments on natural microflora of whole cantaloupes (Phase II)

Experimental design

Cantaloupes from the same provider and production lot were purchased from a local retail store in Manhattan, Kans. Each item was inspected to ensure absence of bruises or lacerations on the surface of the produce. Cantaloupes were washed separately with water (control), a solution of vinegar, and a commercial antimicrobial fruit and vegetable treatment (CAFVT). A whole unwashed and untreated cantaloupe was used to determine initial microflora load. After washing, each cantaloupe was manually cut in half. One half was cut into four visually equal-sized wedges (slices) with the rind intact and the remaining half was cut into cubes with rinds removed carefully. Then cantaloupes were stored in plastic containers with lids at 4 ± 1°C for 6 days. Treated and untreated cantaloupes were tested after washing treatment on day 0 and on day 1, 3, and 6 of storage. Two replications were conducted, and samples of each treatment were analyzed in duplicate. The average was used for statistical analysis.

Washing and storage procedures

Cantaloupes were washed separately with tap water (pH = 9.7; free chlorine = 2.78 ppm) which was used as control, a 9% vinegar solution containing 0.45% acetic acid (pH = 3.02) which was prepared by mixing 12 L of distilled white vinegar (5% acetic acid; The Kroger Co., Cincinnati, Ohio) with 120 L of tap water, and a commercial antimicrobial fruit and vegetable treatment (CAFVT; pH = 2.82; containing lactic acid (1,061 – 1,391 ppm), sodium hydrogensulfate, docecylbezesulfonic acid (76 – 111 ppm); Ecolab, St. Paul, Minn.) for 120 s by

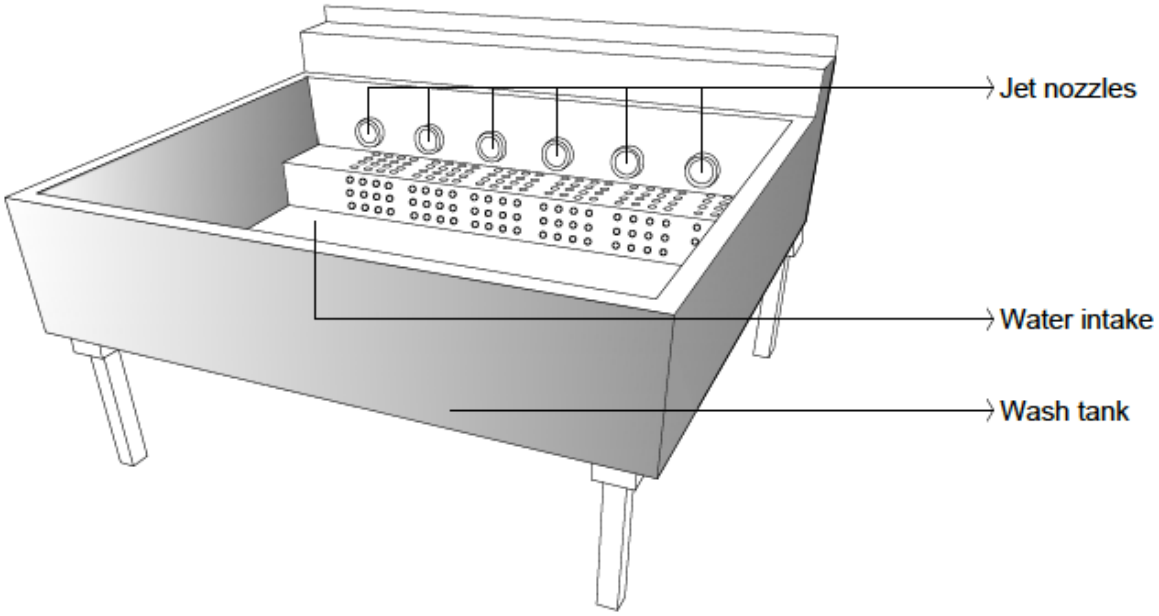
using a continuous water motion system (Model 50PSP66L2B1; Produce Soak by Power Soak Systems, Kansas City, Mo.). Temperature of water used to prepare washing treatments was 18 ± 1°C.

The continuous water motion washing system consisted of a stainless steel two bay wash tank (ca. 150 L), a stainless steel self-draining parallel flow pump, a pump motor connected to the wash tank, water inlet holes which run full length of the back wall of the wash tank, and six low profile wash jets (each bay with 3 low profile jets; average flow rate ca. 10 gpm per jet) located above wash pump inlet holes (Figure 4.1 and Figure 4.2). During the washing operation, the pump located on a side of the wash tank was fed with water in a first direction via a pump inlet connected to an intake port passing through the right side wall of the wash tank, and then water was impelled out from the pump in a second direction substantially parallel to the first direction via a pump outlet connected to an outlet chamber and wash jets (AU Patent No. 2002335694).

After application of washing treatments, the surfaces of cantaloupes were tested for microbial enumeration (day 0). To investigate the effect of washing treatment over storage time, each cantaloupe was manually cut in half and its seeds removed using a sterile knife. One half was cut into four visually equal-sized wedges with the rind intact. The remaining half was further cut into cubes (ca. 2 × 2 cm) with rinds removed carefully. Cantaloupes were stored in plastic containers (184 fl. oz; Polypropylene (PP); Snapware®, Mira Loma, Calif.) at 4 ± 1°C for a total of 6 days and samples of wedged and cubed cantaloupes were separated and tested on days 0, 1, 3, and 6.

Figure 4.1 Front view of the continuous water motion washing system, a) schematic, b) actual washing system, and c) bottom of washing tank

a)

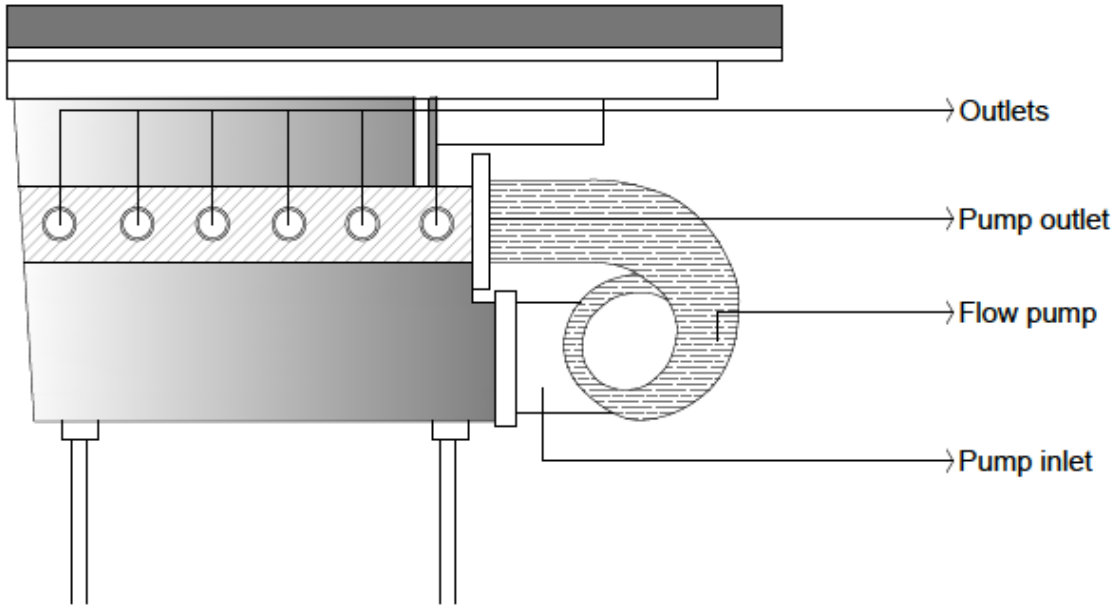


b)

c)

Figure 4.2 Back view of the continuous water motion washing system, a) schematic, b) back of wash tank, and c) pump and pump motor

a)



b)

c)

Sampling and enumeration procedures

Populations of cantaloupe native microflora on treated and untreated cantaloupes were determined. For day 0 sampling only, a sterilized stainless steel cork-borer (ø = 3.8 cm) was used to randomly mark a total of five rind plugs per cantaloupe, then the rind plugs were removed with a sterile scalpel. The procedure to remove the plugs consisted of cutting around the core-borer mark and excising the circular area of rind tissue to a depth of 1 ± 0.5 mm, resulting in a composite sample (56.7 cm^2). The composite sample was placed in a sterile filtered stomacher bag (177mm × 305mm; Fisher Scientific, Pittsburgh, Pa.) in which 50 ml of sterile 0.1% peptone water (Difco; Franklin Lakes, N.J.) was added and then stomached on medium speed for 1 min (Seward 400 Stomacher, Seward Limited; Worthing, Great Britain) and subsequently serially diluted by using 9 ml of 0.1% peptone water blanks. For all other sampling days (1, 3, and 6), pieces of cubed and wedged cantaloupe from each washing treatment were selected and cut with a sterile knife to obtain 30 ± 0.3 g samples.

When preparing wedged cantaloupe samples, it was ensured that each sample (30 ± 0.3 g) included the rind portion attached to the fruit flesh. Then samples were transferred to a sterile stomacher bag in which 300 ml of sterile 0.1% peptone water was added and then stomached on medium speed for 1 min and subsequently serially diluted by using 9 ml of 0.1% peptone water blanks. All samples were surface plated (0.25 ml aliquots in quadruplicate or 0.1 ml aliquots in duplicates) onto tryptic soy agar (Difco; Flankin Lakes, N.J.) and incubated at 37°C for 18 to 24 h.

Statistical analysis

Microbial data (CFU/g) were analyzed after log transformation. The experiment followed a randomized complete block (replication as block factor) with a split-split plot design. Data was analyzed using the PROC GLIMMIX procedures with NOBOUND option of SAS version 9.4,

(SAS Institute, Cary, N.C.; Appendix D). Washing treatments (washed whole cantaloupes) were considered as whole plot factors, and shape type (wedged or cubed cantaloupe pieces) and microbial counts over time were considered as subplot factor and sub-subplot factors, respectively. Appropriate interactions were tested first at a significant level of 0.05, followed by test of main effects. The SLICEDIFF option was used to explore the differences in the level of one effect inside the levels of other effect. Then, appropriate corresponding least squares means were determined and pairwise comparisons were conducted using Bonferroni's adjustment.

Effectiveness of a commercially available fruit and vegetables wash for reducing pathogens on whole cantaloupes (Phase III)

Experimental design

For phase III trials, whole cantaloupes were inoculated with either a five-strain cocktail of *Salmonella* spp. or a three-strain cocktail of *L. monocytogenes*. Cantaloupes were washed separately with tap water (as control) and a commercial produce wash (CPW) at various exposure times (30, 60, or 120 s). *Listeria monocytogenes* inoculated cantaloupes were treated with the commercial produce wash and tap water for only 120 s exposure time. The trials were replicated five times for *Salmonella* spp. inoculated cantaloupes and three times for *L. monocytogenes* inoculated cantaloupes.

Bacterial strains

Mixtures of each pathogen isolated from different sources were used as inocula. *Salmonella* spp. strains used in the study included RM 33363 (serovar Poona), RM 6832 (serovar Newport), RM 2247 (serovar Baildon), RM 6825 (serovar Gaminara), and ATCC 13311 (*Salmonella* Thyphimirum, Manassas, Va.); these strains have been associated with produce outbreaks. *Listeria monocytogenes* strains included RM 3818 (associated with cantaloupes

outbreak), ATCC 19115 (serotype 4b, human isolate, Manassas, Va.), ATCC 19118 (serotype 4e, chicken isolate, Manassas, Va.), and SLR-2249 (laboratory strain with the *ActA* gene removed, St. Cloud, Minn.). All RM strains were kindly provided by Dr. Robert Mandrell (USDA ARS, Albany, Calif.).

Inoculum preparation

For inocula preparation, one loopful of each culture strain was used to inoculate 9 ml of tryptic soy broth (TSB; Difco; Flankin Lakes, N.J.) and incubated at 37°C for 24 h. A final transfer of 0.5 ml was made into 30 ml of TSB, which was incubated at 37°C for 18 to 24 h. Cells of each strain were collected by centrifugation (4,960 × g for 15 min; JA-17 rotor, Model J2-21 M/E; Beckman Coulter, Inc., Pasadena, Calif.) at 4°C. The cell pellets were then resuspended in 30 ml of sterile 0.1% peptone water (Difco; Franklin Lakes, N.J.), and transferred into a small plastic vial equipped with an atomizer to form a mixed strain cocktail. The same procedures were used for the preparation of *Salmonella spp*. and *L. monocytogenes* cocktail inoculums. The cell density of *Salmonella spp*. and *L. monocytogenes* cocktail inoculums was 8.54 and 8.52 $\log_{10}$ CFU/ml, respectively, as determined by plating serial dilutions onto xylose-lysine deoxycholate (XLD; Difco; Franklin Lakes, N.J.) for *Salmonella* spp. or modified Oxford medium (MOX; Difco; Franklin Lakes, N.J.) for *L. monocytogenes*, with incubation at 37°C for 24 h. The inoculum was maintained at 22 ± 2°C and applied to produce within 1 h of preparation.

Procedure of inoculation

Cantaloupes from the same provider and lot were obtained from the K-State Dining Services and local retail stores in Manhattan, Kans. Cantaloupes were stored at 4 ± 1°C for no more than 24 h prior to inoculation; before inoculation, samples were tempered at room temperature (22 ± 2°C). Inside a biosafety cabinet, a fine mist of the inoculum (ca. 8 - 10 ml per

eight or ten full sprays, respectively) was sprayed onto the cantaloupe's surface using a plastic bottle with an atomizer (8 oz, high-density polyethylene (HDPE), The Bottle Crew, West Bloomfield, Mich.). To assure for complete inoculum coverage, cantaloupes were rotated by using a glove-covered hand. After inoculation, cantaloupes were allowed to dry inside the biosafety cabinet for 1 h to permit cell attachment. The same procedure was repeated for all cantaloupes inoculated either with *Salmonella* spp. or *L. monocytogenes*.

Washing procedures

Cantaloupes inoculated with *Salmonella* spp. as described above were washed separately with the commercial produce wash (citric acid, sodium lauryl sulfate, sodium carbonate, magnesium carbonate, and grapefruit oil extract; HealthPro Brands, Cincinnati, Ohio; pH= 3.6) or cold tap water (pH = 9.4; free chlorine = 2.78 ppm; 20 ± 2°C; Chloride = 50 ppm) for three different exposure times (30, 60, and 120 s), while cantaloupes inoculated with *Listeria monocytogenes* were washed with the commercial produce wash and cold tap water (20 ± 2°C) for 120 s. The commercial fruit and vegetables wash treatment was prepared by mixing the produce wash product in powder form (containing citric acid, sodium lauryl sulfate, sodium carbonate, magnesium carbonate, and grapefruit oil extract; 28 g) with 8 L of cold tap water according to the manufacturer's directions (HealthPro Brands, Cincinnati, Ohio). Washing treatments were applied by submerging the cantaloupes under the surface of the wash solutions and stirring with a sterile L spreader to ensure for complete coverage and contact of cantaloupe's surface with wash solution. A metal colander disinfected with 70% ethanol (Ethanol 200 proof, Decon Laboratories, INC., King of Prussia, Pa.) was used to hold cantaloupes during washing. After the treatment application, cantaloupes were rinsed with tap water (1 L per unit) and then allowed to dry for 30 min before sampling.

Sampling and enumeration procedures

A sterilized stainless steel cork-borer (ø = 3.8 cm) was used to randomly mark a total of five rind plugs per cantaloupe, then rind plugs were removed with a sterile scalpel. The procedure to remove the plugs consisted in cutting around the core-borer mark and excising the circular area of rind tissue to a depth of 1 ± 0.5 mm, resulting in a composite sample (56.7 cm^2). The composite sample was placed in a sterile filtered stomacher bag (177mm × 305mm; Fisher Scientific, Pittsburgh, Pa.) and 30 (for *Salmonella* spp. samples) or 50 (for *L. monocytogenes* samples) ml sterile 0.1% peptone water was added to the bags which were then stomached on medium speed for 1 min (Seward 400 Stomacher, Seward Limited; Worthing, Great Britain). Samples were serially diluted by using 9 ml of 0.1% peptone water, and then surface plated (0.1 ml) onto XLD media for *Salmonella* spp. recovery or MOX media for *Listeria monocytogenes* recovery. Additionally, non-inoculated cantaloupes were sampled, using the same procedure, for standard aerobic plate counts. Samples were serially diluted and plated onto tryptic soy agar and incubated at 36°C for 24 h to estimate aerobic plate counts.

Statistical analysis

For phase III trials, a randomized complete block design (RCBD, with replication as block factor) was used to test the effects of washing treatments in combination with exposure time on reducing *Salmonella* spp. populations and a generalized RCBD with repetition day as block factor was used to test the effects of washing treatments on reducing *Listeria monocytogenes* populations. Data sets were analyzed using PROC MIXED in SAS version 9.4 (SAS institute, Cary, N.C.; Appendix E) with washing treatment and exposure time being treated as fixed effects. When pertinent, two-way interactions were tested first at a significant level of 0.05, followed by a test of main effects. The appropriate corresponding least squares means were determined and pairwise comparisons were conducted using Fisher's protected LSD. Mean $\log_{10}$

reductions and associated standard errors were estimated from contrasts of the treatment combination minus the inoculated samples at each trial.

Results and Discussion

Investigation of handling practices for fresh produce (Phase I)

Foodservice personnel identification

A total of 51 people responded to the survey: 61% (31) were school cafeteria employees, 8% (4) worked for a State Agency, and 2% (1) corresponded to USDA personnel. Additionally, 29% (15) of the respondents answered the questionnaire based on observed practices in foodservice facilities as food safety consultants, School Food Authority (SFA), board of education member, school district office-clerk, hospital foodservice, or private school foodservice.

Type of produce

Respondents were asked to indicate all types of fruits and vegetables used in school facilities. Ninety-two percent (47 of 51) of respondents indicated using whole fresh vegetables in their facilities, 82% (42) reported using pre-prepared vegetables, only 10% (5) reported to use fruits and vegetables in other forms (e.g., canned, bulk packaged), 6% (3) reported not using vegetables in their facilities, and 6% (3) did not respond to this question.

Produce washing, preparation, and storage

Fifty-three percent of respondents (27) reported using cold tap water to wash fresh fruit and vegetables, 31% (16) indicated using a washing sink with or without antimicrobial solutions, 10% (5) indicated using antimicrobial products, 4% (2) did not respond to this question, and 2% (1) indicated using other methods. Among the 10% of respondents that indicated using an

antimicrobial solution, it was specified that they used antimicrobials available in the market such as FIT fruit and vegetable wash, Eat Cleaner fruit and vegetable wash, and a generic fruit and vegetable wash.

Questions regarding storage practices after washing of produce showed that 49% (25) of the respondents stored whole fruits and vegetables for next day preparation and consumption. Respondents specified whole fruits and vegetables washed and stored for next day preparation or consumption included: carrots (80%), tomatoes (72%), cantaloupes (64%), romaine lettuce (60%), green leaf lettuce (52%), and others (48%; apples, oranges, bananas, kiwi, grapes, berries, stone fruit, pears, and plums). Forty-three percent (22) reported that their facility does not store whole fruits and vegetables for next day consumption after washing, and 8% (4) did not respond to this question.

Unwashed whole fruits and vegetables were stored for different time periods, 43% (22) of respondents indicated storing unwashed whole fruits and vegetables for up to 7 days, 22% (11) of respondents indicated storing fruits and vegetables for 1 day (or overnight for use the following day), 10% (5) for 6 days, 8% (4) for 3 days, 8% (4) for 2 days, 8% (2) indicated this did not apply to their facility, and 2% (1) did not respond to this question.

Respondents were also asked to identify the type of containers used in their facilities to store fruits and vegetables. Seventy-three percent of respondents (37) indicated they stored fruits and vegetables in plastic containers with lids, 14% (7) used baking sheets covered with plastic bun bags, 6% (3) used baking sheets with racks and covered with plastic bun bags, and 27% (14 of 51) of the respondents reported other means to store fruits and vegetables such as either 4 or 6” steam table pans with or without clear plastic wrap, plastic container without lids, plastic bags, fruit bowls, and boxes or original packaging.

The fruits and vegetables of main focus for our research were cantaloupes, green leaf lettuce, and tomatoes due to their association with foodborne outbreaks in the past (Ackers et al., 1998; Behravesh et al., 2012; CDC, 2011a,b; Taylor et al., 2010; Walsh, Bennet, Mahovic, & Gould, 2014). Therefore, respondents were given a list of prepared fruits and vegetables of interest and asked to indicate if the described fruits and vegetables were prepared in their facilities. Respondents also were asked to indicate for how long and what means were used to store the prepared fruits and vegetables.

Seventy-eight percent (40 of 51) of respondents indicated preparing both sliced tomatoes and leaf lettuce for sandwiches, 59% (30) diced tomatoes, 51% (26) both shredded lettuce and diced cantaloupes, 33% (18) cantaloupe wedges with rind, 22% (11) cantaloupe wedges without rind, 19% (10) other fruits such as oranges, watermelon, melons, kiwi, berries, peaches, and grapes. Among all respondents only 8% (6) of respondents did not answer the question. The prepared produce was reported to be stored for 1 day up to 7 days. Overall, greater than 31% of respondents indicated storing all the prepared produce in plastic containers with lids for next day preparation or consumption (Table 4.1).

Practices identified as factors contributing to outbreaks in school settings as reported by other authors include inadequate handling and improper refrigeration (Daniels et al., 2002; Richards et al., 1993). Within school environments, lunches are prepared using four main production systems: 1) full service or independent kitchens, which prepare and serve all food at the school in which it is located; 2) mostly on-site production kitchens, which prepare and serve food at the school located, but also send food or meals to other schools under the same school

Table 4.1 Type of fruits and vegetables prepared in foodservice facilities and different storage type[1] and length of storage used by produce type.

[a]Response of participant No. (%)	Cubed Cantaloupe	Cantaloupe wedges with rind	Cantaloupe wedges without rind	Sliced tomatoes	Diced tomatoes	Shredded lettuce	Leaf for sandwich
What fruits and vegetables do you prepare in your facility?							
	26(51)	18(33)	11(22)	40(78)	30(59)	26(51)	40(78)
How does your facility store prepared fresh fruits and vegetables?							
PCWL	29(57)	16(31)	18(35)	35(69)	30(59)	25(49)	31(61)
BSCPB	1(2)	2(4)	0	1(2)	0	1(2)	5(10)
BSRCPB	0	0	1(2)	1(2)	1(2)	1(2)	4(8)
Other	7(14)	6(12)	5(10)	5(10)	7(14)	15(29)	9(18)
N/A	14(27)	26(51)	24(47)	8(16)	13(25)	9(18)	7(14)
After preparation and serving, how long would you store fresh fruits and vegetables?							
1 day	20(39)	16(31)	16(31)	22(43)	-	19(37)	31(61)
2 day	6(12)	3(6)	3(6)	10(20)	-	7(14)	4(8)
3 day	6(12)	5(10)	5(10)	5(10)	-	10(20)	6(12)
6 day	1(2)	0	1(2)	1(2)	-	1(2)	0
Up to 7 days	1(2)	1(2)	2(4)	2(4)	-	1(2)	2(4)
N/A	15(29)	22(43)	21(41)	9(18)	-	11(22)	6(12)

[a] N= 51. Respondents could choose more than one answer; thus the total percentage adds to more than 100.

[1] PCWL= Plastic containers with lids; BSCPB= Baking sheets covered with plastic bun bags; BSRCPB= Baking sheets with racks and covered with plastic bun bags; Other included= 4 or 6" steam table pans with or without clear plastic wrap, plastic container without lids, plastic bags, fruit bowls, and boxes or original packaging; NA= not apply.

foodservice account; 3) base or central production kitchens, which prepare and ship food or meals for other schools, either in bulk or pre-portioned, kitchen typically not located in a school; and 4) finishing or satellite kitchens, which receive food or meals from central/base production kitchens, food or meals require minimal preparation to be served (USDA-FNS, 2008).

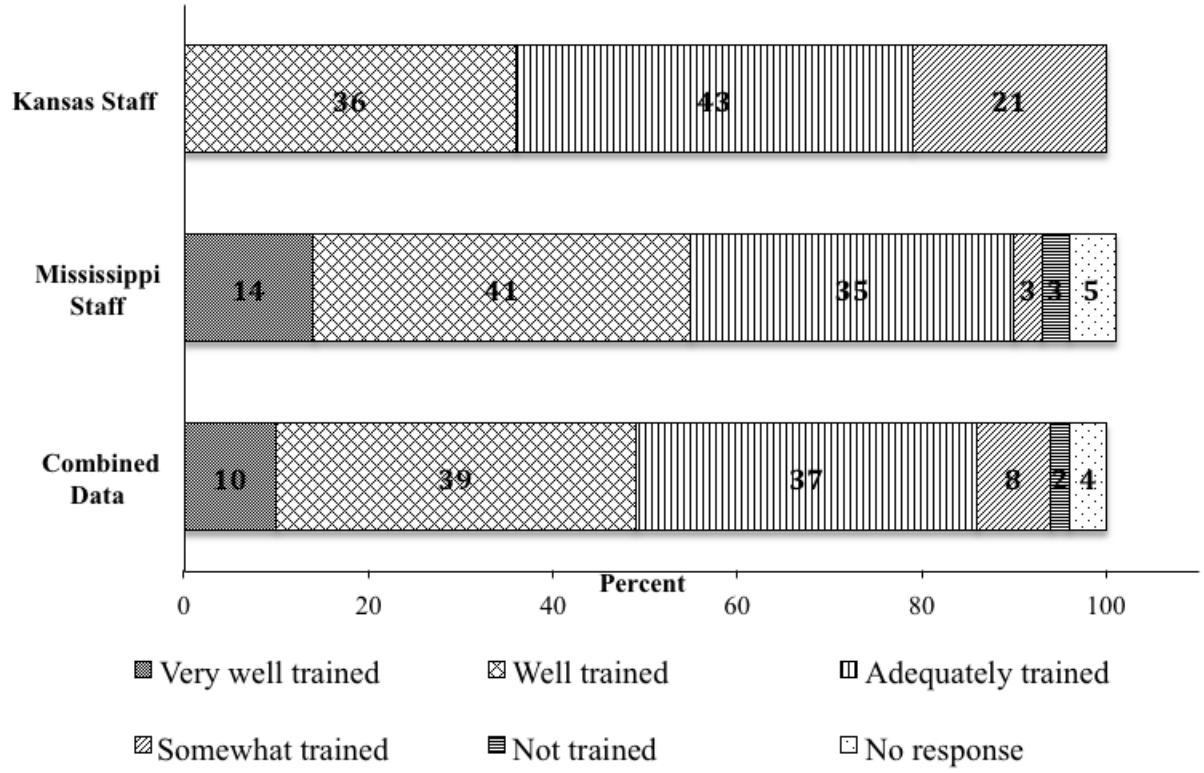
Inadequate employee manipulation of foods increases the potential for cross-contamination. For example, a staphylococcal food poisoning occurred in Rhode Island elementary schools that prepared school lunches through a centralized kitchen production system. Large amounts of *Staphylococcus aureus* were recovered and enterotoxin A was identified in leftover ham (Richards et al., 1993). A food handler of a central kitchen, who tested positive for the implicated enterotoxin strain of *S. aureus*, reported to removing the casings from two of nine warm ham rolls 48 h prior to lunch preparation and service. Although the centralization in the school lunch preparation in the Rhode Island outbreak contributed to the introduction and proliferation of bacteria, it also represents a strategic point to implement food safety interventions to decrease the risk of food contamination (Richards et al., 1993).

Facility personnel and equipment

Respondents were asked to rate staff training with regards to washing and preparing fruits and vegetables and were provided with this scale: very well trained, well trained, adequately trained, somewhat trained, and not trained, in which the anchors "very well trained" and "not trained" corresponded to highest and lowest level, respectively, of training in the scale. Thirty-nine percent (20) of respondents indicated having a well trained staff, 37% (19) indicated staff was adequately trained, 10% (5) indicated having a vey well trained staff, 8% (4) responded that staff were somewhat trained, 2% (1) indicated that staff were not trained, and 4% (2) did not respond to the question. When analyzing responses by state, 43% of respondents from the state

of Kansas indicated their staff or personnel was “adequately trained,” while 41% of respondents from the state of Mississippi indicated personnel was “well trained” (Figure 4.3).

Figure 4.3 Ratings of staff training to wash and prepare fruits and vegetables



Additionally, respondents were asked if their facilities possess adequate equipment dedicated to washing and preparing fresh fruits and vegetables. Sixty-five percent of respondents (33) indicated possessing adequate equipment and 29% of the respondents (15) indicated lacking adequate equipment to wash and prepare fresh fruits and vegetables. Among the 29% of respondents indicating lacking of equipment, 80% of these respondents indicated lacking sinks, 53% indicated lacking countertop space, 47% indicated lacking refrigerators, 27% indicated lacking cutting boards, 13% indicated lacking knives, and 27% indicated lacking other

equipment (e.g., stationers and salad spinners). When asked specifically about refrigerator capacity and space to accommodate fresh fruits and vegetables, 65% of respondents (33) indicated having enough refrigerator capacity and space to accommodate fresh fruits and vegetables, 25% (13) indicated lacking refrigerator capacity and space to accommodate fruits and vegetables, and 10% (5) did not respond to the question.

Respondents were asked to freely comment about their equipment needs and problems. Respondents noted a lack of equipment to perform their job and limited kitchen space. In addition, some mentioned that they have old equipment and very old facilities, serving more students than the facility was designed and built to serve. Some respondents mentioned having three-compartment sinks; however, they lack a sink designated for fruit and vegetable washing and preparation. In addition, one participant noted that their facility has equipment for refrigeration, but the equipment is obsolete and needs to be replaced. One concern was the set-up or layout of the kitchen meeting current food safety recommendations.

The implementation of safety programs based on HACCP principles, strict standards, constant training, and personnel supervision are key factors that could help to reduce the risk of contamination at any level of school meal preparation. Therefore, observational research should be conducted in school settings in order to verify adherence to good manufacturing practices during preparation of school meals, and to evaluate improvement of produce safety handling. Other research efforts should focus on practical interventions to reduce potential cross-contamination in school facilities during preparation and handling, along with prevention efforts on improving personnel training and skills to prepare fruits and vegetables (Daniels et al., 2002; Lee & Greig, 2010).

Efficacy of washing treatments on native microflora of whole cantaloupes (Phase II)

Aerobic plate count (APC) populations of untreated (control) cantaloupe surfaces averaged 3.88 $\log_{10}$ CFU/cm^2. Aerobic plate counts of cantaloupe rinds varied after washing treatments. Washing with tap water showed populations of 3.39 $\log_{10}$ CFU/cm^2, whereas populations after washing with the CAFVT and 9% vinegar solution were 2.98 $\log_{10}$ CFU/cm^2 and 3.01 $\log_{10}$ CFU/cm^2, respectively.

Aerobic bacteria populations transferred from cantaloupe rind surfaces to fresh-cut pieces (wedges or cubes) were determined immediately after preparation on day 0 and then sampled on day 1, 3, and 6 of storage. Cubes from untreated (control) cantaloupes, showed populations of 2.80 and 3.43 $\log_{10}$ CFU/g on day 0 and day 1, respectively, and populations increased significantly on day 3 and day 6 reaching 7.19 and 8.50 $\log_{10}$ CFU/g, respectively. Aerobic plate count populations of cubed and wedged cantaloupes from whole washed and unwashed (untreated) cantaloupes were significantly different over time ($P <$ at $0.05/16 \approx 0.0031$; Table 4.2). Populations of cubes from cantaloupes washed with 9% vinegar solution and CAFVT ranged from 1.01 to 3.30 $\log_{10}$ CFU/g on day 0 to day 3. However by day 6 populations increased significantly, reaching approximately 6.3 and 8.07 $\log_{10}$ CFU/g, respectively. Cubes from cantaloupes washed with tap water showed populations > 2.3 $\log_{10}$ CFU/g on day 0 and day 1, and reached populations > 4.6 $\log_{10}$ CFU/g on day 3. Although populations increased up to 6.62 $\log_{10}$ CFU/g on day 6, these counts were statistically similar to day 3 counts.

Wedges from untreated (control) and CAFVT washed cantaloupes showed populations over 5.6 $\log_{10}$ CFU/g on day 3 and approximately 8 $\log_{10}$ CFU/g on day 6.

Table 4.2 Aerobic plate count populations (APC; $\log_{10}$ CFU/g) on fresh-cut cantaloupe prepared from washed whole cantaloupes after storage at 4 ± 1°C for up to 6 days (n = 2).

Treatment	Surface	Cantaloupe Cubes				Cantaloupe Wedges			
	D0	**D0	D1	D3	D6	D0	D1	D3	D6
Untreated	3.88	2.80^{bx}	3.43^{bx}	7.19^{ax}	8.50^{ax}	4.16^{bx}	4.18^{bxy}	5.61^{abx}	8.09^{ax}
Tap water	3.39	2.56^{bx}	2.37^{bx}	4.61^{abxy}	6.62^{ax}	2.99^{bx}	4.86^{abx}	4.34^{bxy}	7.40^{ax}
9% vinegar solution	3.01	1.07^{bx}	1.01^{bx}	3.30^{by}	6.29^{ax}	2.97^{abx}	1.39^{by}	2.04^{by}	5.20^{ax}
CAFVT	2.98	1.39^{bx}	1.76^{bx}	2.47^{by}	8.07^{ax}	2.03^{cx}	2.91^{bcxy}	5.64^{abx}	7.58^{ax}

CAFVT = commercial antimicrobial fruit and vegetable treatment

^{abc} Means with different superscripts within a row section are significantly different at Bonferroni P=0.05/96 ≈ 0.00052; with ^{a} as the largest and ^{c} as the smallest values.

^{xy} Means with different superscripts within a column are significantly different at Bonferroni P=0.05/96 ≈ 0.00052; with ^{x} as the largest and ^{y} as the smallest values.

*Standard error (SE) = 0.5905; D = day of storage

Wedges from cantaloupes washed with 9% vinegar solution showed populations between 1.39 and 2.97 $\log_{10}$ CFU/g on day 0 to 3, however populations increased continuously and reached 5.20 $\log_{10}$ CFU/g on day 6. Populations of wedges from cantaloupes washed with water ranged between 2.99 and 4.86 $\log_{10}$ CFU/g on day 0 to 3, and increased up to 7.40 $\log_{10}$ CFU/g on day 6.

Significant differences of APC populations among washing treatments were observed only for cubed cantaloupes on day 3 and wedged cantaloupes on day 1 and day 3 ($P <$ at $0.05/16 \approx 0.0031$; Table 4.2). On day 1 sampling, wedges from cantaloupes washed with 9% vinegar solution showed the lowest population with 1.39 $\log_{10}$ CFU/g, while wedges from cantaloupes washed with water showed the highest population, 4.86 $\log_{10}$CFU/g. On day 3 sampling, cubes from cantaloupes washed with 9% vinegar solution and CAFVT showed the lowest APC populations with 3.30 and 2.47 $\log_{10}$ CFU/g, respectively, while cubes from untreated (control) cantaloupes showed the highest population with 7.19 $\log_{10}$ CFU/g. Similarly, wedges from cantaloupes washed with 9% vinegar solution showed the lowest population with 2.04 $\log_{10}$ CFU/g. However, wedges from cantaloupes washed with CAFVT along with wedges from the untreated (control) showed the highest populations with approximately 5.6 $\log_{10}$ CFU/g. Interestingly, on day 0, wedges from CAFVT-washed cantaloupes showed lower APC populations by ≥ 0.96 log when compared to APC populations from wedges obtained from untreated and tap water washed cantaloupes, whereas cubes from 9% vinegar solution and CAFVT-washed cantaloupes showed lower APC populations by ≥ 1.1 log when compared to APC populations from cubes obtained from untreated and tap water washed cantaloupes. This indicates that washing cantaloupes with 9% vinegar solution and CAFVT reduced natural microflora on the surface of cantaloupe, which may have helped reduce the probability of

transferring microorganisms from the rind to the flesh during cutting or transformation from whole cantaloupe to cubes.

It is worth noting that fresh-cut melons prepared at home kitchens have a suggested 7-day shelf life at 5°C (CDC, 2013). However, shelf life for fresh-cut fruits for catering and foodservice is only 1- 2 days (Barth, Hankinson, Zhuang, & Breidt, 2009). Similarly, our results indicated that storage of fresh-cut (wedged and cubed) cantaloupes at refrigeration temperatures (4 ± 1°C) should not exceed 3 days of storage since aerobic plate count populations reached ≥ 5.2 $\log_{10}$ CFU/g on day 6, even though washing treatments were applied prior to preparation. Moreover, it is important to keep in mind that the risk of recontamination can be amplified by further processing steps due to poor employee hygiene or improper handling with poorly sanitized utensils, equipment, or surfaces, among others.

The reduced efficacy of washing treatments on fresh-cut pieces (wedges, slices, and cubes) of cantaloupes over storage time may be due to strong attachment of microorganisms (influenced by cantaloupe surface morphology), and the formation of biofilms enhanced by the availability of nutrients in cantaloupe juices after the fruit was cut (Nguyen-the & Carlin, 1994; Ukuku, Bari, Kawamoto, & Isshiki, 2005; Ukuku & Fett, 2002b).

*Effectiveness of a commercially available fruit and vegetable wash for reducing pathogens (***Salmonell*a spp. and* **L. monocytogenes***) on whole cantaloupes (Phase III)*

Non-inoculated cantaloupes sampled for standard aerobic plate counts during *Salmonella* spp. and *L. monocytogenes* trials had total aerobic plate count populations of 4.70 $\log_{10}$ CFU/cm^2 and 4.80 $\log_{10}$ CFU/cm^2, respectively. No two-way interaction effect was observed (washing treatment × exposure time) on reducing *Salmonella* spp. populations on cantaloupe surface (Table 4.3). However, *Salmonella* spp. populations were affected ($P < 0.05$) by the commercial

produce wash and exposure time (Table 4.3). The average *Salmonella* spp. population on the surface of cantaloupes after washing with tap water and the commercial produce wash solution were 5.50 and 4.87 $\log_{10}$ CFU/cm^2, respectively (Table 4.4). With respect to exposure time, pooled data across washing treatments showed that 60 and 120 s exposure times achieved the lowest *Salmonella* spp. population recovery after washing procedures (Table 4.4), while exposure time of 30 s showed the highest recovery of *Salmonella* spp. populations after washing. Sampling of residual wash treatment water resulted in recovery of 4.30 $\log_{10}$ CFU/ml of *Salmonella* spp. populations from the cold tap water and populations below the detection limit of 1.95 $\log_{10}$ CFU/ml for the commercial produce wash (Table 4.5).

Table 4.3 *P*-values of the main effects and interaction effects for recovered *Salmonella* spp. and *L. monocytogenes* after application of washing treatments.

Effect[a]	*P*-values	
	Salmonella spp.	***L. monocytogenes***
[1]Wash treatment	0.0002	0.0039
[2]Exposure time	0.0354	-
Wash treatment × Exposure time	0.2679	-

[a] Main and/or interaction effect is significant if $P < 0.05$
[1] For both *Salmonella* spp. and *L. monocytogenes* trials commercial produce wash and tap water (as control)
[2] For *Salmonella* spp. trails = 30, 60, 120 s and 120 s for *L. monocytogenes* trails

The inoculated populations of *Salmonella* spp. on the surface of cantaloupes (n = 6) that were not washed averaged 6.13 $\log_{10}$ CFU/cm^2. Compared to the inoculated samples recovery, a reduction of 0.62 $\log_{10}$ CFU/cm^2 was observed on the rind of cantaloupes washed with tap water while reductions of 1.26 $\log_{10}$ CFU/cm^2 ($P < 0.05$) were observed on the rind of cantaloupes washed with the commercial produce wash. This difference in log reductions may be due to a

difference of pH in washing treatments; pH measurements of washing treatments indicated pH values of ca. 3 for the commercial produce wash and ca. 9 for tap water. Various researchers have reported that the antimicrobial action of organic acids is due to pH reduction in the environment, disruption of membrane transport and/or permeability, anion accumulation, or a reduction in internal cellular pH by dissociation of hydrogen ions from acid (FDA, 2013; Rico, Martin-Diana, Barat, & Ryan, 2007; Parish et al., 2003).

Table 4.4 Mean populations of *Salmonella* spp. and *L. monocytogenes* populations (log_{10} CFU/cm^2) after application of washing treatments on cantaloupes.

Effect	Treatment/treatment combination	*Salmonella* spp.[c]	*L. monocytogenes*[d]
Wash treatment	Cold tap water[*]	5.51[a]	5.41[a]
	Commercial produce wash	4.87[b]	4.92[b]
Exposure time	30 s[**]	5.43[x]	-
	60 s	5.19[y]	-
	120 s	4.94[y]	-
Interaction effect	Cold tap water × 30 [***]	5.61	-
	Cold tap water × 60	5.51	-
	Cold tap water × 120	5.41	-
	Commercial produce wash × 30	5.26	-
	Commercial produce wash× 60	4.86	-
	Commercial produce wash × 120	4.47	-

[*] Data pooled for exposure time (n = 18); Standard error (SE) = 0.22
[**] Data pooled for washing treatment (n =18); SE = 0.23
[***] SE = 0.26
[ab] Means or [xy] Means with different superscripts within a column section are significantly different ($P < 0.05$)
[c] The initial population of *Salmonella* spp. on unwashed inoculated samples mean was 6.13 log_{10} CFU/cm^2
[d] The initial population of *L. monocytogenes* on unwashed inoculated cantaloupes was 6.03 log_{10} CFU/cm^2

Exposure time for *L. monocytogenes* inoculated cantaloupes to washing treatments was 120 s. This decision was based on the results obtained from the *Salmonella* spp. trial where application of washing for 120 s showed the lowest *Salmonella* spp. population recovery (Table 4.4). Similarly to *Salmonella* spp., in these set of trials the commercial produce wash had a significant effect ($P < 0.05$) on *L. monocytogenes* populations after washing procedures (Table 4.4). Application of the commercial produce wash for 120 s achieved 1.12 $\log_{10}$ CFU/cm^2 reduction of *L. monocytogenes* population on cantaloupes rind. However, a reduction of 0.63 $\log_{10}$ CFU/cm^2 was achieved by washing with cold tap water for 120 s.

Moreover, sampling of residual water after treatment indicated that *L. monocytogenes*-inoculated cantaloupes transferred the pathogenic load to regular tap water by 4.47 $\log_{10}$ CFU/ml while recovery of microorganisms in the commercial produce wash water after treatment was below the detection limit of 1.95 $\log_{10}$ CFU/ml (Table 4.5).

Table 4.5 Mean ± standard deviation (SD) of *Salmonella* spp. (*n* = 15) and *L. monocytogenes* (*n = 9)* populations recovered from residual water after wash treatments.

Pathogen	Treatment	Log_{10} CFU/ml
Salmonella spp.	Cold tap water	4.30 ± 0.22
	Commercial produce wash	< 1.95 DL[a]
L. monocytogenes	Cold tap water	4.47 ± 0.40
	Commercial produce wash	< 1.95 DL[a]

[a]Detection limits (DL) for residual water samples was 1.95 $\log_{10}$ CFU/ml, respectively

Parnell, Harris, and Suslow (2005) reported *Salmonella* Typhimurium populations log reductions of 0.7 and 1.8 $\log_{10}$ CFU/melon on cantaloupes washed by immersion for 60 s with

water and 200 ppm total chlorine, respectively. Fishburn, Tang, and Frank (2012) evaluated the efficacy of five home-used washing technologies (diluted chlorine bleach, electrolyzed oxidizing water, ozone, veggie wash or running tap water) in reducing *L. monocytogenes* populations on cantaloupes that were washed by submersion for 2 min. Electrolyzed oxidizing water, ozone, veggie wash, and running tap water showed 0.55 log reductions of *L. monocytogenes*, whereas diluted chlorine bleach achieved 1.43 log reduction.

Various researchers have studied the efficacy of a produce wash (FIT) on tomatoes (Beuchat, Harris, Ward, & Kajs, 2001; Harris, Beuchat, Kajs, Ward, & Taylor, 2001), strawberries (Lukasik et al., 2003), and potatoes (Park et al., 2008). Beuchat et al. (2001) reported *Salmonella* reductions of > 6.83 $\log_{10}$ in tomatoes when applying the FIT prototype wash. Harris et al. (2001) found that application of the FIT produce wash resulted in *Salmonella* reductions in tomatoes greater than those achieved with sterile water and Dey and Engley (D/E) broth. Washing strawberries by immersion for 2 min with the FIT produce wash achieved 2 log reductions of *E. coli* O157:H7 and *Salmonella* Montevideo on the surface of strawberries (Lukasik et al., 2003). In another study, flume water enhanced with the FIT produce wash resulted in reductions of enteric pathogens between 1.4 and 1.8 $\log_{10}$ CFU/g on surfaces of potato tubers. Additionally, effectiveness of the produce wash was not affected regardless of water quality (presence of organic material; Park et al., 2008).

In our study, the application of the commercial produce wash achieved *Salmonella* spp. and *L. monocytogenes* reductions of ≥ 1 $\log_{10}$ CFU/cm^2, which are similar results to those reported by Lukasik et al. (2003) and Park et al. (2008). Log reduction results reported by Beuchat et al. (2001) and Harris et al. (2001) were significantly higher than our findings. Therefore, it is important to note that methods used for application of produce wash and recovery

of microorganisms by these researchers were different than those used in the current study. Moreover, the current study focused on methods that are used in foodservice operations and not necessarily methods used in the laboratory settings. Although the commercial washing treatment was capable of achieving ≥ 1 log reduction of *Salmonella* spp. and *L. monocytogenes* populations on cantaloupe rind, these reductions are insufficient to assure microbial safety of cantaloupes. Minimal reduction of pathogenic microorganism populations could be attributed mainly to the characteristics of a cantaloupe's surface, which is a complex meshwork of tissue that provides binding sites that are difficult to reach with sanitizers (Ukuku & Fett, 2002a,b; Wang et al., 2007). However, the commercial produce wash showed significant potential to maintain adequate microbial water quality and reduced the risk of cross-contamination when new produce is introduced to the washing sink or tank.

Conclusions and Implications

In the survey responses, a small percentage of respondents (10%) indicated they use antimicrobial washes for washing produce. Prepared fruits and vegetables of interest (green leaf lettuce, tomato, and cantaloupes) were stored under refrigerated conditions overnight or as long as 7 days. Challenges faced for school foodservice personnel included limitations in existing kitchen equipment and infrastructure, training, and skills of personnel to wash and prepare fruits and vegetables. While schools are the only foodservice environment required to have a food safety program based on HACCP-principles ensuring that directors, managers, and employees fully understand the importance of a properly maintained and managed food safety program will help to prevent food safety hazards that arise during food preparation (receiving, storing, preparing, cooking, cooling, reheating, holding, assembling, packaging, transporting) and service

are adequately controlled. School foodservice managers should be encouraged to reinforce preexisting food safety knowledge through training courses and certifications, and should emphasize proper food safety practices or behaviors in order to create a culture of food safety and reduce the risk of foodborne illnesses outbreaks.

Washing cantaloupes with 9% vinegar solution, CAFVT, or CPW reduced natural microflora or pathogenic populations on the surface of cantaloupes by approximately 1 log. However, it is important to note that the approximate infectious dose of pathogenic microorganisms, such as *L. monocytogenes,* is estimated to be as low as one cell in immunocompromised individuals, and these washing treatments might not be able to ensure cantaloupe safety if pathogenic populations > 1 log are present on the surface or internalized in the produce. Therefore, the use of disinfectants such as 9% vinegar solution, CAFVT, and CPW would assist mainly to maintain process/wash water free of microbial contaminants and reduce the risk of cross-contamination when new produce is introduced to the washing sink or tank.

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Chapter 5
Efficacy of a washing system and commercial produce washes to reduce *Escherichia coli* surrogates on green leaf lettuce surface

Abstract

Our study investigated the efficacy of a continuous water motion washing system and chemical wash solutions for controlling *Escherichia coli* surrogates on the surface of green leaf lettuce and increasing shelf life of green leaf lettuce throughout a 6-day storage period after treatment application. Lettuce leaves were inoculated with a five-strain cocktail mix of rifampicin-resistant derivatives of *E. coli* surrogates and then washed with tap water (as control), 5% vinegar solution, or a commercial antimicrobial for fruits and vegetable treatment (CAFVT) for 120 s with agitation by using a continuous water motion system or by hand (as control). *E. coli* surrogates populations were enumerated on day 0 after washing treatments and on days 1, 4, and 6 of storage (4 ± 1°C). On day 0, log reductions achieved by CAFVT (2.25 $\log_{10}$ CFU/g) were greater (P = 0.0145) than those by water (1.34 $\log_{10}$ CFU/g), but similar to 5% vinegar solution (2.09 $\log_{10}$ CFU/g). Washing lettuce with continuous agitation achieved higher (P = 0.0072) *E. coli* surrogate reductions (2.26 $\log_{10}$ CFU/g) than without agitation (1.53 $\log_{10}$ CFU/g). *E. coli* surrogate populations on lettuce leaves washed with CAFVT and water with agitation remained steady during storage, whereas *E. coli* surrogate populations on lettuce leaves washed with all other treatments slightly decreased over time. In conclusion, *E. coli* populations on day 0 were significantly affected by the wash solution and washing action (agitation), and storage of green leaf lettuce at refrigeration temperatures (4 ± 1°C) after washing reduced the risk of potential proliferation of *E. coli* surrogates.

Keywords: acetic acid, vinegar, lactic acid, lettuce, *Escherichia coli,* produce

Introduction

The increased number of foodborne outbreaks associated with fresh produce has emphasized the need to study new efficient, economical, and effective decontamination technologies to reduce the risk of foodborne illnesses. *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains have been implicated in outbreaks of illnesses associated with consumption of leafy greens such as spinach and lettuce (CDC 2006; CDC 2010; CDC 2012). Leafy greens, and other types of fresh produce are highly perishable food commodities that are generally consumed raw. However, they are washed before consumption, primarily to remove soil, pesticide residues, and spoilage-causing and pathogenic microorganisms. Practices applied during production, harvest, and post-harvest activities may increase the risk of potential contamination with microbial pathogens.

During post-harvest operations, washing is intended to improve produce quality and safety (Pao and others 2012). Thus, maintaining the quality of wash water is essential to minimize potential transmission of pathogenic microorganisms from water to produce. Chlorine-based chemicals are broadly used in fruit and vegetable processing facilities to clean produce, sanitize surfaces within the facility in contact with produce, as well as to maintain microbial quality of wash water (Parish and others 2003). Several sanitizing agents have been studied for their effectiveness in reducing microbial populations in produce. Studies have indicated that use of chlorinated water on leafy greens decreases microbial load by values ranging from < 1 to < 3 $\log_{10}$ CFU/g (Beuchat and others 2004; Bari and others 2005; Akbas and Ölmez 2007; Allende 2009).

Several researchers also have emphasized the antimicrobial potential of organic acids such as citric, lactic, and acetic acids, which have been classified as GRAS (21 RF 184.1005,

1033, 1061). The antimicrobial action of organic acids is due to pH reduction in the environment, disruption of membrane transport and/or permeability, anion accumulation, or a reduction of internal cellular pH by dissociation of hydrogen ions from acid (FDA 2013; Rico and other 2007; Parish and others 2003). Dipping inoculated fresh-cut iceberg lettuce in 0.5% citric acid or 0.5% lactic acid solutions for 2 min showed reductions of approximately 2 $\log_{10}$ CFU/g for *E. coli* populations (Akbas and Ölmez 2007). Washing solutions of 5% acetic acid resulted in 3 log reductions of *E. coli* O157:H7 populations in iceberg lettuce; however, this concentration of acetic acid may be detrimental to sensory characteristics of produce (Chang and Fang 2007).

Additionally, numerous types of produce washing systems have been developed primarily to remove soil debris and pesticide residues from fresh produce. However, it is important to note that the design of most commercial equipment has not taken into account the requirements for reduction of microbial populations on produce surfaces. Moreover, the application of conventional sanitizers with commercial-scale washing equipment has the capability to achieve 1 to 2 log reductions of microbial populations in contaminated produce, however this technology is rarely available for foodservice and consumer applications (Sapers 2006).

Therefore, the objective of this study was to test the efficacy of a continuous water motion washing system for foodservice application combined with chemical wash solutions in reducing rifampicin-resistant *E. coli* surrogates on the surface of green leaf lettuce. A secondary objective was to study shelf life of green leaf lettuce after treatment throughout a 6-day refrigerated storage period.

Materials and Methods

Experimental design

Lettuce heads from the same lot of production were purchased from a local super market in Manhattan, KS. For each replication day, lettuce leaves (1,000 g) were placed in 6 separate plastic containers (or lettuce groups) with the curled or fringed side of leaves facing up. Lettuce groups (1,000 g) were inoculated with a five-strain cocktail of rifampicin-resistant derivatives of *E. coli* surrogates. After inoculation, each group was randomly assigned to a washing treatment combination (wash solution × washing action). Subsamples of lettuce (25 ± 0.3 g) were separated from each plastic container/group prior to inoculation, after inoculation, and after washing procedures. Inoculated lettuce was washed for 120 s with either tap water (as control), a 5% vinegar solution, or a commercial antimicrobial for fruits and vegetables treatment (CAFVT). Washing action was done by using a washing system, which produces continuous agitation of the wash solution, or by hand (as control). Washed lettuce was tested after washing treatment on day 0 and days 1, 4, and 6 of storage. Three replications of the experiment were conducted, samples of washed lettuce were analyzed in duplicates, and the average was used for statistical analysis.

Bacterial strains and inoculum preparation

A five-strain cocktail of rifampicin-resistant derivatives of *E. coli* surrogates was used to inoculate lettuce samples. Rifampicin-resistant derivatives of *E. coli* ATCC-BAA 1427, *E. coli* ATCC-BAA 1428, *E. coli* ATCC-BAA 1429, *E. coli* ATCC-BAA 1430, and *E. coli* ATCC-BAA 1431 (American Type Culture Collection, Manassas, VA) strains were independently grown on tryptic soy agar (TSA) at 37 ± 2°C for 24 ± 2 h. One colony of each strain was used to inoculate 9 mL of tryptic soy broth (TSB) and each broth was incubated at 37 ± 2°C for 24 ± 2 h. A second

transfer occurred by pipetting 0.5 mL of 24-h culture to 30 mL TSB in a centrifuge tube and then incubating at 37± 2°C for 24 ± 2 h.

Cells of each strain were collected by centrifugation (ca. 4,960 × g, for 15 min, 4 °C; JA-17 rotor, Model J2-21 M/E, Beckman Coulter, In., Pasadena, CA) and then resuspended in 20 mL of sterile 0.1% peptone water. They were then transferred into a small plastic bottle equipped with an atomizer (8 oz, high-density polyethylene (HDPE), The Bottle Crew, West Bloomfield, MI) to form a five-strain cocktail inoculum. The initial inoculum concentration was determined by serially diluting the inoculum in 0.1% sterile peptone water and plating on TSA supplemented with 100 μg of rifampicin per mL.

Inoculation procedure

Commercially available unwashed green leaf lettuce was purchased at a local supermarket. Damaged outer leaves and core area (4 – 6 cm) were removed from each head of lettuce by using a knife. Intact lettuce leaves (1,000 g) with the curled or fringed side of leaves facing up were placed in plastic containers (19 L, polypropylene (PP), Sterilite®, Townsend, MA; n = 18) that were disinfected with 70% ethanol (Ethanol 200 proof, Decon Laboratories, INC., King of Prussia, PA), Each container represented a group of green leaf lettuce to be inoculated and subsequently washed. Prior to inoculation, one subsample (25 ± 0.3 g) of each lettuce group was separated for estimation of microbial flora populations. A fine mist of *E. coli* surrogates inoculum (ten full sprays for ca. 10 mL total) was sprayed onto lettuce. The plastic container was covered with its respective lid and manually shaken back and forth for ca. 2 s to assist further inoculum distribution. Inoculated lettuce was allowed to dry for 1 h at room temperature (22 ± 2°C), in a biosafety cabinet, to allow attachment of cells. The same procedure

was repeated for each lettuce group. After drying, two subsamples (25 ± 0.3 g) of each contaminated lettuce group were analyzed to determine the initial *E. coli* surrogates population.

Washing procedures

The efficacy of a continuous water motion washing system (Model 50PSP66L2B1; Produce Soak by Power Soak Systems, Kansas City, MO) in combination with chemical washes was evaluated. Green leaf lettuce groups (ca. 1,000 g) were washed separately with water (pH = 9.43: free chlorine = 2.25 ppm) which was used as control, a 5% vinegar solution containing 0.24% acetic acid (pH = 3.29), or a commercially available antimicrobial fruit and vegetable treatment [CAFVT; pH = 2.84; lactic acid (1,061 – 1,391 ppm), sodium hydrogensulfate, docecylbezesulfonic acid (76 – 111 ppm); Ecolab, St. Paul, MN] for 2 min by using a continuous water motion washing system or manually (as control), for a total of six different treatment combinations. Lettuce groups (ca. 1,000 g) were randomly assigned to the wash treatment combinations and washed in random order for a completely randomized design.

Preliminary studies, using different acetic acid concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5%) on vinegar solutions, were made to establish the best concentration to use based on sensory and quality parameters. The solutions containing 0.2 and 0.3% of acetic acid showed a lower impact on browning edges, off- odor and flavor, and crispiness of lettuce leaves (data not shown). Therefore, a 5% vinegar solution containing 0.24% acetic acid was chosen for further antimicrobial analysis

The 5% vinegar solution was prepared by mixing 6 L of white distilled vinegar (5% acetic acid; The Kroger Co., Cincinnati, OH) with 120 L of tap water. The commercial antimicrobial fruit and vegetable treatment was prepared by using an automatic dispenser to reach 0.75 – 1.00 oz. (22 – 30 mL) of concentrate per gallon (3.7854 L) of water.

The continuous water motion washing system consisted of a stainless steel two bay wash tank (ca. 150 L), a stainless steel self-draining parallel flow pump, a pump motor connected to the wash tank, water inlet holes that run full length of the back wall of the wash tank, and six low profile wash jets (each bay with 3 low profile jets; flow rate 10 gpm per jet) located above wash pump inlet holes (Figure 4.1 and Figure 4.2). During the washing operation, the pump located on a side of the wash tank was fed with water in a first direction via a pump inlet connected to an intake port passing through the right side wall of the wash tank. Then water was impelled out from the pump in a second direction substantially parallel to the first direction, via a pump outlet connected to an outlet chamber and wash jets producing agitation of the wash solution (AU Patent No. 2002335694).

However, for the purpose of this study a portion of the wash pump inlet holes were blocked with an L shape plastic device [24.1 cm L × 8.26 cm W ×12.1 cm H; ultra high molecular weight polyurethane (UHMW)] to obtain an average flow rate ca. 7 gpm per jet (R. McNamara, personal communication, November 5, 2015). This modification decreased damaged to lettuce leaves by decreasing the flow rate from 10 gpm per jet to 7 gpm per jet.

Lettuce groups washed by hand (as control) were submerged in and out of the washing solution (ca. 120 L) for 2 min by glove-covered hands. After washing, lettuce was removed from the wash tank by using a stainless steel basket, shaken to remove excess water, and allowed to air dry for 5 min. Two subsamples per washed lettuce group (25 ± 0.3 g) were separated for enumeration, and the remaining lettuce leaves were stored in plastic containers with lids (184 fl. oz, polypropylene (PP), Snapware®, Mira Loma, CA) at 4 ± 1°C for further sampling at days 1, 4, and 6.

Enumeration

Following washing procedures, lettuce samples (25 ± 0.3 g) from each treatment combination (n = 2 per rep) were diluted with 125 mL of Dey-Engley (DE) neutralizing broth (BD Difco, Franklin Lakes, NJ) and blended for 1 min in a pulse control blender (Ninja Express Chopper NJ 100, Euro-Pro Operating LLC, Newton, MA). The resulting homogenate (0.25 mL aliquot in quadruplicate and 0.1 mL aliquot in duplicate) and serially diluted homogenate in 9 mL of 0.1% sterile peptone water (BD Bacto, Franklin Lakes, NJ; 0.1 mL per plate in duplicate) were surface-plated on TSA supplemented with 100 μg of rifampicin per mL. Plates were incubated at 37 ± 2°C for 24 ± 2 h.

Lettuce samples separated prior (n =1) and after inoculation (n = 2) for estimation of indigenous microbial flora and initial *E. coli* surrogate populations, respectively, were prepared following the same procedures described above, with the exception that these samples were initially diluted with 125 mL of 0.1% sterile peptone water instead of DE neutralizing broth. Additionally, samples for indigenous microbial flora populations were surface-plated onto TSA, and lettuce samples used for estimation of initial *E. coli* surrogate populations were surface-plated onto TSA supplemented with 100 μg of rifampicin per mL.

Statistical Analysis

The experiment was replicated three times and followed a randomized complete block design (RCBD) with a 3 wash solutions (5% vinegar solution, CAFVT, and tap water as control) × 2 wash actions (continuous agitation and by hand as control) factorial arrangement of treatments.

Statistical analysis was divided in four sections (Appendix F).

1) Analysis for indigenous microflora counts from samples tested prior to inoculation: Data were analyzed using the PROC MIXED procedures of SAS version 9.4 (SAS Institute, Cary, NC, USA) and replication was used as blocking factor.

Ho: There are no differences in indigenous microflora populations among lettuce groups [lettuce leaves (1,000 g) assigned to the different washing treatment combinations (wash solution × washing action)] prior to inoculation.

2) Analysis for *E. coli* surrogate populations after inoculation: Data were analyzed using the PROC MIXED procedures of SAS.

Ho: There are no differences in *E. coli* surrogate populations of contamination among lettuce groups [lettuce leaves (1,000 g) assigned to the different washing treatment combinations (wash solution × washing action)] after inoculation.

3) Analysis for *E. coli* surrogate population reductions on day 0 after washing treatment: Data for *E. coli* surrogate log reductions for day 0 were analyzed using the PROC GLIMMIX procedures of SAS, with replication used as blocking factor. Appropriate interactions (wash solution × washing action) were tested first at a significant level of 0.05, followed by test of main effects. The SLICEDIFF option was used to determine the differences in the level of one factor at a fixed level of the other factor. Appropriate corresponding least squares means were determined and pairwise comparisons were conducted using Bonferroni's adjustment.

Ho (interaction effect): Mean log reductions of *E. coli* surrogate populations are not affected by the interaction of wash solution × washing action.

> ***Ho*** *(main effect wash solution)*: There are no differences in mean log reductions of *E. coli* surrogate populations for the different wash solutions (5% vinegar solution, CAFVT, and tap water as control).

***Ho** (main effect washing action)*: There are no differences in mean log reduction of *E. coli* populations for the different washing actions (continuous agitation and motionless).

4) *Escherichia coli* surrogate populations on day 0, 1, 4, and 6 after application of washing treatments: Data for *E. coli* surrogate populations for all days were analyzed using the PROC GLIMMIX, compound symmetry covariance structure (Type = CS), and Kenward-Roger (DDF = KR) procedures of SAS. Day of microbial sampling (4 days) was used as repeated measure and replication as blocking factor. Appropriate interactions were tested first at a significance level of 0.05, followed by test of main effects. The SLICEDIFF option was used to explore the differences in the level of one factor at a fixed level of the other factor. Appropriate corresponding least squares means were determined and pairwise comparisons were conducted using Tukey's adjustment.

Ho (interaction effect): *E. coli* surrogate populations are not affected by the interaction of wash solution × washing action × day after application of washing treatment.

***Ho** (main effect wash solution)*: There are no differences in *E. coli* surrogate populations for the different wash solutions (5% vinegar solution, CAFVT, and water as a control).

***Ho** (main effect washing action)*: There are no differences in *E. coli* surrogate populations for the different washing actions (continuous agitation, and motionless).

***Ho (**main effect day of storage)*: There are no differences in *E. coli* surrogate populations for the different storage days (day 0, 1, 4, and 6).

Results and Discussion

Indigenous microflora

Aerobic plate counts of samples tested prior to inoculation averaged ca. 5.14 $\log_{10}$ CFU/g. Statistical analysis of APC populations indicated there were no differences ($P > 0.05$) on the surfaces of green leaf lettuce indigenous microflora prior to inoculation and assignment of treatment combinations (wash solution × washing action).

Inoculated samples

Escherichia coli surrogate populations on surfaces of green leaf lettuce samples tested after inoculation averaged ca. 6.57 $\log_{10}$ CFU/g. Statistical analysis of *E. coli* surrogates populations indicated there were no differences ($P > 0.05$) among lettuce groups assigned to the different washing treatment combinations (wash solution × washing action) before washing.

Efficacy of wash treatments against E. coli *surrogates*

On day 0 after application of washing treatments, mean log reductions of *E. coli* surrogate populations were not affected by the interaction of wash solution × washing action ($P = 0.2259$; Table 5.1); however mean log reductions of *E. coli* populations were significantly affected by the wash solutions ($P = 0.0145$) and washing actions ($P = 0.0072$; Table 5.1). Therefore, data were pooled across wash solutions (tap water, 5% vinegar solution, and CAFVT) and then across wash actions (continuous agitation and by hand) to determine statistical differences.

Reductions of *E. coli* surrogate populations achieved by CAFVT (2.25 $\log_{10}$ CFU/g) were significantly greater than those by water (1.34 $\log_{10}$ CFU/g), but similar to 5% vinegar solution (2.09 $\log_{10}$ CFU/g). Moreover, no differences ($P > 0.05$) existed between water and 5% vinegar solution washing treatments with respect to mean log reductions of *E. coli* surrogate populations.

Application of the wash solutions with continuous agitation, as provided by the washing system, achieved higher ($P < 0.05$) *E. coli* surrogate reductions (2.26 $\log_{10}$ CFU/g) than application of wash solutions by hand (1.53 $\log_{10}$ CFU/g; Table 5.2).

Table 5.1 *P*-values of the main effects and interaction effects for *E. coli* surrogate $\log_{10}$ reductions after application of washing treatments on day 0.

Effect	*P*-Value
Wash Solution	0.0145*
Washing Action	0.0072*
Wash Solution × Washing Action	0.2259

* Main and/or interaction effect is significant ($P < 0.05$).

Although wash solution and washing action did not significantly interact to affect mean log reduction of *E. coli* surrogate populations, treatment combinations (wash solution × washing action) achieved log reductions ranging from 0.87 to 2.64 $\log_{10}$ CFU/g (Table 5.2). In fact, application of the CAFVT with agitation or by hand reached 2.34 and 2.17 log reductions of *E. coli* surrogate populations, respectively. Washing surfaces of green leaf lettuce with 5% vinegar solution with continuous agitation achieved log reductions of 2.64 $\log_{10}$ CFU/g, while washing with 5% vinegar solution by hand achieved 1.55 $\log_{10}$ CFU/g. Moreover, *E. coli* surrogate log reductions of 1.82 $\log_{10}$ CFU/g were achieved when washing with tap water with continuous agitation when using the washing system, while reductions of 0.87 $\log_{10}$ CFU/g were achieved when washing with tap water by hand. Washing green leaf lettuce with water or 5% vinegar solution incorporating continuous agitation improved *E. coli* surrogate log reductions by approximately 1 log (Table 5.2).

Other researchers have reported the effect of agitation and wash treatments in removing

bacteria from produce surface. For example, Sapers and others (2002) investigated means to improve efficacy of hydrogen peroxide washes in reducing *E. coli* on contaminated apples. Their findings showed that applying 0.5% hydrogen peroxide at 50°C with vigorous agitations improved reduction of *E. coli* populations by 1 log. Nastou and others (2012) reported that agitation improved the efficacy of acetic acid against *L. monocytogenes* on lettuce by approximately 1 $\log_{10}$ CFU/cm^2.

Table 5.2 Mean log reductions of *E. coli* surrogate populations on green leaf lettuce after application of washing treatments on day 0.

Effect	Treatment	Log Reduction[c]
[1]Main Effect of Wash Solution	Tap Water	1.34[b]
	5% Vinegar Solution	2.09[ab]
	[2]CAFVT	2.25[a]
[3]Main Effect of Washing Action	Agitation	2.26[x]
	By Hand	1.53[y]
[4]Interaction Effect of Wash Solution × Washing Action	Tap Water × Agitation	1.82
	Tap Water × By Hand	0.87
	5% Vinegar Solution × Agitation	2.64
	5% Vinegar Solution × By Hand	1.55
	CAFVT × Agitation	2.34
	CAFVT × By Hand	2.17

[1] Data pooled for washing action (n = 12); Standard error (SE) = 0.19.
[2] CAFVT Commercial antimicrobial for fruits and vegetable treatment.
[3] Data pooled for wash solution (n =12); SE = 0.15.
[4] n= 6; SE = 0.27.
[ab] Means or [xy] Means with different superscripts within a column section are significantly different ($P < 0.05$).
[c] The initial mean population of *E. coli* on unwashed inoculated samples was ~ 6.5 $\log_{10}$ CFU/g.

Additionally, Wang and others 2007 studied the effect of flow hydrodynamics (flow velocity and agitation rate) and exposure time on the reduction of *E. coli* O157:H7 from surfaces

of cantaloupes and cut apples. Their findings indicated that *E. coli* O157:H7 reductions were improved with the increase in flow velocity and agitation rate. For example, when cantaloupes and cut apples were washed with peroxyacetic acid (POAA; 80 ppm) for 3 min, an increase in flow velocity from 0.0 (soaking in motionless sanitizer) to 0.8 (soaking in flowing sanitizer) m/min improved *E. coli* O157:H7 reductions on cantaloupes and cut apples surfaces by approximately 1 log. In addition, two agitation modes, A and B, were used to wash cantaloupes and cut apples. Mode A of agitation (conducted below the fruit sample in the water) reduced *E. coli* O157:H7 by 1.2 log CFU/cm^2 in 3 min, whereas Mode B agitation (conducted above the fruit sample in the water) reduced *E. coli* O157:H7 only by 0.8 log CFU/cm^2.

Generally, the ability of organic acids to inhibit microbial growth has been associated to lipid permeability, which allows them to easily penetrate the lipid membrane of the bacterial cell and equilibrate across the membrane by simple diffusion (Booth and Stratford 2003). Once internalized into the neutral pH of the cell's cytoplasm, acids dissociate into anions and protons (Booth and Stratford 2003; Hirshfield and others 2003). High accumulations of protons can overcome the cytoplasmic buffering capacity and ultimately lead to a decline in cytoplasmic pH that cancel cell function capabilities (Booth and Stratford 2003). Inhibitory effects of organic acids do not solely rely on the reduction of cell internal pH, as other factors such as ratio of undissociated species of the acid, chain length, cell physiology and metabolism play an important role (Akbas and Ölmez 2007; Nastou 2012). For example, high accumulation of the acid anions in the cytoplasm can have an effect on the osmotic and metabolic processes that occur in the cytoplasm (Hirshfield and others 2003).

The improvement in log reductions caused by agitation might be attributed to the increase in sheer force to which produce was subjected (Wang and others 2007). This mechanical force

during washing may have exposed *E. coli* cells attached to inaccessible sites on the surface of lettuce to the wash solution.

Changes of E. coli *surrogate populations over storage time*

After washing treatments on day 0, lettuce leaves were stored in plastic containers with lids at 4 ± 1°C for further sampling on days 1, 4, and 6 of storage. Analysis of data indicated that wash solution, washing action, and day of storage had a marginally significant interaction effect ($P = 0.0482$) on *E. coli* surrogate populations (Table 5.3). Therefore, analysis of simple effect comparisons was conducted.

The analysis indicated that within day of sampling the effectiveness of wash treatments was significantly different only on days 0 and 6 (Table 5.4). Indeed, for lettuce leaves that were washed with the washing system (agitation wash), on day 0 lower ($P < 0.05$) *E. coli* surrogate populations were observed on surfaces of lettuce leaves treated with 5% vinegar solution than those treated by tap water. However, *E. coli* surrogate populations on surfaces of lettuce leaves treated with 5% vinegar solution were similar to those on lettuce leaves treated with CAFVT. On day 6, *E. coli* surrogate populations on the surface of lettuce leaves treated with 5% vinegar solution were lower ($P < 0.05$) than those treated with CAFVT or tap water.

For lettuce leaves that were washed by hand, on day 0 lower ($P < 0.05$) *E. coli* surrogate populations were observed on surfaces of lettuce leaves treated with CAFVT than those treated with tap water. However, populations on surfaces of lettuce leaves treated with CAFVT were similar to those on lettuce leaves treated with 5% vinegar solution. On day 6, lettuce leaves washed with CAFVT or 5% vinegar solution showed lower ($P < 0.05$) *E. coli* surrogate populations than lettuce leaves treated with tap water alone.

Furthermore, within wash solution, *E. coli* surrogate populations were significantly

different over the sampling days (0, 1, 4, and 6) for lettuce leaves washed with 5% vinegar solution with agitation and for lettuce leaves washed by hand with tap water, 5% vinegar solution, and CAFVT (Table 5.4). *Escherichia coli* surrogate populations on the surface of lettuce leaves washed with continuous agitation and 5% vinegar solution remained steady from day 0 to day 4, however from day 4 to day 6 populations decreased significantly.

Populations on the surface of lettuce leaves washed by hand with tap water significantly decreased from day 1 to day 4, however by day 6 populations recovered. Conversely, for lettuce leaves washed by hand with 5% vinegar solution was observed that populations remained steady from day 0 to day 4, and then populations significantly decreased from day 4 to day 6. Moreover, populations on lettuce leaves washed by hand with CAFVT decreased from day 0 to day 6 (Table 5.4). In addition, is important to point out that by day 1 of storage quality defects such as wilting, browning edges, and bruising were observed for lettuce leaves washed with the 5% vinegar solution and CAFVT (Appendix G , Appendix H, Appendix I).

Table 5.3 P-values of the main effects and interaction effects for *E. coli* surrogate populations for repeated measures of *E. coli* surrogate populations on lettuce leaves after washing treatments and storage at 4°C.

Effect	***P*-Value**
Wash Solution	0.0083*
Washing Action	0.0646
Wash Solution × Washing Action	0.1272
Day	< 0.0001*
Wash Solution × Day	0.0885
Washing Action × Day	0.0199*
Wash Solution × Washing Action × Day	0.0482*

* Main and/or interaction effect is significant ($P < 0.05$).

Table 5.4 *Escherichia coli* surrogate populations (log_{10} CFU/g) on lettuce leaves after application of washing treatments and storage at 4°C for up to 6 days (n = 6).[*]

Wash Solution	Agitation Wash					By Hand				
	Day					Day				
	Untreated	0	1	4	6	Untreated	0	1	4	6
Tap Water	6.79	4.98^{ax}	4.50^{ax}	4.72^{ax}	4.27^{ax}	6.58	5.72^{ax}	5.21^{ax}	4.37^{bx}	4.92^{abx}
5% Vinegar Solution	6.53	3.89^{aby}	3.79^{abx}	4.21^{ax}	3.25^{by}	6.42	4.87^{axy}	4.85^{ax}	4.21^{ax}	3.91^{by}
CAFVT	6.47	4.13^{axy}	4.67^{ax}	4.18^{ax}	4.38^{ax}	6.65	4.48^{ay}	4.33^{abx}	4.32^{abx}	3.62^{by}

CAFVT = commercial antimicrobial fruit and vegetable treatment.

[ab] Means with different superscripts within a row section are significantly different ($P < 0.05$) with [a] as the largest and [b] as the smallest values.

[xy] Means with different superscripts within a column section are significantly different ($P < 0.05$) with [x] as the largest and [y] as the smallest values.

[*]Standard error (SE) = 0.3036

The behavior of *E. coli* surrogate populations on green leaf lettuce over time in our study is similar to other studies that evaluated pathogenic *E. coli*, where no changes or slight decreases in *E. coli* O157:H7 populations were observed after storage of leafy greens at refrigeration temperatures. For example, Luo and others (2009) reported that *E. coli* O157:H7 populations on spinach samples stored at 1 and 5°C decreased over time, with significant reduction of populations noted within 3 days of storage. Moreover, Luo and others (2010) in another study on the effect of storage temperature on *E. coli* O157:H7 indicated that storage at 5°C of commercially packaged lettuce limited the growth or slightly decreased *E. coli* O157:H7 populations. Lopez-Velasco and others (2010) reported a slight decrease of *E. coli* O157:H7 populations after 15 days of storage at 4°C. Interestingly, *E. coli* populations decreased between day 0 and day 5, and by day 15 populations recovered to levels similar to initial populations. Lopez-Velasco and others (2010) suggested that this behavior might be due to the adaptability of the microorganism to the low temperatures and the recovery of injured cells.

Although the washing treatments (wash solution × washing action) used in this experiment achieved *E. coli* surrogate reductions ranging from 0.87 to 2.64 $\log_{10}$ CFU/g, none of the treatments were capable of completely eliminating *E. coli* surrogate contamination. However, storage of green leaf lettuce at refrigeration temperatures (4 ± 1°C) reduced the risk of potential proliferation of *E. coli* surrogates, but was not capable of eliminating the inoculated microorganism on the surface of green leaf lettuce.

Conclusions

Data from this study indicates that overall incorporation of chemical wash solutions (CAFVT or 5% vinegar solution) improved the reduction of *E. coli* surrogates population on the

surface of green leaf lettuce by 0.7 to 0.9 log, and agitation of wash solutions improves reductions by 0.7 log. In addition, storage of washed green leaf lettuce at refrigeration temperatures (4 ± 1°C) limited the growth and slightly decreased *E. coli* surrogates population during 6 days of storage. Based on these results washing with 5% white distilled vinegar solution represents a good alternative at foodservices or home use to decrease the potential microbial contamination on the surface of green leaf lettuce. However, when using chemical wash solutions it is important to maintain adequate concentrations to avoid possible negative quality and sensory defects due to over use. Agitation during washing is also advised as it can enhance the ability of the chemical wash solutions to reduce the microbial load on the surface of green leaf lettuce.

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Chapter 6
Summary and Implications

Consumption of raw produce has been associated with foodborne-disease outbreaks due to contamination with pathogenic microorganisms. Generally, produce commonly associated in foodborne-disease outbreaks are "salads," leafy vegetables, sprouts, tomatoes, and melons (Sivapalasingam and others 2004; Callejón and others 2015). For fresh produce, the risk of contamination begins at the field during production and harvesting. Washing plays an important role on fresh produce quality and safety. Washing during post-harvest processing is used mainly to improve produce quality (e.g. remove soil, chemical residues, and other debris from produce surfaces) and safety. The wash solutions used in our studies demonstrated antimicrobial activity against pathogens (*E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes), E. coli* surrogates, and indigenous microflora on the surface of fresh green leaf lettuce, tomatoes, and cantaloupes. These wash solutions represent an alternative for produce decontamination and prevention of cross-contamination during washing. Overall, incorporation of wash solutions or agitation (washing system) in the washing process compared to tap water alone reduced greater ($P < 0.05$) APC populations, pathogens, or *E. coli* surrogate populations from lettuce, tomato, and cantaloupe surfaces. However, none of the washing treatments were capable of completely eliminating microbial contamination.

Specific findings in our research indicate:

- The application of the chemical wash treatment (0.35% citric acid) was capable of reducing *E. coli* O157:H7 and *Salmonella* spp. by approximately 3 logs on the surface of green leaf lettuce and tomatoes. Post-treatment residual water with the chemical wash treatment contained populations below detection limits of 1.95 and 0.95 log CFU/ml for lettuce and tomatoes, respectively.

- The chemical wash treatment (0.35% citric acid) was capable of reducing ≤ 1.2 logs of *Salmonella* spp. and *L. monocytogenes* on inoculated cantaloupes. The lowest mean population of recovered *Salmonella* spp. was observed when wash treatment was applied for 60 or 120 s.
- Washing cantaloupes with tap water, 9% vinegar solution, or a commercial antimicrobial fruit and vegetable treatment (CAFVT) for 120 s by using a continuous motion water system reduced natural microflora on the surface of cantaloupes by approximately 1 log, which assists in reducing the probability of transferring microorganisms from rind to the flesh during slicing and cutting. However storage of fresh-cut cantaloupes at 4 ± 1°C should not exceed 3 days, since microbial populations were ≥ 5.2 $\log_{10}$ CFU/g on day 6.
- Washing lettuce with CAFVT or 5% vinegar solution reduced *E. coli* surrogate populations by approximately 2 logs. The continuous water motion washing system improved *E. coli* surrogate log reductions (P = 0.0072) by approximately 1 log compared to washing by hand. For example, washing lettuce with 5% vinegar solution with continuous agitation achieved log reductions of 2.64 $\log_{10}$ CFU/g, while 5% vinegar solution by hand achieved 1.55 $\log_{10}$ CFU/g. Overall, CAFVT and 5% vinegar solution applied with agitation showed the highest log reductions, 2.64 and 2.34 $\log_{10}$ CFU/g, respectively. Storage of green leaf lettuce for 6 days at 4 ± 1°C after washing reduced the risk of potential proliferation of *E. coli* surrogates.

Further research exploring the impact of these wash solutions on sensory and quality attributes need to be addressed. Since water quality plays an important role in the efficacy of sanitizers, further research needs to be conducted to understand the relationship between the wash solution pH, water temperature, agitation (sheer force), and organic matter content of water

on the effectiveness of these wash solutions. Moreover, in depth research on the synergetic effect of washing methods that involve mechanical action and novel antimicrobials with higher lethality needs to be conducted for different produce surfaces.

Appendix A
Statistical Analysis Program Code for Data Analyzed in Chapter 3: Validation of Washing Treatments to Reduce *Escherichia coli* O157:H7 and *Salmonella* spp. on the Surface of Green Leaf Lettuce and Tomatoes

The data is organized in six columns. Column 1 is the replication number of the entire experiment; Colum 2 is the washing treatment applied, where zero=inoculated sample no treated, ctrl=water, and CWT= chemical wash treatment; Column 3 is the exposure time in s; Column 4 is the type of produce; Column 5 is the sample number; and Column 6 is the log CFU average count for two duplicate plates.

Data

Rep	trt	time	product	sample	log;
1	zero	0	lettuce	1	7.785329835
1	zero	0	lettuce	2	7.40654018
1	CWT	30	lettuce	1	3.954242509
1	CWT	30	lettuce	2	5.45484486
1	CWT	60	lettuce	1	5.875061263
1	CWT	60	lettuce	2	5.354108439
1	CWT	120	lettuce	1	3.977723605
1	CWT	120	lettuce	2	5.122215878
1	ctrl	30	lettuce	1	6.58546073
1	ctrl	30	lettuce	2	6.511883361
1	ctrl	60	lettuce	1	5.752048448
1	ctrl	60	lettuce	2	5.832508913
1	ctrl	120	lettuce	1	5.744292983
1	ctrl	120	lettuce	2	5.854306042
2	zero	0	lettuce	1	7.812913357
2	zero	0	lettuce	2	7.841984805
2	CWT	30	lettuce	1	4.204119983
2	CWT	30	lettuce	2	5.469822016
2	CWT	60	lettuce	1	4.618048097
2	CWT	60	lettuce	2	6.093421685
2	CWT	120	lettuce	1	4.041392685
2	CWT	120	lettuce	2	4.612783857
2	ctrl	30	lettuce	1	6.174641193
2	ctrl	30	lettuce	2	5.051152522
2	ctrl	60	lettuce	1	6.053078443
2	ctrl	60	lettuce	2	5.984527313
2	ctrl	120	lettuce	1	5.949390007
2	ctrl	120	lettuce	2	6.303196057
3	zero	0	lettuce	1	7.986771734
3	zero	0	lettuce	2	7.638489257

3	CWT	30	lettuce	1	4.51851394
3	CWT	30	lettuce	2	4.190331698
3	CWT	60	lettuce	1	.
3	CWT	60	lettuce	2	4.740362689
3	CWT	120	lettuce	1	5.511883361
3	CWT	120	lettuce	2	3.929418926
3	ctrl	30	lettuce	1	4.190331698
3	ctrl	30	lettuce	2	4.970811611
3	ctrl	60	lettuce	1	4.716003344
3	ctrl	60	lettuce	2	4.698970004
3	ctrl	120	lettuce	1	4.414973348
3	ctrl	120	lettuce	2	4.176091259
1	zero	0	tomato	1	3.873511279
1	zero	0	tomato	2	3.105825462
1	CWT	30	tomato	1	1.260727422
1	CWT	30	tomato	2	2.618662269
1	CWT	60	tomato	1	0.42
1	CWT	60	tomato	2	0.42
1	CWT	120	tomato	1	0.42
1	CWT	120	tomato	2	0.42
1	ctrl	30	tomato	1	0.42
1	ctrl	30	tomato	2	0.42
1	ctrl	60	tomato	1	0.561757418
1	ctrl	60	tomato	2	0.862787413
1	ctrl	120	tomato	1	2.406855458
1	ctrl	120	tomato	2	0.561757418
2	zero	0	tomato	1	3.637304379
2	zero	0	tomato	2	3.070599314
2	CWT	30	tomato	1	0.42
2	CWT	30	tomato	2	0.42
2	CWT	60	tomato	1	0.42
2	CWT	60	tomato	2	0.42
2	CWT	120	tomato	1	0.42
2	CWT	120	tomato	2	0.42
2	ctrl	30	tomato	1	0.42
2	ctrl	30	tomato	2	0.561757418
2	ctrl	60	tomato	1	2.464847405
2	ctrl	60	tomato	2	2.894195878
2	ctrl	120	tomato	1	0.42
2	ctrl	120	tomato	2	0.42
3	zero	0	tomato	1	4.493723532
3	zero	0	tomato	2	3.122471463
3	CWT	30	tomato	1	1.260727422
3	CWT	30	tomato	2	2.618662269
3	CWT	60	tomato	1	0.42
3	CWT	60	tomato	2	0.42

3	CWT	120	tomato	1	0.42
3	CWT	120	tomato	2	0.42
3	ctrl	30	tomato	1	0.42
3	ctrl	30	tomato	2	0.42
3	ctrl	60	tomato	1	0.561757418
3	ctrl	60	tomato	2	0.862787413
3	ctrl	120	tomato	1	2.406855458
3	ctrl	120	tomato	2	0.561757418

Statistical analysis program

```
options nodate pageno=1;
libname dat “C:|\Users\CIVahl\Documents\KSU Consulting\Keyla_Lopez”;

data lett1; set dat.lettuce;
treat=1;
if trt="ctrl" and time=30 then treat=2;
if trt="ctrl" and time=60 then treat=3;
if trt="ctrl" and time=120 then treat=4;
if trt="CWT" and time=30 then treat=5;
if trt="CWT" and time=60 then treat=6;
if trt="CWT" and time=120 then treat=7;
run;
data lett2; set dat.lettuce;
if TRT='zero' then delete;
run;

data tom1; set dat.tomato;
treat=1;
if trt="ctrl" and time=30 then treat=2;
if trt="ctrl" and time=60 then treat=3;
if trt="ctrl" and time=120 then treat=4;
if trt="CWT" and time=30 then treat=5;
if trt="CWT" and time=60 then treat=6;
if trt="CWT" and time=120 then treat=7;
log2=log;
log3=log;
if log = 0.42 then log2=0.42/2;
if log = 0.42 then log3 = 0;
run;
data tom2; set dat.tomato;
if TRT='zero' then delete;
log2=log;
log3=log;
if log = 0.42 then log2=0.42/2;
if log = 0.42 then log3 = 0;
run;
```

```
ods rtf file="C:\Users\CIVahl\Documents\KSU Consulting\Keyla_Lopez\output_3.rtf";
ods graphics on;
title 'Split-plot analysis of Lettuce -- Treatment Comparisons';
proc mixed data=lett2;
  class rep trt time sample;
  model log=trt time trt*time sample trt*sample time*sample trt*time*sample/ddfm=kr;
  random rep rep*trt*time;
  lsmeans trt/pdiff cl;
  lsmeans trt*time/slice=trt pdiff cl;
run;
quit;

title 'Split-plot analysis of Lettuce with Time Zero -- Estimates of Log Reduction';
proc mixed data=lett1;
  class rep treat sample;
  model log=treat sample treat*sample/ddfm=kr;
  random rep rep*treat;
  lsmeans treat/pdiff cl;
  estimate 'ctrl 30' treat 1 -1 0 0 0 0 0/cl;
  estimate 'ctrl 60' treat 1 0 -1 0 0 0 0/cl;
  estimate 'ctrl 120' treat 1 0 0 -1 0 0 0/cl;
  estimate 'CWT 30' treat 1 0 0 0 -1 0 0/cl;
  estimate 'CWT 60' treat 1 0 0 0 0 -1 0/cl;
  estimate 'CWT 120' treat 1 0 0 0 0 0 -1/cl;
  estimate 'ctrl' treat 3 -1 -1 -1 0 0 0 /divisor=3 cl;
  estimate 'CWT' treat 3 0 0 0 -1 -1 -1/divisor=3 cl;
run;
quit;

title 'Split-plot analysis of Tomato with Values <LOD Set to LOD -- Treatment Comparisons';
proc mixed data=tom2;
  class rep trt time sample;
  model log=trt time trt*time sample trt*sample time*sample trt*time*sample/ddfm=kr;
  random rep rep*trt*time;
  lsmeans trt*time/pdiff cl;
  lsmeans time*sample trt*sample/pdiff cl;
run;
quit;

title 'Split-plot analysis of Tomato with Values <LOD Set to LOD with Time Zero -- Estimates
of Log Reduction';
proc mixed data=tom1;
  class rep treat sample;
  model log=treat sample treat*sample/ddfm=kr solution;
  random rep rep*treat;
```

```
  estimate 'ctrl 30' treat 1 -1 0 0 0 0 0/cl;
  estimate 'ctrl 60' treat 1 0 -1 0 0 0 0/cl;
  estimate 'ctrl 120' treat 1 0 0 -1 0 0 0/cl;
  estimate 'CWT 30' treat 1 0 0 0 -1 0 0/cl;
  estimate 'CWT 60' treat 1 0 0 0 0 -1 0/cl;
  estimate 'CWT 120' treat 1 0 0 0 0 0 -1/cl;
  estimate 'ctrl' treat 3 -1 -1 -1 0 0 0 /divisor=3 cl;
  estimate 'CWT' treat 3 0 0 0 -1 -1 -1/divisor=3 cl;
  estimate 'ctrl samp1' treat 6 -2 -2 -2 0 0 0 sample -3 3 treat*sample 3 3 -2 0 -2 0 -2 0 0 0 0 0 0 0
/divisor=6 cl;
  estimate 'ctrl samp2' treat 6 -2 -2 -2 0 0 0 sample 3 -3 treat*sample 3 3 0 -2 0 -2 0 -2 0 0 0 0 0 0
/divisor=6 cl;
  estimate 'CWT samp1'  treat 6 0 0 0 -2 -2 -2 sample -3 3 treat*sample 3 3 0 0 0 0 0 0 -2 0 -2 0 -
2 0 /divisor=6 cl;
  estimate 'CWT samp2'  treat 6 0 0 0 -2 -2 -2 sample 3 -3 treat*sample 3 3 0 0 0 0 0 0 0 -2 0 -2 0
-2 /divisor=6 cl;
  estimate '30' treat 2 -1 0 0 -1 0 0 /divisor=2 cl;
  estimate '60' treat 2 0 -1 0 0 -1 0 /divisor=2 cl;
  estimate '120' treat 2 0 0 -1 0 0 -1 /divisor=2 cl;
  estimate '30 samp1' treat 2 -1 0 0 -1 0 0 sample -1 1 treat*sample 1 1 -1 0 0 0 0 0 -1 0 0 0 0 0
/divisor=2 cl;
  estimate '30 samp2' treat 2 -1 0 0 -1 0 0 sample 1 -1 treat*sample 1 1 0 -1 0 0 0 0 0 -1 0 0 0 0
/divisor=2 cl;
  estimate '60 samp1' treat 2 0 -1 0 0 -1 0 sample -1 1 treat*sample 1 1 0 0 -1 0 0 0 0 0 -1 0 0 0
/divisor=2 cl;
  estimate '60 samp2' treat 2 0 -1 0 0 -1 0 sample 1 -1 treat*sample 1 1 0 0 0 -1 0 0 0 0 0 -1 0 0
/divisor=2 cl;
  estimate '120 samp1' treat 2 0 0 -1 0 0 -1 sample -1 1 treat*sample 1 1 0 0 0 0 0 -1 0 0 0 0 0 0 -1 0
/divisor=2 cl;
  estimate '120 samp2' treat 2 0 0 -1 0 0 -1 sample 1 -1 treat*sample 1 1 0 0 0 0 0 0 -1 0 0 0 0 0 0 -1
/divisor=2 cl;
run;
quit;
ods rtf close;
```

Appendix B
Exception Letter and Consent Form of Study # 7203

KANSAS STATE UNIVERSITY | University Research Compliance Office

TO: Kelly Getty
ASI
216 Call Hall

Proposal Number: 7203

FROM: Rick Scheidt, Chair
Committee on Research Involving Human Subjects

DATE: 06/05/14

RE: Proposal Entitled, "Validation of washing treatments to reduce microbial contamination on fresh produce: Effect of organic acids and storage time on quality paramenters"

The Committee on Research Involving Human Subjects / Institutional Review Board (IRB) for Kansas State University has reviewed the proposal identified above and has determined that it is EXEMPT from further IRB review. This exemption applies only to the proposal - as written – and currently on file with the IRB. Any change potentially affecting human subjects must be approved by the IRB prior to implementation and may disqualify the proposal from exemption.

Based upon information provided to the IRB, this activity is exempt under the criteria set forth in the Federal Policy for the Protection of Human Subjects, **45 CFR §46.101, paragraph b, category: 6, subsection:** .

Certain research is exempt from the requirements of HHS/OHRP regulations. A determination that research is exempt does not imply that investigators have no ethical responsibilities to subjects in such research; it means only that the regulatory requirements related to IRB review, informed consent, and assurance of compliance do not apply to the research.

Any unanticipated problems involving risk to subjects or to others must be reported immediately to the Chair of the Committee on Research Involving Human Subjects, the University Research Compliance Office, and if the subjects are KSU students, to the Director of the Student Health Center.

203 Fairchild Hall, Lower Mezzanine, Manhattan, KS 66506-1103 | 785-532-3224 | fax: 785-532-3278 | k-state.edu/research/comply

KANSAS STATE UNIVERSITY
INFORMED CONSENT FORM
(You must be over 18 in order to participate)

PROJECT TITLE: Validation of washing treatments to reduce microbial contamination on fresh produce: Effect of organic acids and storage time on quality paramenters.
PRINCIPAL INVESTIGATOR: Dr. Kelly J.K. Getty, Department Animal Sciences and Industry, 216 Call Hall, Manhattan, KS 66506
CONTACT NAME AND PHONE FOR ANY PROBLEMS/QUESTIONS*:* If you have any questions about this research project, please feel free to contact Dr. Kelly J.K. Getty at (785) 532- 2203 or kgetty@k-state.edu
IRB CHAIR CONTACT/PHONE INFORMATION. For additional information regarding your rights as a research subject, please feel free to contact Rick Scheidt, Chair, Committee on Research Involving Human Subjects, 203 Fairchild Hall, Kansas State University, Manhattan, KS 66506, (785) 532-3224 or Jerry Jaax, Associate Vice President for Research Compliance and University Veterinarian, 203 Fairchild Hall, Kansas State University, Manhattan, KS 66506, (785) 532-3224.
PURPOSE OF THE RESEARCH: To determine how the use of tap water and alternative antimicrobial solutions as washing treatments affect quality attributes on green leaf lettuce, tomatoes, and cantaloupes and to determine how food service facilities wash, prepare, and store produce.
PROCEDURES OR METHODS TO BE USED: You will be asked to respond to questions about fresh produce safety practices. ***Please carefully read each question and do not leave any items blank***. Individual responses will be completely anonymous. Please be assured that your responses will be confidential and all data will be reported as group data.
RISKS OR DISCOMFORTS ANTICIPATED: There are no anticipated risks or discomforts. You may discontinue your participation at any point.
BENEFITS ANTICIPATED: If you decide to participate in this study there may be no direct benefit to you. It is hoped that the information gained in this study will be utilized to understand which quality attributes are affected by the use of experimental washing treatments for produce.
EXTENT OF CONFIDENTIALITY: Only the researchers whom designed this study will have access to this information. Also, the data recorded will be held by a state entity and therefore are subjected to disclosure if required by law.
TERMS OF PARTICIPATION: ***I understand this project is research, and that my participation is completely voluntary. I also understand that if I decide to participate in this study, I may withdraw my consent at any time, and stop participating at any time without explanation, penalty, or loss of benefits, or academic standing to which I may otherwise be entitled.***
I verify that my signature below indicates that I have read and understand this consent form, and willingly agree to participate in this study under the terms described, and that my signature acknowledges that I have received a signed and dated copy of this consent form.

Participant name:

Participant signature: _____ *Date:* _____

Witness to Signature: (Project staff) _____ *Date:* _____

Appendix C
Fresh Produce Questionnaire for Study # 7203

Dear Participants,

Fresh fruits and vegetables undergo minimal processing in order to maintain quality and nutrirional attributes. Minimal processing causes greater perishability of fresh produce. Therefore, refrigerated temperatures are required to ensure shelf-life, quality, and safety of these products. Extension of shelf-life and quality of fresh produce is relevant due to its economic impact. For these reasons, there is a need to study new alternatives to maintaining quality, while inhibiting undesirable microbial growth in fresh fruits and vegetables.

Below, you will be asked to respond to questions about fresh produce safety practices performed in your food service facility. **If you currently do not work in a food service facility, but you have previously worked in food service or inspect facilities, please base your responses on those experiences.** ***Please answer your questions to the best of your ability.*** Individual responses will be completely anonymous. Please be assured that your responses will be confidential and all data will be reported as group data.

Your response is very important to the success of this study and to the quality of future food safety education. By participating in this survey you will be providing valuable information that will help us better understand the effect of minimal processing on the quality, safety, and shelf life of fresh produce. If you have any questions about this research project, please feel free to contact Dr. Kelly J.K. Getty at (785) 532- 2203 or kgetty@k-state.edu. For additional information regarding your rights as a research subject, please feel free to contact the University Research Compliance Office at 203 Fairchild Hall, Kansas State University, Manhattan, KS 66506, (785) 532-3224.

Thank you for your time in assisting us with this research project. IF YOU CANNOT COMPLETE THE SURVEY IN TIME, PLEASE MAIL TO KELLY GETTY AT 216 CALL HALL, MANHATTAN, KS 66506.

THANKS AGAIN,

Kelly J.K. Getty, Ph.D.
Associate Professor

Fresh Produce Survey

1. I work for the following food service facility (Please check one)
   ____School cafeteria
   ____State Agency
   ____USDA
   ____Other (specify) _____

2. What type of fresh fruits and vegetables are used in your facility (Please check ALL that apply)
   ____Fresh (Whole)
   ____Pre-prepared (i.e. fresh-cut, fresh wash, bagged)
   ____We do not use fresh vegetables in our facility.
   ____Other _____

3. How well is your staff trained to correctly wash and prepare (i.e. wash, clean, cut, store) fruits and vegetables (Please check ONE)
   _____Very well trained
   _____Well trained
   _____Adequately trained
   _____Somewhat trained
   _____Not trained

4. In your opinion, does your facility have adequate equipment dedicated to wash and prepare fresh fruits and vegetables?
   _____Yes
   _____No

   If your reply above was NO, please indicate what equipment you are lacking, lacking in capacity, or needing improvement (Please check ALL that apply)
   ____Refrigerators
   ____Sinks
   ____Counter tops
   ____Cutting boards
   ____Knives
   ____Other (specify) _____

   Please feel free to comment about your equipment needs and problems.
   _____
   _____
   _____
   _____
   _____

5. Does your facility have enough refrigerator capacity and other space to accommodate fresh fruits and vegetables?
   _____Yes
   _____No

6. How does your facility wash fresh fruits and vegetables?
   ____Cold tap water
   ____Antimicrobial wash (Please specify) _____
   ____Washing sink with or without antimicrobial
   ____Other (Please specify) _____

7. After washing does your facility store WHOLE fruits and vegetables that will be prepare the next day?
   _____Yes (If YES check ALL fruits and vegetables that apply)
   _____No

   ____Green leaf lettuce
   ____Romaine lettuce
   ____Tomatoes
   ____Carrots
   ____Cantaloupes
   ____Other (specify) _____

8. How long do you store WHOLE fruits and vegetables?
   _____ 1 day (overnight for use the following day)
   _____ 2 days
   _____ 3 days
   _____ 6 days
   _____ up to 7 days
   _____Not Applicable to our facility

9. What type(s) of containers are used to store fruits and vegetables (Please check ALL that apply)
   _____ Plastic containers with lids
   _____ Baking sheets covered with plastic bun bags
   _____ Baking sheets with racks and covered with plastic bun bags
   _____Other_____

10. What type fruits and vegetables do you prepare in your facility? (Please check ALL that apply)
    _____ Diced tomatoes
    _____ Sliced tomatoes
    _____ Shredded lettuce
    _____ Leaf lettuce for sandwich
    _____ Diced cantaloupes

_____ Cantaloupes wedges with rind
_____ Cantaloupe wedges without rind
_____Other (Specify)_____

11. How does your facility store prepared **leaf lettuce** for sandwiches?
_____ Plastic containers with lids
_____ Baking sheets covered with plastic bun bags
_____ Baking sheets with racks and covered with plastic bun bags
_____Other_____
_____Not Applicable to our facility

12. How does your facility store **shredded lettuce**?
_____Plastic containers with lids
_____ Baking sheets covered with plastic bun bags
_____ Baking sheets with racks and covered with plastic bun bags
_____Other_____
_____ Not Applicable to our facility

13. How does your facility store **sliced tomatoes**?
_____ Plastic containers with lids
_____ Baking sheets covered with plastic bun bags
_____ Baking sheets with racks and covered with plastic bun bags
_____Other_____
_____ Not Applicable to our facility

14. How does your facility store **diced tomatoes**?
_____ Plastic containers with lids
_____ Baking sheets covered with plastic bun bags
_____ Baking sheets with racks and covered with plastic bun bags
_____Other_____
_____ Not Applicable to our facility

15. How does your facility store **cubed cantaloupes**?
_____Plastic containers with lids
_____ Baking sheets covered with plastic bun bags
_____ Baking sheets with racks and covered with plastic bun bags
_____Other_____
_____ Not Applicable to our facility

16. How does your facility store **cantaloupe wedges with rind**?
_____ Plastic containers with lids
_____ Baking sheets covered with plastic bun bags
_____ Baking sheets with racks and covered with plastic bun bags
_____Other_____
_____ Not Applicable to our facility

17. How does your facility store **cantaloupe wedges without rind**?
    _____ Plastic containers with lids
    _____ Baking sheets covered with plastic bun bags
    _____ Baking sheets with racks and covered with plastic bun bags
    _____Other_____
    _____ Not Applicable to our facility

18. After preparation and serving, how long would you store **prepared leaf lettuce for sandwiches**?
    _____ 1 day (overnight for use the following day)
    _____ 2 days
    _____ 3 days
    _____ 6 days
    _____ up to 7 days
    _____Not Applicable to our facility

19. After preparation and serving, how long would you store **shredded lettuce**?
    _____ 1 day (overnight for use the following day)
    _____ 2 days
    _____ 3 days
    _____ 6 days
    _____ up to 7 days
    _____Not Applicable to our facility

20. After preparation and serving, how long would you store **sliced tomatoes for sandwiches**?
    _____ 1 day (overnight for use the following day)
    _____ 2 days
    _____ 3 days
    _____ 6 days
    _____ up to 7 days
    _____Not Applicable to our facility

21. After preparation and serving, how long would you store **cubed cantaloupes**?
    _____ 1 day (overnight for use the following day)
    _____ 2 days
    _____ 3 days
    _____ 6 days
    _____ up to 7 days
    _____Not Applicable to our facility

22. After preparation and serving, how long would you store cantaloupe wedges with rind?
    _____ 1 day (overnight for use the following day)
    _____ 2 days
    _____ 3 days
    _____ 6 days

_____ up to 7 days
_____Not Applicable to our facility

23. After preparation and serving, how long would you store **cantaloupe wedges without rind**?
    _____ 1 day (overnight for use the following day)
    _____ 2 days
    _____ 3 days
    _____ 6 days
    _____ up to 7 days
    _____Not Applicable to our facility

THANK YOU FOR YOUR PARTICIPATION!

Appendix D
Data and SAS Program for Efficacy of Washing Treatments on Native Microflora of Whole Cantaloupes (Phase II) of Chapter 4

The data is organized in five columns. Column 1 is the replication number of the entire experiment; Colum 2 is the cantaloupe sample type or shape; Colum 3 is the washing treatment applied, where untreated= non washed, tap water=control, Vinegar = 9% vinegar solution, and CAFVT= commercial antimicrobial for fruits and vegetables treatment; Column 4 is the day of storage and sampling; and Column 5 is the log CFU average count for two duplicate plates.

Rep	Sample_type	Treatment	Day	Log _CFU
1	Wedge	Untreated	0	5.066699
1	Wedge	Untreated	1	2.833784
1	Wedge	Untreated	3	5.109579
1	Wedge	Untreated	6	7.886491
1	Wedge	Tap water	0	3.907089
1	Wedge	Tap water	1	4.615424
1	Wedge	Tap water	3	4.674861
1	Wedge	Tap water	6	7.155336
1	Wedge	CAFVT	0	1.041393
1	Wedge	CAFVT	1	3.50974
1	Wedge	CAFVT	3	5.301464
1	Wedge	CAFVT	6	7.579
1	Wedge	Vinegar	0	3.353852
1	Wedge	Vinegar	1	1.740363
1	Wedge	Vinegar	3	2.43
1	Wedge	Vinegar	6	5.844166
1	Cubed	Untreated	0	2.187521
1	Cubed	Untreated	1	2.840733
1	Cubed	Untreated	3	6.591621
1	Cubed	Untreated	6	8.754042
1	Cubed	Tap water	0	3.138303
1	Cubed	Tap water	1	2.308564
1	Cubed	Tap water	3	4.654177
1	Cubed	Tap water	6	7.262807
1	Cubed	CAFVT	0	1.740363
1	Cubed	CAFVT	1	1.518514
1	Cubed	CAFVT	3	2.472756
1	Cubed	CAFVT	6	7.575419
1	Cubed	Vinegar	0	0.5
1	Cubed	Vinegar	1	0.5
1	Cubed	Vinegar	3	2.472756
1	Cubed	Vinegar	6	5.151982
2	Wedge	Untreated	0	3.261501
2	Wedge	Untreated	1	5.529238

```
2	Wedge	Untreated	3	6.111431
2	Wedge	Untreated	6	8.291813
2	Wedge	Tap water	0	2.082785
2	Wedge	Tap water	1	5.120574
2	Wedge	Tap water	3	4.007534
2	Wedge	Tap water	6	7.63799
2	Wedge	CAFVT	0	3.028164
2	Wedge	CAFVT	1	2.320146
2	Wedge	CAFVT	3	5.985307
2	Wedge	CAFVT	6	7.579212
2	Wedge	Vinegar	0	2.585461
2	Wedge	Vinegar	1	1.041393
2	Wedge	Vinegar	3	1.643453
2	Wedge	Vinegar	6	4.559907
2	Cubed	Untreated	0	3.406881
2	Cubed	Untreated	1	4.009876
2	Cubed	Untreated	3	7.781755
2	Cubed	Untreated	6	8.238673
2	Cubed	Tap water	0	1.995635
2	Cubed	Tap water	1	2.421604
2	Cubed	Tap water	3	4.566437
2	Cubed	Tap water	6	5.975891
2	Cubed	CAFVT	0	1.04
2	Cubed	CAFVT	1	1.995635
2	Cubed	CAFVT	3	2.47
2	Cubed	CAFVT	6	8.565139
2	Cubed	Vinegar	0	1.643453
2	Cubed	Vinegar	1	1.518514
2	Cubed	Vinegar	3	4.124178
2	Cubed	Vinegar	6	7.434089
/*
Notes: Added Graphics to visualize Data & Calculated CIs
*/
/* SAS Macros */
*Creates line plots for LSMeans of Trt*Shape*Time and Trt*Time Terms;
 %macro line_plots(lsm_list);
 *Creates a Lattice Line Plot for the estimates of the Three-Way Interaction Terms;
  data LSM_TSD;
    set &lsm_list;
    where Effect="Treatme*Sample_T*Day" & Alpha=0.05;
  run;

  proc sgpanel data=LSM_TSD;
    panelby Day Sample_Type / layout=lattice columns=4 rows=2;
    scatter y=Treatment x=Estimate / group=Treatment xerrorlower=Lower xerrorupper=Upper;
    rowaxis label="Treament";
```

```
        colaxis label="Log CFU" Min=-1 Max=11 Values=(0 to 10 by 2);
        title3 'Response Variable: Log_CFU';
        title4 'Treatment*Sample_Type*Day Interaction Plot';
 run;

 proc sgpanel data=LSM_TSD;
  panelby Treatment Sample_Type / layout=lattice columns=4 rows=2 novarname;
  scatter y=Day x=Estimate / group=Day xerrorlower=Lower xerrorupper=Upper;
  rowaxis label="Day";
        colaxis label="Log CFU" Min=-1 Max=11 Values=(0 to 10 by 2);
        title3 'Response Variable: Log_CFU';
        title4 'Treatment*Sample_Type*Day Interaction Plot';
 run;

*Creates a Lattice Line Plot for the estimates of the Two-Way Interaction Treatment*Day Term;
 data LSM_TD;
  set &lsm_list;
  where Effect="Treatment*Day" & Alpha=0.05;
 run;

 proc sgpanel data=LSM_TD;
  panelby Day / columns=4 rows=1;
  scatter y=Treatment x=Estimate / group=Treatment xerrorlower=Lower xerrorupper=Upper;
  rowaxis label="Treament";
        colaxis label="Log CFU" Min=-1 Max=11 Values=(0 to 10 by 2);
        title3 'Response Variable: Log_CFU';
        title4 'Treatment*Day Interaction Plot';
 run;
 proc sgpanel data=LSM_TD;
  panelby Treatment / columns=4 rows=1;
  scatter y=Day x=Estimate / group=Day xerrorlower=Lower xerrorupper=Upper;
  rowaxis label="Treament";
        colaxis label="Log CFU" Min=-1 Max=11 Values=(0 to 10 by 2);
        title3 'Response Variable: Log_CFU';
        title4 'Treatment*Day Interaction Plot';
 run;
%mend line_plots;

/* SAS Analysis */
ods rtf file = "C:\Users\bloedow\Documents\Consulting\Clients\Keyla Lopez (Summer 2015-
Present)\Analysis\SAS Output\Statistical Analysis on Cantaloupe Project (06_04_15).doc";

title 'Cantaloupe Project (Keyla Lopez)';

 *Imports the Full Cantaloupe Dataset from Excel into SAS;
  proc import out=Cantaloupe
```

```
   datafile='C:\Users\bloedow\Documents\Consulting\Clients\Keyla Lopez (Summer 2015-
Present)\Data\CANTALOUPE DATA TO BE ANALYZED.xlsx'
   dbms=xlsx
   replace;
   sheet="SAS Data";
   getnames=yes;
  run;

 *Creates truncated variables from the Full Cantaloupe Dataset;
  data Cantaloupe;
   set Cantaloupe;
   Log_CFU=round(Log_CFU,0.000001);
         where Sample_Type~="Surface";
  run;

 *Prints out the Full Cantaloupe Dataset;
  proc print data=Cantaloupe;
   title2 'Print-out of the Full Dataset';
  run;

 *Creates a Lattice Line Plot for the Full Cantaloupe Dataset;
  proc sgpanel data=Cantaloupe;
   panelby Day Sample_Type / layout=lattice columns=4 rows=2;
   scatter y=Treatment x=Log_CFU / group=Rep;
   rowaxis label="Treament";
         colaxis label="Log CFU" Min=-1 Max=11 Values=(0 to 10 by 2);
         title2 'Response Variable: Log CFU';
         title3 'Treatment*Sample_Type*Day Interaction Plot';
         title4 ;
  run;

  proc sgpanel data=Cantaloupe;
   panelby Treatment Sample_Type / layout=lattice columns=4 rows=2;
   scatter y=Day x=Log_CFU / group=Rep;
   rowaxis label="Day";
         colaxis label="Log CFU" Min=-1 Max=11 Values=(0 to 10 by 2);
         title2 'Response Variable: Log CFU';
         title3 'Treatment*Sample_Type*Day Interaction Plot';
         title4 ;
  run;

/* RCBD w/ Split-Split Plot Analysis */
  title2 'RCBD w/ Split-Split Design';
 *Corrects for non-estimable issue (Rep & Rep*Trt) while performing GLIMMIX analysis using
the setup of the experimental design;
  proc glimmix data=Cantaloupe nobound;
```

```
   class   Rep Treatment Sample_Type Day;
         model   Log_Cfu = Treatment|Sample_Type|Day ;
         random  Rep Rep*Treatment Sample_Type*Rep(Treatment);
   lsmeans Treatment|Sample_Type|Day  / cl ;
   lsmeans Treatment*Sample_Type*Day  / slice=Treatment*Sample_Type
slice=Sample_Type*Day slicediff=Treatment*Sample_Type slicediff=Sample_Type*Day;
   lsmeans Treatment*Day  / slice=Treatment slice=Day slicediff=Treatment slicediff=Day;
         title3 'Response Variable: Log_CFU';
         title4 'GLIMMIX Model (NOBOUND)';
   ods output lsmeans=RCBD_GLIMMIX_NOBOUND;
  run;

  %line_plots(RCBD_GLIMMIX_NOBOUND);

ods rtf close;
```

Appendix E
Data and SAS Programs for Effectiveness of a Commercially Available Fruit and Vegetables Wash for Reducing Pathogens (*Salmonella* spp. and *L. monocytogenes*) on Whole Cantaloupes (Phase III) of Chapter 4

Cantaloupes inoculated with Salmonella spp.

The data is organized in five columns. Column 1 is the replication number of the entire experiment; Colum 2 is the washing treatment applied, where Attachment= non washed, CTRL=control, and CPW= commercial produce wash; Colum 3 is the time of exposure, Column 4 is the produce, and Column 5 is the log CFU average count for two duplicate plates.

```
data cantaloupe; set dat.cantaloupe;
Input Rep trt time product log;
Datalines;
2       Attachment 1  0       Cantaloupe    6.592676004
2       CTRL          30      Cantaloupe    5.675797732
2       CTRL          60      Cantaloupe    6.026420843
2       CTRL          120     Cantaloupe    5.940310894
2       CPW           30      Cantaloupe    5.455114875
2       CPW           60      Cantaloupe    5.284806584
2       CPW           120     Cantaloupe    4.827888715
3       Attachment 1  0       Cantaloupe    6.02012661
3       CTRL          30      Cantaloupe    5.900439382
3       CTRL          60      Cantaloupe    5.646834036
3       CTRL          120     Cantaloupe    4.702949978
3       CPW           30      Cantaloupe    5.545218728
3       CPW           60      Cantaloupe    4.812824969
3       CPW           120     Cantaloupe    4.966191413
4       Attachment 1  0       Cantaloupe    6.199376388
4       CTRL          30      Cantaloupe    5.983776588
4       CTRL          60      Cantaloupe    5.791891062
4       CTRL          120     Cantaloupe    5.837648552
4       CPW           30      Cantaloupe    5.67789409
4       CPW           60      Cantaloupe    5.313158323
4       CPW           120     Cantaloupe    4.951591905
5       Attachment 1  0       Cantaloupe    6.111292748
5       CTRL          30      Cantaloupe    5.237514482
5       CTRL          60      Cantaloupe    5.065249631
5       CTRL          120     Cantaloupe    5.010510841
5       CPW           30      Cantaloupe    4.452779953
5       CPW           60      Cantaloupe    4.150577771
5       CPW           120     Cantaloupe    2.702949978
6       Attachment 1  0       Cantaloupe    5.717826747
6       CTRL          30      Cantaloupe    5.229759017
```

```
6       CTRL        60      Cantaloupe      5.035694387
6       CTRL        120     Cantaloupe      5.539504245
6       CPW         30      Cantaloupe      5.171207773
6       CPW         60      Cantaloupe      4.774943501
6       CPW         120     Cantaloupe      4.920832541
run;

data cant1; set cantaloupe;
treat=1;
if trt="CTRL" and time=30 then treat=2;
if trt="CTRL" and time=60 then treat=3;
if trt="CTRL" and time=120 then treat=4;
if trt="CPW" and time=30 then treat=5;
if trt="CPW" and time=60 then treat=6;
if trt="CPW" and time=120 then treat=7;
run;

data cant2; set cantaloupe;
  if trt="Attachment 1" then delete;
run;

*ods rtf file="C:\Users\CIVahl\Documents\KSU Consulting\Keyla_Lopez\cantaloupe_v1.rtf";
ods graphics on;

Title 'RCBD Analysis of Cantaloupe -- Treatment Comparisons';
proc mixed data=cant2 plots=all;
   class rep trt time;
   model log=trt time trt*time;
   random rep ;
   lsmeans trt|time/pdiff cl;
   lsmeans trt*time/pdiff adjust=bonferroni;
   lsmeans trt*time/pdiff adjust=scheffe;
   lsmeans trt*time/pdiff adjust=tukey;
run;
quit;

title 'RCBD Analysis of Cantaloupe with Time Zero -- Estimates of Log Reduction';
proc mixed data=cant1 plots=all;
   class rep treat;
   model log=treat;
   random rep;
   lsmeans treat/pdiff cl;
   estimate 'CTRL 30' treat 1 -1 0 0 0 0 0/cl;
   estimate 'CTRL 60' treat 1 0 -1 0 0 0 0/cl;
   estimate 'CTRL 120' treat 1 0 0 -1 0 0 0/cl;
   estimate 'CPW 30' treat 1 0 0 0 -1 0 0/cl;
```

```
   estimate 'CPW 60' treat 1 0 0 0 0 -1 0/cl;
   estimate 'CPW 120' treat 1 0 0 0 0 0 -1/cl;
   estimate 'CTRL' treat 3 -1 -1 -1 0 0 0 /divisor=3 cl;
   estimate 'CPW' treat 3 0 0 0 -1 -1 -1/divisor=3 cl;
run;
quit;
*ods rtf close;
```

Cantaloupes inoculated with *Listeria monocytogenes*

The data is organized in five columns. Column 1 is day in which the experiment was run; Column 2 is the replication number of the entire experiment is the washing treatment applied, Colum 3 is the washing treatment applied, where Attachment= non washed, CTRL=control, and CPW= commercial produce wash, Column 4 is the exposure time, and Column 5 is the log CFU average count for two duplicate plates.

```
Data cantaloupeLM;
input day rep trt$ time log;
datalines ;
1       1       ATTACHMENT      0       6.059911196
1       2       ATTACHMENT      0       6.155394953
2       3       ATTACHMENT      0       5.6363091s97
2       4       ATTACHMENT      0       5.705945125
3       5       ATTACHMENT      0       5.589501417
3       6       ATTACHMENT      0       7.038009354
1       1       CTRL            120     5.221524631
1       2       CTRL            120     5.609035112
1       3       CTRL            120     5.161072086
2       4       CTRL            120     4.744403377
2       5       CTRL            120     5.097403006
2       6       CTRL            120     5.22882387
3       7       CTRL            120     5.799626168
3       8       CTRL            120     6.163883063
3       9       CTRL            120     5.623858888
1       1       CPW             120     5.204000286
1       2       CPW             120     5.376671886
1       3       CPW             120     5.161072086
2       4       CPW             120     4.193659453
2       5       CPW             120     4.034249205
2       6       CPW             120     3.7588812
3       7       CPW             120     5.432943853
3       8       CPW             120     5.663747445
3       9       CPW             120     5.416031134
;
run;
data cant1; set cantaloupe;
```

```
treat=1;
if trt="CTRL" and time=120 then treat=2;
if trt="CPW" and time=120 then treat=3;
run;

data cant2; set cantaloupe;
  if trt="ATTACHME" then delete;
run;

ods rtf file="C:\Users\CIVahl\Documents\KSU Consulting\Keyla_Lopez\cantaloupe_v2.rtf";
ods graphics on;
Title 'GRCBD Analysis of Cantaloupe -- Treatment Comparisons';
proc mixed data=cant2;
   class day trt;
   model log=trt/ ddfm= satterth;
   random day;
   lsmeans trt/pdiff cl;
run;
quit;

title 'GRCBD Analysis of Cantaloupe with Time Zero -- Estimates of Log Reduction';
proc mixed data=cant1;
   class day treat;
   model log=treat/ddfm= satterth;
   random day;
   lsmeans treat/pdiff cl;
   estimate 'CTRL 120' treat 1 -1 0 /cl;
   estimate 'CPW 120' treat 1 0 -1 /cl;
run;

quit;
ods rtf close;
```

Appendix F
Statistical Analysis Program Code Utilized to Analyze Data in Chapter 5: Efficacy of a Washing System and Commercial Produce Washes to Reduce *Escherichia coli* Surrogates on Green Leaf Lettuce

Background samples data

The background data is organized in five columns. Column 1 is the replication number of the entire experiment; Colum 2 is the washing treatment applied, where water=control, Vinegar= 5% vinegar solution and water mix, and CAFVT= commercial antimicrobial for fruits and vegetables treatment; Colum 3 is the washing action applied to produce; Column 4 is the cantaloupe sample; Column 4 is the colony forming unit (CFU); and Column 5 is the log CFU.

Rep	Solution	Action	Sample	CFU	Log CFU
1	CAFVT	Hand	S1	10500	4.021189299
1	Water	Agitation	S1	2700	3.431363764
1	Water	Hand	S1	4800	3.681241237
1	CAFVT	Agitation	S1	34800	4.541579244
1	Vinegar	Hand	S1	2100	3.322219295
1	Vinegar	Agitation	S1	45600	4.658964843
2	Water	Hand	S1	468000	5.670245853
2	Vinegar	Hand	S1	2370000	6.374748346
2	CAFVT	Hand	S1	381000	5.580924976
2	Water	Agitation	S1	354000	5.549003262
2	CAFVT	Agitation	S1	642000	5.807535028
2	Vinegar	Agitation	S1	804000	5.905256049
3	Water	Hand	S1	864000	5.936513742
3	Vinegar	Hand	S1	402000	5.604226053
3	CAFVT	Agitation	S1	237000	5.374748346
3	Vinegar	Agitation	S1	1119000	6.048830087
3	Water	Agitation	S1	414000	5.617000341
3	CAFVT	Hand	S1	254400	5.405517107

Contaminated/Inoculated samples data

The contaminated data is organized in five columns. Column 1 is the replication number of the entire experiment; Colum 2 is the washing treatment applied, where water=control, Vinegar= 5% vinegar solution and water mix, and CAFVT= commercial antimicrobial for fruits and vegetables treatment; Colum 3 is the washing action applied to produce; Column 4 is the cantaloupe sample; Column 4 is the colony forming unit (CFU); and Column 5 is the log CFU.

Rep	Solution	Action	Sample	CFU	Log CFU
1	CAFVT	Hand	S1	6360000	6.803457116
1	CAFVT	Hand	S2	5130000	6.710117365
1	Water	Agitation	S1	11520000	7.061452479
1	Water	Agitation	S2	3000000	6.477121255
1	Water	Hand	S1	1590000	6.201397124
1	Water	Hand	S2	6450000	6.809559715
1	CAFVT	Agitation	S1	9180000	6.962842681
1	CAFVT	Agitation	S2	1380000	6.139879086
1	Vinegar	Hand	S1	10560000	7.023663918
1	Vinegar	Hand	S2	510000	5.707570176
1	Vinegar	Agitation	S1	3330000	6.522444234
1	Vinegar	Agitation	S2	5400000	6.73239376
2	Water	Hand	S1	2340000	6.369215857
2	Water	Hand	S2	9450000	6.975431809
2	Vinegar	Hand	S1	6930000	6.840733235
2	Vinegar	Hand	S2	3270000	6.514547753
2	CAFVT	Hand	S1	1590000	6.201397124
2	CAFVT	Hand	S2	3420000	6.534026106
2	Water	Agitation	S1	8190000	6.913283902
2	Water	Agitation	S2	2760000	6.440909082
2	CAFVT	Agitation	S1	2280000	6.357934847
2	CAFVT	Agitation	S2	2610000	6.416640507
2	Vinegar	Agitation	S1	2130000	6.328379603
2	Vinegar	Agitation	S2	3540000	6.549003262

3	Water	Hand	S1	5910000	6.771587481
3	Water	Hand	S2	2400000	6.380211242
3	Vinegar	Hand	S1	780000	5.892094603
3	Vinegar	Hand	S2	3270000	6.514547753
3	CAFVT	Agitation	S1	2580000	6.411619706
3	CAFVT	Agitation	S2	3390000	6.530199698
3	Vinegar	Agitation	S1	870000	5.939519253
3	Vinegar	Agitation	S2	12480000	7.096214585
3	Water	Agitation	S1	8070000	6.906873535
3	Water	Agitation	S2	9270000	6.967079734
3	CAFVT	Hand	S1	4350000	6.638489257
3	CAFVT	Hand	S2	10890000	7.03702788

Log CFU data before and after washing treatments

The data is organized in eight columns. Column 1 is the replication number of the entire experiment; Colum 2 is the day of storage or sampling; Colum 3 is the washing treatment applied, where water=control, Vinegar= 5% vinegar solution and water mix, and CAFVT= commercial antimicrobial for fruits and vegetables treatment; Colum 4 is the washing action applied to produce; Column 5 is the sample status of washing; Column 6 is the lettuce sample; Column 7 is the colony forming unit (CFU); and Column 8 is the log CFU.

Rep	Day	Solution	Action	Status	Sample	CFU	Log CFU
1	0	CAFVT	Hand	Untreated	S1	6360000	6.803457
1	0	CAFVT	Hand	Untreated	S2	5130000	6.710117
1	0	Water	Agitation	Untreated	S1	11520000	7.061452
1	0	Water	Agitation	Untreated	S2	3000000	6.477121
1	0	Water	Hand	Untreated	S1	1590000	6.201397
1	0	Water	Hand	Untreated	S2	6450000	6.809560
1	0	CAFVT	Agitation	Untreated	S1	9180000	6.962843
1	0	CAFVT	Agitation	Untreated	S2	1380000	6.139879
1	0	Vinegar	Hand	Untreated	S1	10560000	7.023664
1	0	Vinegar	Hand	Untreated	S2	510000	5.707570
1	0	Vinegar	Agitation	Untreated	S1	3330000	6.522444
1	0	Vinegar	Agitation	Untreated	S2	5400000	6.732394
2	0	Water	Hand	Untreated	S1	2340000	6.369216
2	0	Water	Hand	Untreated	S2	9450000	6.975432
2	0	Vinegar	Hand	Untreated	S1	6930000	6.840733
2	0	Vinegar	Hand	Untreated	S2	3270000	6.514548
2	0	CAFVT	Hand	Untreated	S1	1590000	6.201397
2	0	CAFVT	Hand	Untreated	S2	3420000	6.534026
2	0	Water	Agitation	Untreated	S1	8190000	6.913284
2	0	Water	Agitation	Untreated	S2	2760000	6.440909
2	0	CAFVT	Agitation	Untreated	S1	2280000	6.357935
2	0	CAFVT	Agitation	Untreated	S2	2610000	6.416641
2	0	Vinegar	Agitation	Untreated	S1	2130000	6.328380

2	0	Vinegar	Agitation	Untreated	S2	3540000	6.549003
3	0	Water	Hand	Untreated	S1	5910000	6.771587
3	0	Water	Hand	Untreated	S2	2400000	6.380211
3	0	Vinegar	Hand	Untreated	S1	780000	5.892095
3	0	Vinegar	Hand	Untreated	S2	3270000	6.514548
3	0	CAFVT	Agitation	Untreated	S1	2580000	6.411620
3	0	CAFVT	Agitation	Untreated	S2	3390000	6.530200
3	0	Vinegar	Agitation	Untreated	S1	870000	5.939519
3	0	Vinegar	Agitation	Untreated	S2	12480000	7.096215
3	0	Water	Agitation	Untreated	S1	8070000	6.906874
3	0	Water	Agitation	Untreated	S2	9270000	6.967080
3	0	CAFVT	Hand	Untreated	S1	4350000	6.638489
3	0	CAFVT	Hand	Untreated	S2	10890000	7.037028
1	0	CAFVT	Hand	Treated	S1	22200	4.346353
1	0	CAFVT	Hand	Treated	S2	83400	4.921166
1	0	Water	Agitation	Treated	S1	195000	5.290035
1	0	Water	Agitation	Treated	S2	501000	5.699838
1	0	Water	Hand	Treated	S1	894000	5.951338
1	0	Water	Hand	Treated	S2	83700	4.922725
1	0	CAFVT	Agitation	Treated	S1	5310	3.725095
1	0	CAFVT	Agitation	Treated	S2	3570	3.552668
1	0	Vinegar	Hand	Treated	S1	104100	5.017451
1	0	Vinegar	Hand	Treated	S2	146700	5.166430
1	0	Vinegar	Agitation	Treated	S1	642	2.807535
1	0	Vinegar	Agitation	Treated	S2	12030	4.080266
1	1	CAFVT	Hand	Treated	S1	14340	4.156549
1	1	CAFVT	Hand	Treated	S2	57900	4.762679
1	1	Water	Agitation	Treated	S1	213000	5.328380
1	1	Water	Agitation	Treated	S2	13350	4.125481
1	1	Water	Hand	Treated	S1	125400	5.098298
1	1	Water	Hand	Treated	S2	34200	4.534026

1	1	CAFVT	Agitation	Treated	S1	53100	4.725095
1	1	CAFVT	Agitation	Treated	S2	41400	4.617000
1	1	Vinegar	Hand	Treated	S1	177000	5.247973
1	1	Vinegar	Hand	Treated	S2	36900	4.567026
1	1	Vinegar	Agitation	Treated	S1	2040	3.309630
1	1	Vinegar	Agitation	Treated	S2	3060	3.485721
1	4	CAFVT	Hand	Treated	S1	1110	3.045323
1	4	CAFVT	Hand	Treated	S2	5280	3.722634
1	4	Water	Agitation	Treated	S1	258000	5.411620
1	4	Water	Agitation	Treated	S2	107400	5.031004
1	4	Water	Hand	Treated	S1	15570	4.192289
1	4	Water	Hand	Treated	S2	4500	3.653213
1	4	CAFVT	Agitation	Treated	S1	14610	4.164650
1	4	CAFVT	Agitation	Treated	S2	2400	3.380211
1	4	Vinegar	Hand	Treated	S1	49800	4.697229
1	4	Vinegar	Hand	Treated	S2	83400	4.921166
1	4	Vinegar	Agitation	Treated	S1	33300	4.522444
1	4	Vinegar	Agitation	Treated	S2	6000	3.778151
1	6	CAFVT	Hand	Treated	S1	5670	3.753583
1	6	CAFVT	Hand	Treated	S2	180	2.255273
1	6	Water	Agitation	Treated	S1	30000	4.477121
1	6	Water	Agitation	Treated	S2	37500	4.574031
1	6	Water	Hand	Treated	S1	59100	4.771587
1	6	Water	Hand	Treated	S2	10680	4.028571
1	6	CAFVT	Agitation	Treated	S1	30000	4.477121
1	6	CAFVT	Agitation	Treated	S2	870	2.939519
1	6	Vinegar	Hand	Treated	S1	2220	3.346353
1	6	Vinegar	Hand	Treated	S2	19800	4.296665
1	6	Vinegar	Agitation	Treated	S1	210	2.322219
1	6	Vinegar	Agitation	Treated	S2	690	2.838849
2	0	Water	Hand	Treated	S1	1050000	6.021189

2	0	Water	Hand	Treated	S2	327000	5.514548
2	0	Vinegar	Hand	Treated	S1	20400	4.309630
2	0	Vinegar	Hand	Treated	S2	82500	4.916454
2	0	CAFVT	Hand	Treated	S1	86700	4.938019
2	0	CAFVT	Hand	Treated	S2	6570	3.817565
2	0	Water	Agitation	Treated	S1	28200	4.450249
2	0	Water	Agitation	Treated	S2	72300	4.859138
2	0	CAFVT	Agitation	Treated	S1	13350	4.125481
2	0	CAFVT	Agitation	Treated	S2	3750	3.574031
2	0	Vinegar	Agitation	Treated	S1	6750	3.829304
2	0	Vinegar	Agitation	Treated	S2	6360	3.803457
2	1	Water	Hand	Treated	S1	67500	4.829304
2	1	Water	Hand	Treated	S2	495000	5.694605
2	1	Vinegar	Hand	Treated	S1	20400	4.309630
2	1	Vinegar	Hand	Treated	S2	27600	4.440909
2	1	CAFVT	Hand	Treated	S1	2370	3.374748
2	1	CAFVT	Hand	Treated	S2	31200	4.494155
2	1	Water	Agitation	Treated	S1	6300	3.799341
2	1	Water	Agitation	Treated	S2	23400	4.369216
2	1	CAFVT	Agitation	Treated	S1	49500	4.694605
2	1	CAFVT	Agitation	Treated	S2	42300	4.626340
2	1	Vinegar	Agitation	Treated	S1	744	2.871573
2	1	Vinegar	Agitation	Treated	S2	5640	3.751279
2	4	Water	Hand	Treated	S1	5700	3.755875
2	4	Water	Hand	Treated	S2	11490	4.060320
2	4	Vinegar	Hand	Treated	S1	1320	3.120574
2	4	Vinegar	Hand	Treated	S2	8550	3.931966
2	4	CAFVT	Hand	Treated	S1	61200	4.786751
2	4	CAFVT	Hand	Treated	S2	102000	5.008600
2	4	Water	Agitation	Treated	S1	6210	3.793092
2	4	Water	Agitation	Treated	S2	71400	4.853698

2	4	CAFVT	Agitation	Treated	S1	25200	4.401401
2	4	CAFVT	Agitation	Treated	S2	50100	4.699838
2	4	Vinegar	Agitation	Treated	S1	7110	3.851870
2	4	Vinegar	Agitation	Treated	S2	17700	4.247973
2	6	Water	Hand	Treated	S1	120000	5.079181
2	6	Water	Hand	Treated	S2	28200	4.450249
2	6	Vinegar	Hand	Treated	S1	5760	3.760422
2	6	Vinegar	Hand	Treated	S2	9150	3.961421
2	6	CAFVT	Hand	Treated	S1	4920	3.691965
2	6	CAFVT	Hand	Treated	S2	1320	3.120574
2	6	Water	Agitation	Treated	S1	12690	4.103462
2	6	Water	Agitation	Treated	S2	1830	3.262451
2	6	CAFVT	Agitation	Treated	S1	53100	4.725095
2	6	CAFVT	Agitation	Treated	S2	62700	4.797268
2	6	Vinegar	Agitation	Treated	S1	1050	3.021189
2	6	Vinegar	Agitation	Treated	S2	660	2.819544
3	0	Water	Hand	Treated	S1	1176000	6.070407
3	0	Water	Hand	Treated	S2	669000	5.825426
3	0	Vinegar	Hand	Treated	S1	124800	5.096215
3	0	Vinegar	Hand	Treated	S2	48600	4.686636
3	0	CAFVT	Agitation	Treated	S1	63900	4.805501
3	0	CAFVT	Agitation	Treated	S2	102600	5.011147
3	0	Vinegar	Agitation	Treated	S1	28500	4.454845
3	0	Vinegar	Agitation	Treated	S2	22500	4.352183
3	0	Water	Agitation	Treated	S1	84000	4.924279
3	0	Water	Agitation	Treated	S2	45000	4.653213
3	0	CAFVT	Hand	Treated	S1	21600	4.334454
3	0	CAFVT	Hand	Treated	S2	34200	4.534026
3	1	Water	Hand	Treated	S1	384000	5.584331
3	1	Water	Hand	Treated	S2	327000	5.514548
3	1	Vinegar	Hand	Treated	S1	1164000	6.065953

3	1	Vinegar	Hand	Treated	S2	30600	4.485721
3	1	CAFVT	Agitation	Treated	S1	19200	4.283301
3	1	CAFVT	Agitation	Treated	S2	122400	5.087781
3	1	Vinegar	Agitation	Treated	S1	21300	4.328380
3	1	Vinegar	Agitation	Treated	S2	93300	4.969882
3	1	Water	Agitation	Treated	S1	45000	4.653213
3	1	Water	Agitation	Treated	S2	55200	4.741939
3	1	CAFVT	Hand	Treated	S1	37200	4.570543
3	1	CAFVT	Hand	Treated	S2	44700	4.650308
3	4	Water	Hand	Treated	S1	294000	5.468347
3	4	Water	Hand	Treated	S2	130500	5.115611
3	4	Vinegar	Hand	Treated	S1	10560	4.023664
3	4	Vinegar	Hand	Treated	S2	35700	4.552668
3	4	CAFVT	Agitation	Treated	S1	5340	3.727541
3	4	CAFVT	Agitation	Treated	S2	53400	4.727541
3	4	Vinegar	Agitation	Treated	S1	65400	4.815578
3	4	Vinegar	Agitation	Treated	S2	10350	4.014940
3	4	Water	Agitation	Treated	S1	46200	4.664642
3	4	Water	Agitation	Treated	S2	36600	4.563481
3	4	CAFVT	Hand	Treated	S1	17400	4.240549
3	4	CAFVT	Hand	Treated	S2	136500	5.135133
3	6	Water	Hand	Treated	S1	489000	5.689309
3	6	Water	Hand	Treated	S2	321000	5.506505
3	6	Vinegar	Hand	Treated	S1	2040	3.309630
3	6	Vinegar	Hand	Treated	S2	60900	4.784617
3	6	CAFVT	Agitation	Treated	S1	23400	4.369216
3	6	CAFVT	Agitation	Treated	S2	91500	4.961421
3	6	Vinegar	Agitation	Treated	S1	6060	3.782473
3	6	Vinegar	Agitation	Treated	S2	52200	4.717671
3	6	Water	Agitation	Treated	S1	14520	4.161967
3	6	Water	Agitation	Treated	S2	112200	5.049993

3	6	CAFVT	Hand	Treated	S1	20400	4.309630
3	6	CAFVT	Hand	Treated	S2	36600	4.563481

Statistical analysis code

```
ods rtf file = "C……..doc";

title 'Lettuce Wash Project (Keyla Lopez)';

/* Statistical Analysis for Background Dataset */
title2 'Background Dataset';
*Creates truncated variables from the Full Lettuce Background Dataset;
data LW_Background;
set LW_Background;
Log_CFU=round(Log_CFU,0.000001);
Trt=trim(Wash_Solution)||"_"||Washing_Action;
run;
*Prints out the Full Lettuce Background Dataset;
proc print data=LW_Background;
run;
*Performs MIXED analysis using the setup of the experimental design;
proc mixed data=LW_Background;
class Rep Wash_Solution Washing_Action;
model Log_CFU = Wash_Solution|Washing_Action;
random Rep;
lsmeans Wash_Solution Washing_Action / cl;
lsmeans Wash_Solution*Washing_Action / cl pdiff adjust=tukey;

/* Statistical Analysis for Contaminated Dataset */
title2 'Contaminated Dataset';
*Creates truncated variables from the Full Lettuce Contaminated Dataset;
data LW_Contaminated;
set LW_Contaminated;
Log_CFU=round(Log_CFU,0.000001);
run;
*Prints out the Full Lettuce Contaminated Dataset;
proc print data=LW_Contaminated;
run;
*Performs MIXED analysis using the setup of the experimental design;
proc mixed data=LW_Contaminated;
class Rep Wash_Solution Washing_Action Sample;
model Log_CFU = Wash_Solution|Washing_Action;
random Rep Rep*Wash_Solution*Washing_Action;
lsmeans Wash_Solution Washing_Action / cl;
lsmeans Wash_Solution*Washing_Action / cl pdiff adjust=tukey;

/* Statistical Analysis for Reduction Dataset */
title2 'Reduction Dataset';
*Creates truncated variables from the Full Lettuce Reduction Dataset;
data LW_Reduction;
set LW_Reduction;
Log_CFU=round(Log_CFU,0.000001);
```

```
run;
*Sorts the Full Lettuce Reduction Dataset;
proc sort data=LW_Reduction;
by descending Status  Rep Washing_Action Wash_Solution Day Sample;run;
*Prints out the Full Lettuce Reduction Dataset;
proc print data=LW_Reduction;
run;
*Creates a Full Lettuce Treated Reduction Dataset;
data LW_Reduct_Trt;
set LW_Reduction;
where Status="Treated";
CFU_After=CFU;
Log_CFU_After=Log_CFU;
drop Status CFU Log_CFU;
run;
*Sorts the Full Lettuce Treated Reduction Dataset;
proc sort data=LW_Reduct_Trt;
by Rep Washing_Action Wash_Solution Sample Day;
run;
*Creates a Full Lettuce Untreated Reduction Dataset;
data LW_Reduct_Untrt;
set LW_Reduction;
where Status="Untreated";
CFU_Before=CFU;
Log_CFU_Before=Log_CFU;
drop Status CFU Log_CFU;
run;
*Sorts the Full Lettuce Untreated Reduction Dataset;
proc sort data=LW_Reduct_Untrt;
by run;
*Creates the Full Combined Lettuce Reduction Dataset;
data LW_Reduct;
merge LW_Reduct_Untrt LW_Reduct_Trt;
by Rep Washing_Action Wash_Solution Sample;
run;
*Sorts the Full Combined Lettuce Reduction Dataset;
proc sort data=LW_Reduct;
by Rep Washing_Action Wash_Solution Day Sample;
run;
proc means data=LW_Reduct noprint;
var Log_CFU_Before Log_CFU_After;
by Rep Washing_Action Wash_Solution Day;
output out=LW_Reduct_Avg(drop=_TYPE_ drop=_FREQ_) mean= ;
run;
*Creates new Reduction Factor variable for the Combined Lettuce Reduction Dataset;
data LW_Reduct_Avg;
set LW_Reduct_Avg;
RF=Log_CFU_Before-Log_CFU_After;
run;
*Prints out the the Combined Lettuce Reduction Dataset;
proc print data=LW_Reduct_Avg;
run;

title2 'Reduction Factor (All Days)';
 *Performs GLIMMIX Model Analysis using Compound Symmetry RM Covariance Structure;
proc glimmix data=LW_Reduct_Avg ;
```

```
class Rep Wash_Solution Washing_Action Day;
model RF = Wash_Solution|Washing_Action|Day / ddfm=KR;
random Rep ;
random Day / residual subject=Rep*Wash_Solution*Washing_Action type=CS;              lsmeans
Wash_Solution|Washing_Action|Day / cl;
lsmeans Wash_Solution*Washing_Action*Day / slice=Washing_Action*Day slicediff=Washing_Action*Day
slice=Wash_Solution*Washing_Action              slicediff=Wash_Solution*Washing_Action adjust=tukey;
ods output lsmeans=LW_Reduct_Avg_Glimmix;
title3 'GLIMMIX Model (RCBD w/ Repeated Measures)';
title4 'Compound Symmetry RM Covariance Structure';
run;

title2 'Log CFU After Wash (All Days)';

*Performs GLIMMIX Model Analysis on LogCFU using Compound Symmetry RM Covariance Structure;
proc glimmix data=LW_Reduct_Avg ;
class Rep Wash_Solution Washing_Action Day;
model Log_CFU_After = Wash_Solution|Washing_Action|Day / ddfm=KR;
random Rep ;
random Day / residual subject=Rep*Wash_Solution*Washing_Action type=CS;
lsmeans Wash_Solution|Washing_Action|Day / cl;
lsmeans Wash_Solution*Washing_Action*Day / slice=Washing_Action*Day slicediff=Washing_Action*Day
slice=Wash_Solution*Washing_Action     slicediff=Wash_Solution*Washing_Action adjust=Tukey;
ods output lsmeans=LW_Reduct_Avg_Glimmix;
title3 'GLIMMIX Model (RCBD w/ Repeated Measures)';
title4 'Compound Symmetry RM Covariance Structure';
run;

title2 'Reduction Factor (Day 0 Only)';
*Creates truncated variables from the Full Lettuce Background Dataset;
data LW_Reduct_Avg_Day0;
set LW_Reduct_Avg;
where Day=0;
run;
*Performs GLIMMIX analysis using the setup of the experimental design;
proc glimmix data=LW_Reduct_Avg_Day0;
class Rep Wash_Solution Washing_Action;
model RF = Wash_Solution|Washing_Action;
random Rep;
lsmeans Wash_Solution Washing_Action / cl pdiff=all lines adjust=Tukey;
lsmeans Wash_Solution*Washing_Action / cl pdiff=all lines  slice=Washing_Action slicediff=Washing_Action
slice=Wash_Solution                                        slicediff=Wash_Solution adjust=Tukey;
title3 'GLIMMIX Analysis (RCBD)';
ods output lsmeans=LW_Reduct_Avg_Day0_Glimmix;
run;
ods rtf close;
```

Appendix G
Appearance of Lettuce Samples After Washing with Water and After 1, 4, and 5 Days of Storage at 4 ± 1°C

Figure G.1 Lettuce washed with tap water after day 0 of storage

Figure G.2 Lettuce washed with tap water after day 1 of storage

Figure G.3 Lettuce washed with tap water after day 4 of storage

Figure G.4 Lettuce washed with tap water after day 5 of storage

Appendix H
Appearance of Lettuce Samples after Washing with 5% Vinegar Solution and After 1, 4, and 5 Days of Storage at 4 ± 1°C

Figure H.1 Lettuce washed with 5% vinegar solution after day 0 of storage

Figure H.2 Lettuce washed with 5% vinegar solution after day 1 of storage

Figure H.3 Lettuce washed with 5% vinegar solution after day 4 of storage

Figure H.4 Lettuce washed with 5% vinegar solution after day 5 of storage

Appendix I
Appearance of Lettuce Samples After Washing with the Commercial Antimicrobial for Fruit and Vegetable Treatment (CAFVT) and After 1, 4, and 5 Days of Storage at 4 ± 1°C

Figure I.1 Lettuce washed with CAFVT after day 0 of storage

Figure I.2 Lettuce washed with CAFVT after day 1 of storage

Figure I.3 Lettuce washed with CAFVT after day 4 of storage

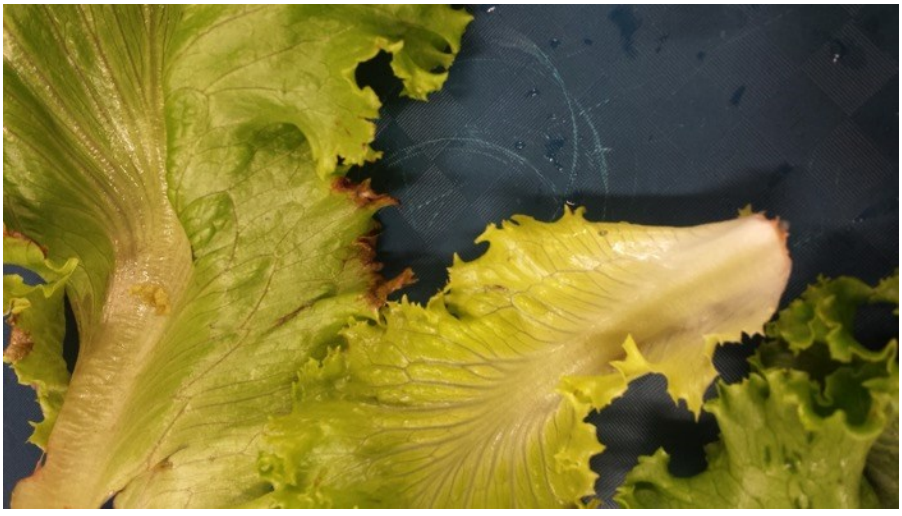

Figure I.4 Lettuce washed with CAFVT after day 5 of storage