

SALMONELLA TYPHIMURIUM LT2 TRANSFER AND REDISTRIBUTION ON BABY
SPINACH AND CILANTRO DURING PILOT-SCALE PROCESSING

By

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ABSTRACT

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Several recent outbreaks traced to baby spinach and cilantro have been hypothesized to involve cross-contamination during washing and processing. Consequently, this study aimed to assess the redistribution of *Salmonella* Typhimurium LT2 during pilot-scale production of fresh-cut baby spinach and cilantro. Four inoculated:uninoculated product weight ratios (0.5:100, 1:100, 5:100, and 10:100) and three different inoculation levels (10^3 , 10^1 , and 10^{-1} CFU/g) were used with spot-inoculated red leaf lettuce serving as a colored surrogate for baby spinach and cilantro washing. Sanitizer-free wash water was used for all trials and a chlorine-based sanitizer was used at 60 ppm available chlorine only for the highest inoculation level (10^3 CFU/g) and the three highest weight ratios (1:100, 5:100, and 10:100). Overall, initial inoculation level had a greater impact on the amount of *Salmonella*-positive samples than the weight ratios examined for both commodities. The number of positive samples concurrently decreased as the initial inoculation level of the surrogate decreased. Within each inoculation level, no significant differences ($P > 0.05$) were found among the four product ratios. This is the first study to assess the spread of *Salmonella* from incoming product to baby spinach and cilantro during processing. These results will provide important data for microbial risk assessments associated with leafy greens.

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To my parents, Susan and Chet
Thank you for always supporting my dreams

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KEY TO ABBREVIATIONS

ANOVA – Analysis of Variance

°C – degrees Celsius

CDC – Centers for Disease Control and Prevention

CFU – Colony Forming Unit(s)

cm – centimeter

ERS – Economic Research Service

FAO – Food and Agriculture Organization of the United Nations

FDA – U.S. Food and Drug Administration

FSMA – Food Safety Modernization Act

g – gram

GAPs – Good Agriculture Practices

GMPs – Good Manufacturing Practices

GN Broth – Gram negative broth

kg – kilogram

L – liter

LOD – Limit of Detection

min – minute

mL – milliliter

nm – nanometers

PBS – Phosphate Buffered Solution

ppm – parts per million

QPRAM – Quantitative Predictive Risk Assessment Model

h – hour

RTE – Ready-to-Eat

RH – Relative Humidity

rpm – revolutions per minute

RV Broth – Rappaport-Vassilidas Broth

s – second

SD – Standard Deviation

SSOPs – Standard Sanitation Operating Procedures

TSA-YE – Trypticase Soy Agar with 0.6% Yeast Extract

TSB-YE – Trypticase Soy Broth with 0.6% Yeast Extract

TT Broth – Tetrathionate Broth

μL – microliter

U.S. – United States

USDA – United States Department of Agriculture

WHO – World Health Organization

INTRODUCTION

Fruits and vegetables comprise a principal part of the diet for overall health and wellness. As per the USDA MyPlate guidelines, it is recommended that adults consume 1½ - 2 cups of fruit and 2½ - 3 cups of vegetables per day (USDA 2017). Therefore, these foods are consumed every day, multiple times per day. However, since many of these foods are ready-to-eat (RTE) or minimally cooked, the opportunity for reducing bacterial or pathogenic loads that may be present on these foods is significantly less than for foods that are thoroughly cooked prior to consumption. Given the high year round consumer demand for fresh fruits and vegetables, the importation of produce, some of which is invariably contaminated, has also increased (Merriweather 2015). Between the years 1996-2008, produce has been linked to 82 foodborne outbreaks, of which leafy greens have accounted for 34% of the total (FDA 2009). Pathogens linked to outbreaks related to fresh produce include *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, Hepatitis A, *Campylobacter jejuni*, and Norovirus, among others. Produce linked to multistate outbreaks include salads, leafy vegetables (romaine, spinach, shredded lettuce), tomatoes, sprouts, carrots, onions, cucumber, berries (strawberry, blueberry, raspberry), melons (cantaloupe, honeydew, watermelon), and grapes, among many others (Callejon et al. 2015).

Although commonly associated with egg and poultry outbreaks, *Salmonella* also poses a threat to the safety of fresh produce. Between 2004 and 2012, *Salmonella* was responsible for the most multi-state produce-related outbreaks in the United States and was also the most common pathogen associated with contamination of sprouts (Callejon et al. 2015). The economic burden caused by *Salmonella* is \$3.7 billion, which is 24% of the total annual burden faced by the industry from foodborne outbreaks.

Produce is prone to contamination at any point in the farm to fork continuum. Cross contamination is often the catalyst, amplifying the spread of contamination during pre- and post-harvest practices. The initial contaminant load does not have to be high for significant cross contamination to occur (Buchholz et al. 2012; Buchholz et al. 2014; Davidson et al. 2013). Vehicles for transmission during pre-harvest and harvest include: irrigation water, soil, manure, wild or domestic animals, field workers, harvesting equipment and even insects (FDA 1998; FDA 2013; FDA 2009). During post-harvest, produce can become compromised through direct contact with other contaminated produce and contamination can spread to previously uncontaminated produce through the wash water, mechanical equipment, or plant workers (FDA 1998; FDA 2009; FDA 2015a). Consumers can also cross-contaminate products in the home from cutting boards, knives, and hands that have been in contact with raw meats (Buchholz et al. 2011; FDA 2015a).

The Food and Drug Administration utilizes risk assessment tools in order to determine preventative methods that can be applied to ensure the safe handling of produce throughout the distribution chain (FDA 2015b). Specific tools used by the produce industry include FDA iRISK and the Quantitative Produce Risk Assessment Model (QPRAM) (FDA 2015b). These tools are invaluable to the FDA when determining areas of improvement for the safe handling and distribution of fresh produce. The FDA is also utilizing these same risk assessment tools to develop guidelines of the Food Safety Modernization Act (FDA 2016). Under this act, the industry is taking a preventative as opposed to a reactive approach to foodborne outbreaks. In order to establish better preventative controls for the fresh produce industry, the FDA is utilizing scientific data and risk assessment when establishing new or revising previous guidelines (FDA 2016a).

In order to ensure safer production of fresh produce and reduce the number of related foodborne outbreaks, data gaps need to be filled in the QPRAM and FDA iRISK tools. An accurate account of cross contamination occurring when a contaminant is introduced during flume washing of leafy greens and other produce does not yet exist. Therefore, the need for quantifying pathogen redistribution during this specific step in production is pertinent.

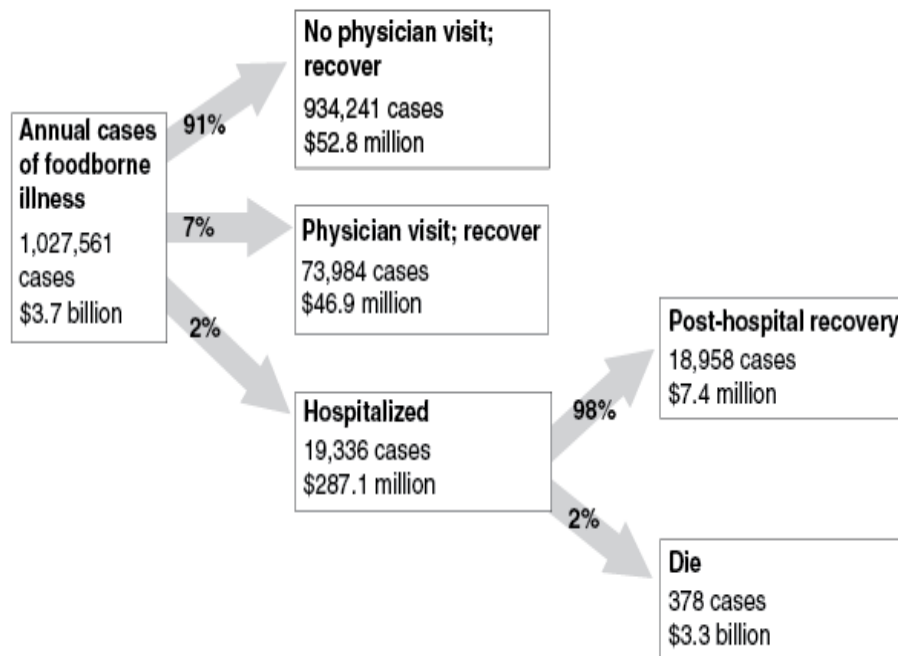
Consequently, the objectives for this research study were to:

1. Quantify *Salmonella* Typhimurium LT2 transfer and redistribution when realistically low initial inoculation levels and weight ratios of inoculated surrogate are introduced to uninoculated baby spinach and cilantro during post-harvest processing, in sanitizer-free wash water.
2. Assess the efficacy of a chlorine-based sanitizer (60 ppm available chlorine, acidified to pH 6.5) when the highest inoculation level 10^3 CFU/g and 1:100, 5:100, and 10:100 weight ratios of inoculated surrogate to uninoculated baby spinach and cilantro are used.

CHAPTER 1:
REVIEW OF PERTINENT LITERATURE

1.1 Economic burden of foodborne illness

It is estimated by the CDC that 48 million people in the United States are sickened annually by foodborne illness but only 9.4 million (20%) of these cases result from identifiable pathogens (Hoffman et al. 2015). Of the 9.4 million identifiable cases, the USDA Economic Research Service estimated the annual economic burden at \$15.5 billion, although due to difficulty in identifying cases, this value could be much higher (Hoffman 2015). As of 2015, the five pathogens responsible for 90% of the economic burden included *Salmonella*, *Toxoplasma gondii*, *Listeria monocytogenes*, Norovirus, and *Campylobacter* (Hoffman et al. 2015). Of these five, *Salmonella* is responsible for \$3.7 billion (24%) in annual economic losses which is the highest of the top 15 foodborne illness-causing pathogens (Hoffman et al. 2015). *Salmonella* also ranked 6th with per case monetary cost of \$1,896 (Roos 2010; Hoffman et al. 2015). The monetary amount lost per year from pathogens is determined from the severity of annual cases, loss of productivity from missed work, associated medical costs such as physician office visits, emergency room visits, outpatient clinic visits, hospitalizations, and premature death (Figure 1.1) (Roos 2010; Hoffman et al. 2015). The average amount of money lost by a grower from a foodborne outbreak is $\geq$ \$100,000, usually due to litigation costs and/or lost profit (Guitierrez-Rodriguez 2015).



(Source: USDA, Economic Research Service)

Figure 1.1: Breakdown of the economic costs caused by a *Salmonella*-related illness.

1.2 *Salmonella*

The genus *Salmonella* is found in the family *Enterobacteriaceae*, which contains rod-shaped bacteria that are Gram-negative, motile with flagella or non-motile, and facultatively anaerobic (FDA 2012; Motarjemi 2013). Because *Salmonella* are Gram-negative, they are more resistant to antibiotics and sanitizers than Gram-positive bacteria. This is primarily due to their thin peptidoglycan layer, which is located between two thin membranes. The thin outer membrane surrounding the peptidoglycan layer is impermeable and resists toxic materials that could damage the cell (Mitchell 2015; Silhavy et al. 2010).

The two serotypes of *Salmonella* most commonly associated with foodborne illness in the United States are *S. Typhimurium* and *S. Enteritidis* (CDC 2014; WHO 2013; Lawley 2013; Motarjemi 2013). Depending on the serotype, there are two primary illnesses caused by *Salmonella*: non-typhoidal salmonellosis and typhoid fever. Non-typhoidal salmonellosis - estimated at 1,027,561 illnesses annually, is most commonly associated with foodborne outbreaks (Hoffman et al. 2015). The infectious dose of *Salmonella* is 10-100,000 cells but depending on strain characteristics and health of the individual exposed, the dose can be as low as one cell (FDA 2015b; FDA 2012; Fellows 2009). Symptoms of illness will appear 6 - 72 hours following exposure, last 1 - 2 days if acute or 4 - 7 days if greater exposure occurs. Mortality in healthy humans is about 1%, but if elderly, immunocompromised, or infants are exposed, mortality increases to 3.6% (FDA 2015b).

Salmonella grows optimally at 37°C, but can also grow in temperatures between 7 - 45°C (Motarjemi 2013). This pathogen can survive but will not grow at refrigeration or freezing temperatures. *Salmonella* can grow at a pH range of 4.5 - 9.5, with optimal growth occurring at 6.5 - 7.5. While unable to grow at a water activity value of < 0.93, *Salmonella* can survive in many low moisture foods, such as spices (Motarjemi 2013).

Animals are common asymptomatic carriers of *Salmonella* with this organism residing in the intestinal tract and feces (Hoffman et al. 2015; WHO 2013; FDA 2012). Sources of *Salmonella* include the intestinal tracts of livestock (cattle, pigs, and chickens), wildlife (snails, insects, birds, and rodents), and household pets (dogs, cats, turtles) (WHO 2013; Lawley 2013; FDA 2012; Hoffman et al. 2015). Pond water sediment can also be a potential source of the bacteria (FDA 2012). Any contact with *Salmonella*-contaminated water or animals/animal feces and subsequent contact with food or a food contact surface will spread the bacteria leading to a

potential illness (Hoffman et al. 2015). Person-to-person transmission can also occur through the fecal-oral route (FDA 2012).

1.2.1 *Salmonella* Typhimurium LT2. *Salmonella* Typhimurium LT2 belongs to subspecies I of *S. Enterica*, which causes 99% of *Salmonella* infections in humans and is naturally found in mammals and birds (Selander et al. 1996; Popoff 2000). *Salmonella* Typhimurium LT2 was first isolated in the 1940s and is a primary strain for investigating cellular and molecular biology in *Salmonella* (Neidhardt 1996). In a study dedicated to the genomic sequencing of *Salmonella* Typhimurium LT2, it was found that 11% of the *S. Typhimurium* LT2 genes are missing from *S. enterica* serovar Typhi (*S. Typhi*), and 29% are missing from *Escherichia coli* K12 (McClelland et al. 2001). *Escherichia coli* K12 is a member of the closest known genus to *Salmonella*, which is why the aforementioned comparison is relevant (Perna et al. 2001). Since *S. Typhimurium* LT2 shares 352 genes with *S. enterica*, this strain is useful for epidemiological, host specificity, and pathogenicity studies (Selander et al. 1996). Specific regions of the genome encode for various virulence genes which are important in pathogenicity. For *Salmonella*, these regions are referred to as *Salmonella* pathogenicity islands (SPIs) (Perna et al. 2001; Ochman et al. 2000). SPI of *S. Typhimurium* LT2 strain 55% similar to the SPI of *S. Enterica* (McClelland et al. 2001). Another important virulence factor in enteric bacteria is the RpoS sigma factor. Specific to *Salmonella*, RpoS controls expression of the *Salmonella* plasmid virulence genes, which are necessary for systemic infection (Nickerson and Curtiss 1997). However, the *spv* gene is not expressed in the LT2 strain since the RpoS sigma factor is absent (Nickerson and Curtiss 1997). This characteristic, as well as the genomic sequence of LT2, is what make this strain avirulent.

1.3 Fresh produce consumption and produce outbreaks in the U.S.

Between 1970 and 2004, the per capita consumption of produce consistently increased, likely due to the active promotion of fruits and vegetables as components of a healthy diet (FDA 2015c). Between 1982 and 1997, per capita consumption rose most dramatically with an increase of 32% from 91.2 kg to 121.1 kg (FDA 2015c). From 1997-2004, the increase in consumption slowed more gradually and eventually saw a 7% decline from 2009-2014 (Produce for Better Health Foundation 2015). A 5% increase in per capita consumption is projected from 2015 to 2020 (Produce for Better Health Foundation 2015). This increase in consumption has also led to more produce-related outbreaks of illness primarily because more food was consumed outside of the home, such as at buffets and restaurants, with consumption of fresh, RTE produce items like bagged lettuce and pre-sliced fruits also increasing (FDA 2015c). These factors, coupled with increased globalization for the fresh produce trade is likely to increase consumer exposure to a broad range of foodborne pathogens (FDA 2015c).

The FAO and WHO declared that RTE leafy green vegetables and fresh herbs present the greatest concern for microbiological hazards (FAO and WHO 2008). This is due to the lack of a kill step during processing and in the home to eliminate pathogens that may be present. Between 1996 and 2008, produce has been linked to 82 foodborne outbreaks, of which leafy greens have accounted for 34% of the total (FDA 2009). *Salmonella*, *E. coli* O157:H7, *Bacillus cereus*, and *Yersinia enterocolitica* have been linked to sprouts, mung beans, and alfalfa seed outbreaks (CDC 2016). Other produce implicated in multistate outbreaks involving *E. coli* O157:H7 include strawberries (2001), RTE salads (2013), spring mix (2012), romaine lettuce (2011), and spinach (2006). Produce implicated in multistate outbreaks involving *L. monocytogenes* include frozen vegetables (2016), cantaloupes (2011), sprouts (2014), caramel apples (2015), and

strawberries (2011) (CDC 2016; Kniel 2014). *Salmonella*, *L. monocytogenes*, *Campylobacter*, Norovirus and *E. coli* O157:H7 have been linked to several multi-state melon outbreaks (CDC 2016; Castillo et al. 2014; Chapman 2005). Bagged lettuce products have also been a major source of outbreaks; spring mix lettuce and spinach blend contaminated with *E. coli* O157:H7 caused 33 cases of illness and 13 hospitalizations (CDC 2012, cited in Matthews 2014). In 2013 and 2012, bagged and RTE lettuce was linked to two *E. coli* O157:H7 outbreaks, respectively (CDC 2016).

Outbreaks caused by *Salmonella* have involved all major food groups with fresh produce now the leading contributor to this foodborne illness. Tomatoes have been most commonly associated with *Salmonella* with 5,324 cases of illness in the U.S. and 35 outbreaks between 1990 and 2012 (Center for Science in the Public Interest Outbreak Database 2013; Wang et al 2013). *Salmonella* was also responsible for outbreaks involving grapes, cabbage, lettuce, sprouts, herbs, leafy green salads, and coleslaw between 2000 and 2007 (UGA's Center for Food Safety; CDC 2016). A *Salmonella* Copenhagen DT 104b outbreak in Finland was linked to field-contaminated iceberg lettuce originating from Spain that caused 60 confirmed illnesses (Eurosurveillance 2005). Imported cucumbers from Mexico presumably contaminated in the field were responsible for three *Salmonella* outbreaks in 2013, 2014, and 2015 (CDC 2016). In 2006, *S. Oranienburg* was found in cut fruit salads distributed to 10 northeastern U.S. states and one Canadian province in 2006 causing 41 illnesses (CDC 2007). This outbreak most likely resulted from cross contamination during the cutting process of fruit (CDC 2007). *Salmonella* was also responsible for outbreaks involving mangoes in 2012, cantaloupe in 2008 and 2011, and sprouts in 2011, 2013, 2014, and 2015 (CDC 2016).

1.4 Baby spinach and cilantro outbreaks

Cilantro (*Coriandrum sativum*), which is botanically and anatomically closely related to parsley, has a long stem with small feathered leaves (Foley et al 2003). Cilantro is grown and harvested close to the ground, which increases the probability of pathogen contamination from the soil or groundwater (FDA 2001). Specific to cilantro, *Salmonella* has caused 8 multi-state outbreaks in the past twenty years and caused two Class I recalls in 2011 and 2012 after random FDA and USDA samples were *Salmonella*-positive (CDC 2016; US Foods 2016). In 1999, *Salmonella* Thompson contaminated cilantro caused 41 confirmed cases after patrons had visited a restaurant in California (Campbell et al. 2001). Poor record keeping of the distributor used by the restaurant prevented a confirmed source to be found, however cilantro may have been originally contaminated in the field (Campbell et al. 2001).

As per the Produce & Imported Foods Safety Initiative of 1997, the FDA was required to gather data on specific fresh produce commodities imported at the highest volumes (FDA 2003). Among the samples analyzed, 9% (n=177) of random imported cilantro samples were positive for *Salmonella* or *Shigella* (FDA 2003). As a follow up to this study, 3.3% of imported samples (n=33) were *Salmonella*-positive the following year (FDA 2003). Due to the high levels of contaminated imported cilantro and other commodities, the FDA initiated a domestic follow-up study in 2001 in which 1.2% (n=81) of domestically grown cilantro samples were positive for *Salmonella* (FDA 2003). From 2002-2009 the FDA randomly tested 2510 cilantro samples for the presence of enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), and *Salmonella* and found 0.64% (n=16) to be positive for the presence of one of these pathogens (FDA 2015a). Of the commodities tested, the 0.64% positive rate for cilantro was the second highest, with spinach producing the highest positive rate of 0.74%.

In 2012, 2013, 2014 and 2015, cilantro was also responsible for a multistate *Cyclospora* outbreak, causing over 1,310 illnesses of the four outbreaks combined (CDC 2016; FDA 2016c). Investigations into these outbreaks revealed that the cilantro was imported from Puebla, Mexico, where poor maintenance of hand washing stations and poor worker hygiene was the source of the outbreak (CDC 2016). In 2014, the U.S. imported 6,518,000 metric tons of fresh vegetables and 10,870,000 metric tons of fresh fruit. Mexico has been the leading exporter of fresh produce to the United States since 1995 (USDA ERS 2016). In response to the recent contamination issues with cilantro, the FDA has advised those who grow, harvest, sort, pack, or distribute cilantro to be especially vigilant of areas in their supply chain that could be improved to reduce hazards and improve safety (FDA 2016c).

From 1995-2006, 22 outbreaks have been associated with fresh-cut lettuce or spinach (Wendel et al. 2009; Cooley et al. 2007; Khalil and Frank 2009). The most notorious multi-state outbreak involving baby spinach occurred in 2006. Spinach originating from a farm in Salinas, CA was contaminated with *E. coli* O157:H7 and sickened 199 people, causing 3 deaths (Hoffman et al. 2015). This outbreak most likely resulted from fecal contamination from wild pigs or water runoff from a nearby livestock operation (Edward et al. 2015). From 2002-2009, the FDA randomly tested 4433 spinach samples for the presence of enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), and *Salmonella* and found 0.74% to be positive for the presence of one of these pathogens, which was the highest positive rate of the commodities tested (FDA 2015a). Random sampling of baby spinach from farms in Spain found 5.2% (n=38) of collected samples to be contaminated with *Salmonella* following harvest (Garcia-Villanova Ruiz et al. 1987). Spinach originating from the Netherlands caused a widespread *Salmonella* outbreak across Europe in 2007 with 354 reported cases (Eurosurveillance 2005).

Domestically, *Salmonella*-contaminated spinach was implicated in one multi-state outbreak in the United States (CDC 2016). More recently in 2011 and 2015, flat leaf spinach originating from suppliers in California was recalled due to suspected *Salmonella* contamination after random sampling resulted in a *Salmonella*-positive sample (US Foods 2016).

1.5 Previous cross contamination studies on pathogen transfer and redistribution during processing

Numerous studies have examined pathogen cross contamination of fresh produce and redistribution to previously uncontaminated produce during subsequent washing, slicing and dicing. In order to provide vital information for future risk assessments, washing inoculated produce in sanitizer-free water has been studied to generate baseline data. These studies have generally found that a localized amount of contaminated produce can spread to contaminate a larger amount of produce, regardless of initial amount of contamination. However, the results from these studies are impacted by many variables including water quality, sanitizer concentration and contact time, decontamination treatment(s), population and physiological state of target microorganism(s), method of detection, type of produce and surface characteristics, and the time interval between initial contamination and washing (Gil et al. 2009). Together, these factors account for the similarities and differences found in past studies looking into pathogen cross contamination and different sanitizing practices.

A pilot-scale study conducted by Buchholz and others (2012) examined the amount of cross contamination that would occur during simulated commercial washing of romaine and iceberg lettuce in sanitizer-free wash water. Dip-inoculated lettuce (22.7 kg) containing 10^6 , 10^4 , or 10^2 CFU/g *E. coli* O157:H7 was washed for 90 s. After washing, the water used was drained

from the system and the inoculated lettuce was removed from the process line. Next, 90.8 kg of uninoculated lettuce was washed for 90s in the same manner as the inoculated lettuce. Results indicated cross contamination occurred throughout the previously uninoculated 90.8 kg batch, regardless of inoculation level. Following washing, uninoculated lettuce had *E. coli* O157:H7 counts of 2.9, 0.7, and -1.1 log CFU/g for the 10^6 , 10^4 , and 10^2 CFU/g inoculation levels, respectively. Water samples collected after washing the inoculated lettuce had 1.9, 2.2, 0.3 log CFU/mL at the 10^6 , 10^3 and 10^2 inoculation levels, respectively. Water samples collected after washing the uninoculated lettuce, had 0.9, 0.0, and 1.8 log CFU/mL *E. coli* O157:H7 at the 10^6 , 10^3 and 10^2 inoculation levels, respectively. The cell counts in water samples after processing both the inoculated and uninoculated lettuce demonstrate water can be a vehicle of cross contamination well after initial localized contamination. The inoculated lettuce transferred *E. coli* O157:H7 to all major equipment surfaces (shredder, conveyor, flume tank, dewatering shaker, and centrifuge) and the wash water. Highest levels of cross contamination occurred during shredding and conveying, regardless of inoculation level. This study demonstrated water and equipment contact surfaces can contribute to significant pathogen cross contamination during pilot-scale processing of lettuce.

Radicchio was used as a colored surrogate to track the spread of *E. coli* O157:H7 contaminated product to iceberg lettuce in a pilot-scale processing line consisting of a mechanical shredder, conveyor, flume tank, dewatering shaker, and centrifugal dryer. Uninoculated iceberg lettuce was processed, followed by 9.1 kg of dip-inoculated surrogate, and then 90.7 kg uninoculated iceberg lettuce. Flume water was drained from the system following surrogate washing and refilled prior to processing the uninoculated iceberg lettuce. Mean *E. coli* O157:H7 populations on radicchio following processing were 5.0 ± 0.4 log CFU/g, showing

there was ~1 unit log reduction of *E. coli* O157:H7 from radicchio leaves. Iceberg lettuce had *E. coli* O157:H7 populations ranging from -0.1 to 3.0 log CFU/g with an average of 1.2 log CFU/g. Processing equipment had statistically similar *E. coli* O157:H7 populations, with populations of 2.4 ± 0.4 log CFU/100 cm² on the mechanical shredder, 3.2 ± 0.7 log CFU/100 cm² on the conveyor belt, 2.3 ± 0.5 log CFU/100 cm² on the flume tank, and 1.9 ± 0.4 log CFU/100 cm² on the dewatering shaker. Populations in the flume water samples ranged from 1.6 log CFU/mL to -2.0 log CFU/mL (limit of detection). The authors noted that the radicchio, as could be seen on the equipment surfaces and comingled with iceberg lettuce, visually demonstrated the spread of contaminated product during processing, further emphasizing the risk of cross contamination during leafy green processing. This study demonstrates the spread of *E. coli* O157:H7 contamination during sanitizer-free washing of leafy greens.

A previous pilot-plant scale study performed by Davidson and others (2013) examined the efficacy of five different commercial chemical sanitizers when 5.4 kg of iceberg lettuce inoculated with 10⁶ CFU/g *E. coli* O157:H7 was washed for 90 s. Wash treatments included water alone (control), 50 ppm peroxyacetic acid, 50 ppm of mixed peracid, 50 ppm available chlorine alone or treated with citric acid acidified to pH 6.5 or treated with T-128 (an acidifier). Results showed that no sanitizer was significantly more effective than water alone ($P > 0.05$) at reducing *E. coli* O157:H7 populations on the iceberg lettuce (Davidson et al. 2013). Using peracid, *E. coli* O157:H7 populations were reduced by 1.4 log CFU/g however this reduction was not significantly different than using water alone, which reduced populations by 0.75 log CFU/g (Davidson et al. 2013). All five sanitizers significantly ($P < 0.05$) reduced *E. coli* O157:H7 populations on the flume tank and shaker table compared to using water alone. Nine water samples (50 mL each) were also taken in 10 s intervals during the 90 s washing cycle to

assess the spread of *E. coli* O157:H7 in wash water when different sanitizers were used. The chlorine alone, chlorine plus citric acid, and chlorine plus T-128 were generally more effective than the other two sanitizer treatments in reducing *E. coli* O157:H7 populations from wash water with reductions of 3.79, 5.47, and 5.37 log CFU/mL, respectively (Davidson et al. 2013). The authors noted that the organic load in the study was lower than what may be seen in industry and the higher organic load in commercial processing conditions would further reduce the effectiveness of commercial sanitizers (Davidson et al. 2013). Therefore, proper maintenance of wash water and consistent replenishment of sanitizer concentrations in wash water is essential to mitigate cross contamination.

The change in the free chlorine concentration of wash water prepared both with and without an acidulant (T128), was examined by Luo and others (2012). Baby spinach was inoculated with *E. coli* O157:H7 at 2×10^5 CFU/g and washed with large amounts of uninoculated iceberg lettuce in the presence of a chlorine-based sanitizer. At free chlorine concentrations ≤ 1 mg/L, *E. coli* O157:H7 was recovered from the wash water and uninoculated iceberg lettuce. However when free chlorine concentration was > 1 mg/L in the presence of T128, cross contamination to uninoculated iceberg lettuce was reduced. The researchers recommended the importance of consistent monitoring of wash water quality and concentration of free chlorine as ways to mitigate cross contamination during washing (Luo et al. 2012).

In other work, iceberg lettuce was inoculated with *E. coli* O157:H7 at 10^6 CFU/g and washed in either sanitizer-free wash water, water containing sodium hypochlorite, or water containing chlorine dioxide as the sanitizing agent (Lopez-Galvez et al. 2009). When inoculated iceberg lettuce was washed prior to uninoculated iceberg lettuce, cross contamination occurred to the uninoculated lettuce. In the sanitizer-free trial, the uninoculated lettuce had *E.coli* O157:H7

populations up to 3.4 log CFU/g after washing and the inoculated lettuce experienced a 0.5 +/- 0.1 log reduction. When sanitizer was used, similar increased *E. coli* O157:H7 reductions were seen for both inoculated and uninoculated lettuce compared to the sanitizer-free trials. The sanitizers were effective in mitigating *E. coli* O157:H7 presence in the water as no *E. coli* O157:H7 was detected (Lopez-Galvez et al. 2009). This finding suggests that sanitizers are effective in mitigating cross contamination that may occur through wash water (Lopez-Galvez et al. 2009).

In another study, Nou and Luo (2010) examined the extent of cross contamination occurring when romaine lettuce was sanitized before versus after mechanical shredding. When romaine lettuce leaves were spot inoculated with 6.3 CFU/g of *E. coli* O157:H7 and washed for 60 s in water containing 70 ppm available chlorine, lettuce sliced prior to washing experienced a 1.1 log decrease in *E. coli* O157:H7 populations. However, romaine lettuce sliced after washing experienced a 1.9 log decrease in *E. coli* O157:H7 (Nou and Luo 2010). When lettuce was washed before slicing, *E. coli* O157:H7 populations were 0.6-1.3 log lower compared to that seen when lettuce was sliced prior to washing (Nou and Luo 2010). The same study also evaluated the extent of cross contamination occurring when radicchio was inoculated with 3.5×10^3 CFU/g *E. coli* O157:H7 and washed with uninoculated romaine lettuce in chlorinated water for ~90 s. Cross contamination decreased when lettuce was washed before slicing, as 1 out of 12 red leaf samples and 2 out of 60 romaine lettuce samples tested positive for *E. coli* O157:H7 (Nou and Luo 2010). When lettuce was washed after slicing, 12 out of 121 red leaf lettuce samples and 58 out of 60 romaine lettuce samples tested positive for *E. coli* O157:H7 (Nou and Luo 2012). The increased log reductions achieved when lettuce was washed before shredding in both assessments indicates the importance of adequate washing procedures prior to further leafy green

processing. Similar results were found in a study examining the same order of washing/slicing during post-harvest processing of iceberg lettuce as the Nou and Luo (2010) study. A 0.79-0.80 log CFU/g improvement in reduction of *E. coli* O157:H7 was found when artificially inoculated iceberg lettuce was washed before cutting as opposed to washed following cutting (Palma-Salgado et al. 2014).

The quantification and spread of *Listeria monocytogenes* to previously uncontaminated product during mechanical dicing of celery and growth during storage was assessed by Kaminski and others (2014). To quantify *L. monocytogenes* transfer during mechanical dicing of celery, 275 g of inoculated celery containing 5.6 log CFU/g *Listeria monocytogenes* was diced prior to 15 batches of 250 g uninoculated celery (Kaminski et al. 2014). The first (out of 15) uninoculated celery batch diced after the inoculated batch had similar *L. monocytogenes* levels as the contaminated celery of 5.2 ± 0.1 log CFU/g. Batches two and three had significantly lower populations than batch one, with batches 8-15 containing about 2.0 log CFU/g of *Listeria*. Also assessed in this study was the spread of *L. monocytogenes*-contaminated celery by weight during mechanical dicing using Swiss chard as the colored surrogate. Swiss chard was dip-inoculated to achieve 6.8 ± 0.4 log CFU/g. After initial dicing, the populations of *L. monocytogenes* on Swiss chard remained relatively unchanged at 6.6 ± 0.7 log CFU/g. Diced pieces of Swiss chard were allowed to comingle with diced pieces from the 15 batches of subsequently diced uninoculated celery. Swiss chard was removed from all diced uninoculated celery pieces. *L. monocytogenes* was found throughout the 15 batches of uninoculated celery with batches one and two having the highest counts of 4.7 ± 0.3 log CFU/g and 3.9 ± 0.2 log CFU/g, respectively, and the remaining batches having significantly lower *L. monocytogenes* counts of 1.7 log CFU/g. The amount of contaminated product spread during mechanical dicing was measured by analyzing the weight of

contaminated surrogate found in diced uninoculated celery batches. Dicing of 250 g batches of celery and the Swiss chard surrogate was performed, followed by 15 250 g batches of uninoculated celery. All visible pieces of Swiss chard were removed from each batch of celery and weighed. Overall, 25.0% (62.5 g) of Swiss chard was retained in the first batch of diced celery, which was significantly more than what was retrieved from the remaining batches of celery, which contained an average of 0.25% (0.6 g) of the surrogate in batches 2 through 15. The weight amount of surrogate remaining on equipment was 4.33% (10.8 g). Changes in populations of *L. monocytogenes* were analyzed based on storage temperature of diced celery (4, 7, and 10°C) over a 7 day period. For the storage study, inoculated celery with 3.2 ± 0.3 log CFU/g *L. monocytogenes* was diced and stored at 4, 7, or 10°C. During storage, *L. monocytogenes* populations reached maximum populations of 3.3 ± 0.6 , 3.8 ± 0.3 , and 5.2 ± 0.7 log CFU/g for the 4, 7, and 10°C storage temperatures, respectively. This study demonstrates the ability for pathogens to cross contaminate to previously uncontaminated product during mechanical dicing, especially when produce is temperature abused. Therefore, it is imperative proper mitigation efforts are used during mechanical dicing of fresh produce and produce is held at proper refrigeration temperatures in order to best prevent potential foodborne outbreaks.

Spanish yellow onions were diced at the pilot scale level to assess cross contamination of *Listeria monocytogenes* (Scollon 2014). Peeled onions (2.2 kg) were inoculated with *L. monocytogenes* at 5.9 or 4.2 log CFU/50 g to represent high and low inoculation levels and were diced using a pilot scale dicer followed by 10 batches of uninoculated onions, each weighing 2.2 kg. *L. monocytogenes* was found on all surfaces of the dicer following the dicing of the inoculated batch and after dicing of tenth uninoculated batch (Scollon 2014). Samples of uninoculated onions from the first, fifth, and tenth batches were then analyzed for *Listeria*

presence. At the high inoculation level, 4.6, 3.0, and 2.3 log CFU/50 g was found on uninoculated onions, respectively. At the low inoculation level, at least one out of three of the samples collected in triplicate yielded *L. monocytogenes* (Scollon 2014). The diced onions were then washed with chlorine sanitizer at 80 ppm free chlorine. After washing with a sanitizer, *L. monocytogenes* populations decreased 1.4 log on the onions, which was significantly greater compared to washing in sanitizer-free water (Scollon 2014). This study demonstrated that if proper sanitation is not maintained during mechanical dicing of onions, then high volumes of contamination can spread to previously uncontaminated product, even with a sanitizing wash step implemented later in the process flow.

1.6 Inoculation methods

Three primary inoculation methods have been used to artificially contaminate produce samples: dip, spray, and spot. Each method is unique and can provide different information in regards to pathogen recovery, attachment, and elimination during washing or storage. The specific inoculation method selected for studies should mimic real world contamination conditions as closely as possible. These conditions include: location of contamination on the produce, time of contamination in the production chain (pre-harvest, harvest, post-harvest, packing), and surface characteristics of the produce studied such as surface area and weight (Annous et al. 2005; Beuchat et al. 2001; Gil et al. 2009).

Spot inoculation tends to yield more consistent starting populations than dip and spray inoculation because a known number of CFUs is applied to the sample (Annous et al. 2005; Lang et al. 2004). This method most closely replicates random fecal contamination from animals or biological soil amendments (e.g. manure) in the field rather than exposure to contaminated

irrigation water, where dip inoculation would be more appropriate (Land et al 2004; Gil et al. 2009).

Dip inoculation involves submerging the sample into a liquid containing the inoculum. Bacteria are suspended in this liquid, typically water or a buffer solution, with the entire surface of the sample exposed to the inoculum. This method is used to imitate a larger contamination event such as during irrigation, hydrocooling, or flume washing (Annous et al. 2005). The dip method is not as precise as spot inoculation because the amount of inoculum on each piece of produce varies due to the nature of the application (Lang et al. 2004; Gil et al. 2009).

Spray inoculation is most commonly used to imitate contamination occurring during spray irrigation (Erickson 2010). This method involves suspending the inoculum in a liquid and atomizing the cocktail through a spray bottle or similar instrument. The primary disadvantage of using this method is the inoculum may not reach the sample surface and the delivered spray may not contain the targeted inoculum (Lang et al. 2004).

1.7 Previous microbial studies on fresh produce and spot inoculation

A study performed by Koseki and others (2003) analyzed the effectiveness of two sanitizing methods in reducing the pathogen load on spot and dip inoculated lettuce pieces. This study was conducted in order to assess if there was a correlation between inoculation method and sanitizer efficacy. Acidic electrolyzed water and 200 ppm free chlorine were used to decrease the levels of *E. coli* O157:H7 and *Salmonella* spp. Ten inner and outer leaf lettuce pieces were either spot or dip inoculated to contain 7.3-7.8 log CFU/g. Spot inoculating yielded greater log reductions for the inner as compared to outer leaves, presumably because the cells were able to penetrate into leaf stomata, decreasing their accessibility to the sanitizer. Dip inoculation resulted

in reductions of ≤ 1 log CFU/g compared to the 2.5-2.7 CFU/g log reduction obtained using spot inoculation (Koseki et al 2003). Sanitizer efficacy was impacted by the inoculation method and location of inoculum on the leaves; however neither sanitizer was capable of completely inactivating either pathogen from the lettuce.

Another study assessed the effect of inoculation method and inoculum drying time on recovery of *E. coli* O157:H7, *Salmonella*, and *Listeria* following washing of lettuce and parsley (Lang et al. 2004). Produce samples were either dip, spot, or spray inoculated and allowed to dry using one of two drying methods: 2 h at 22°C (single) or 2 h at 22°C followed by 22 h at 4°C (dual). Spot inoculation yielded the most consistent recovery rates for lettuce and parsley after the dual drying method. Based on the consistency of results collected for spot inoculation method and dual drying time, the authors recommended this method be used to determine the efficacy of chlorine sanitizers in order to enhance the reproducibility of results.

1.8 Contamination of produce in the field

The most notable sources for pre-harvest contamination of fruits and vegetables include fecal contaminated irrigation water from domestic or wild animals, water runoff from nearby livestock operations, ground soil, inadequately composted manure, field workers, and cleanliness of tools (Sapers and Doyle 2014; Gil et al. 2009).

Outbreaks involving *Salmonella*, *E. coli* O157:H7, *Giardia*, and *Cryptosporidium* on fresh produce have been traced back to contaminated irrigation water (Gerba and Rock 2014; Thurston et al. 2012; Duffey et al. 2005; Materon et al. 2007). Irrigation water contaminated with *E. coli* O157:H7 was implicated in two multi-state outbreaks involving lettuce in 1995 and 1996 (Hillborn et al. 1999). Contributing factors to the contamination of irrigation water include feces

originating from waterfowl or wild animals, poor storm water drainage, recent rainfall, and runoff from nearby livestock operation(s) (Gerba and Rock 2014). Contamination of produce through irrigation is affected by the irrigation method, frequency of irrigation, surface characteristics of the edible portion of the plant, and the location of the edible portion of the plant in relation to distance from the soil (Gerba and Rock 2014). Edible produce grown near the soil is more likely to become contaminated than tree or vine crops (Manshadi et. al 2013). Produce surface characteristics can also influence the extent of contamination. For example, plants that can retain water in crevices or leaves are more likely to become contaminated, whereas plants having a smooth hydrophobic waxy surface are more resistant to water pooling and consequently less likely to be contaminated (Gerba and Rock 2014).

Another factor that may influence the likelihood of contamination is the irrigation method. The three methods of irrigation commonly practiced are drip, surface, and overhead sprinkler irrigation (Brouwer et al. 2016; Gerba and Rock 2014). Irrigation frequency in relation to time of harvest also becomes an issue if the irrigation water is contaminated due to enhanced pathogen survival. One study conducted by Soloman and others (2003) found that lettuce sprayed either once or intermittently with irrigation water inoculated with *E.coli* O157:H7 at 10^4 CFU/mL or 10^2 CFU/mL resulted in contaminated produce, regardless of inoculation level or irrigation frequency (2003). The only time *E. coli* O157:H7 contamination was not seen at harvest was when the lettuce was irrigated once with water containing 10^2 CFU/mL at the start of the 30-day trial (Soloman et al. 2003). The 30-day time period between initial irrigation in combination with the low contamination level was long enough for the pathogen to be removed from the system. This therefore demonstrates that increased time between irrigation and harvest can reduce the likelihood of contamination if the initial contamination load is low.

Animal manure is commonly used as a crop fertilizer, especially since it helps maintain overall soil quality by recycling nutrients and organic matter (Arthurson et al. 2011). However, if manure is not treated correctly prior to field application, pathogenic bacteria may become introduced to the soil and become disseminated throughout the field (Arthurson et al. 2011). Another threat to field contamination is if improperly treated manure or feces from a nearby livestock field comes into contact with produce (Millner 2014). Reports have shown that livestock and wild animal feces may naturally contain between 10^2 and 10^5 CFU/g *E. coli* and between 10^2 and 10^7 CFU/g *Salmonella* spp. (Himathongkham et al., 1999). Depending on the animal source, manure can contain between 10^2 and 10^7 CFU/g *Salmonella* spp. (Pell 1997). A study performed by Solomon and others (2002) found that *E. coli* O157:H7 from contaminated manure and irrigation water can internalize into lettuce through the root system and lead to decreased efficacy of sanitizers in wash water during post-harvest processing. Contamination of produce can also occur from nearby cattle, swine, poultry, and sheep farms. A study conducted on a dairy farm in Virginia reported 4.7% (n=531) of fecal, feed, water, and various environmental samples contained *Salmonella* isolates (Warnick et al. 2001). Of the isolates, *Salmonella* Typhimurium was the serotype found most frequently in studied herds (Warnick et al. 2001). The data from the aforementioned study may be indicative of the amount of *Salmonella* isolates present on other farms, which could be problematic if these farms are located near produce fields since runoff or dust containing a pathogenic load can be transferred between areas. The manure of ruminants such as cattle and sheep are considered the main sources of *Salmonella* and *E. coli* O157:H7 (Pell 1997). Studies have shown that *Salmonella* can survive in manure for at least 60 days when held at 4°C and 20°C, respectively and for up to 19 days when held at 37°C (Himathongkham et al. 1999). Other studies have demonstrated *Salmonella* can

persist in soil for over 300 days following high initial loads ($\sim 10^6$ CFU/g) of contaminated manure application (Jones 1986; Baloda et al. 2001; Islam et al. 2004). The FDA has established guidelines for growers on proper manure management and treatment such as using active or passive heat treatments for manure, preventing wildlife from getting into livestock housing units and water troughs, and utilizing GAPs will contribute keeping manure used in crop fertilization safe (Millner 2014; FDA 1998).

Maintenance of soil conditions is also pertinent to overall safety and quality of produce. However, the same conditions required to grow robust crops are also prime for pathogen survival and persistence. In terms of soil, water permeability, nutrient content, and the level of native microflora present can all influence the proliferation of pathogens (Millner 2014). An important factor is the amount of nitrogen present in the soil, as the application of fertilizers can enhance the survival of pathogens, such as *E. coli* O157:H7 (Gagliardi and Karns 2000). A risk assessment study detected *Salmonella* in soil samples from produce-growing regions in California and New York State at 2.0% and 2.6%, respectively (Strawn et al. 2013). The same study also found that farm management and irrigation practices influence the presence of pathogens. In produce-growing regions of New York State, 6.1% and 17.5% of fields (n=263) and 11% and 30% of water samples (n=74) were positive for *Salmonella* and *L. monocytogenes*, respectively. A majority of the water samples positive for pathogens were from non-irrigation surface water such as nearby ponds, rivers, and ditches (Strawn et al. 2013). The study also reported that the odds of a *Salmonella*-positive field increased when manure application had been used within 12 months and the odds of *L. monocytogenes*-positive fields increased when irrigation and wildlife presence both occurred within 3 days of each other (Strawn et al. 2013).

Other pre-harvest sources of pathogen contamination include insects, dust, pesticides, field workers, and equipment (Sapers and Doyle 2014). Contaminated equipment may transfer bacteria to produce at the time of harvest (Patel et al. 2011). Any bins used to transport fresh produce following harvest must also be properly washed and sanitized. Pesticides are capable of transferring human pathogens if the water used to prepare the pesticide was previously contaminated (Gerba and Rock 2014). *Salmonella* was isolated from pesticide spray prepared from contaminated irrigation water in Japan (Izumi et al. 2008). Field workers can easily transfer human pathogens to produce if proper sanitation practices are not followed such as ready access to hand washing and bathroom facilities. Outbreaks of *Cyclospora* were caused by field contamination of cilantro in 2012, 2013, 2014, and 2015 due to poor maintenance of bathroom and handwashing facilities for field workers in Mexico (FDA 2016c).

Insects, flies and rodents are capable of transferring human pathogens to produce in the field (Leliveld and Holah 2014). A case study of filth flies near leafy green growing regions was performed in the Salinas and Imperial Valleys of California. Reports found that 90% of the filth flies collected in the Imperial Valley and $\leq 1\%$ of filth flies in the Salinas Valley were positive for *E. coli* O157:H7 (Wayadande and Talley 2010). Also collected in the same study were spinach leaves where flies had left regurgitation spots. Spinach leaves were analyzed twice, one day and one week after the flies had landed on the leaf surface. One day after landing, *E. coli* O157:H7 populations were extremely low however evidence of bacterial replication on the surfaces was evident after one week (Wayadande and Talley 2010). The researchers concluded that flies are capable of transmitting *E. coli* O157:H7 due to the pathogen's ability to survive in the gut of flies and colonize the spinach phyllosphere after fly regurgitation (Wayadande and Talley 2010). In another study by Pace (2013), house and blow flies artificially inoculated with

E. coli O157:H7 or *Salmonella* Enterica reportedly contaminated all analyzed lettuce samples after 10 and 30 seconds of fly contact to the plant surface. The findings from the aforementioned studies indicate that flies are potential sources of contamination to produce in the field.

1.8.1 Harvesting of baby spinach and cilantro. During harvest, both cilantro and baby spinach are mechanically cut from the root of the plant (Smith et al. 2016; FDA 2013; Gunes and Dogu 2010). The blades of the mechanical harvester can serve as a source of contamination (FDA 2013). Opportunity for contamination occurs due to the close proximity the harvester blades are to the soil since soil and contaminated plant material can cross-contaminate the harvester blades (Patel et al. 2011; FDA 2013). Bacteria that become attached to the blades can form biofilms utilizing exudates released from cut spinach, or other produce (Patel 2013 et al.). Once formed, biofilms harbor bacteria that are protected from chemical sanitizers (Patel et al. 2011). When harvester blades were inoculated with *E. coli* O157:H7 at 1 log CFU/blade and held at dynamic temperatures of 30 and 20°C to mimic day and night temperatures in the field respectively, populations of attached bacteria increased to 6.09 log CFU/blade after 24 h (Patel et al. 2011). This highlights the ability of *E. coli* O157:H7 and other enteric pathogens to persist on machinery in the field.

For large commercial scale harvesting, cilantro is mechanically cut either directly below the soil surface or 1.5 - 2 inches above the crown of the plant (Smith et al. 2016). In smaller operations, cilantro is manually cut using the same measurements (Smith et al. 2016). Once cut, cilantro is either placed into bins and taken for further processing or bundled into small bunches and packed into 10 lb. boxes in the field (Smith et al. 2016). Once harvested and placed into bins or boxes, cilantro is taken to a holding area where it is cooled using direct application of ice or cool air to remove field heat prior to distribution (Smith et al. 2016; FDA 2013).

At the time of harvest, spinach leaves are mechanically cut 1 inch above the soil using a lawn mower-type machine and then collected into bins or totes in the field (Gunes and Dogu 2010). Although effective for harvesting, this machine can introduce microorganisms and perhaps pathogens into the product due to contact between the machine and soil, especially if manure is present in the soil (Buchholz et al. 2012). In order to prevent moisture loss and wilting from occurring, most produce is harvested in the morning when the air temperature is cooler (Gunes and Dogu 2010). To prevent leaves from becoming crisp and breaking, it is also good practice to not harvest soon after a heavy rainfall. Once spinach leaves have been removed from the root, it is collected into bins and taken for further processing.

1.9 Post-harvest processing

Post-harvest operations also pose a risk of cross contamination. Washing and fluming are key post-harvest processing steps that can spread contaminants, even if initial levels are low (Farrar and Guzewich 2014; Buchholz et al. 2012; Buchholz et al. 2014; Gil et al 2009; Luo et al. 2012). Proper maintenance and monitoring of the water quality is vital to reducing the risk of pathogens and cross contamination.

Produce will come in contact with various equipment surfaces during post-harvest processing. The most commonly used food contact surface in the industry is stainless steel because it is nonporous, easy to clean, and robust (Fellows 2009; Holah and Thorpe1990). Other equipment surfaces may include conveyor belt materials made from different plastic polymers (Buchholz et al. 2011). However, pathogens have the ability to attach to the surface of stainless steel and other equipment surfaces and form biofilms. Biofilms represent an accumulation of surface-attached microorganisms within a matrix of exopolymers that affords protection from the

external environment and allows for their survival (Costerton et al. 1999). A related study found that *Salmonella* Enteritidis survived on a stainless steel surface for 4 days when the initial contamination level was 10^7 CFU/100 cm², for 24 h when initial contamination was 10^5 CFU/100 cm², and for 1 h when initial contamination was 10^3 CFU/100 cm² (Kusumanigum 2003). These results highlight the importance of frequent equipment surface monitoring using swab analysis, or other method, in order to monitor the occurrence of pathogenic loads and eliminate them immediately. Studies dedicated to the mitigation of biofilms on equipment and food contact surfaces strongly emphasized the importance of proper sanitation techniques.

1.9.1 Shredding and cutting. Shredding is a value added processing step in the industry because it is not mandatory and primarily done for consumer convenience (Buchholz et al. 2011). An example of a value added product is bagged, shredded lettuce because the product is ready to use by the consumer with no further preparation steps needed. During mechanical shredding of leafy greens, a high speed, rotating blade reduces the product to small, consistently sized pieces. The mechanical act of shredding breaks the natural protective surface of produce, releases nutrient rich exudates, and increases the cut surface area exposed to the environment (Brackett 1994; Allende et al. 2009). Under these conditions, sanitizer efficacy is significantly reduced and the likelihood of pathogen transfer and cross contamination increases (Seo and Frank 1999; Khalil and Frank 2010).

A study tracking the transfer of *E. coli* O157:H7 through a pilot-scale leafy green processing line found the greatest amount of *E. coli* O157:H7 transfer occurred from inoculated lettuce to the mechanical shredder (Buchholz et al. 2012; 2014). Poor cleaning and sanitizing of the shredding equipment was implicated as the source of a *Salmonella* Bovismorbificas phage type 32 outbreak involving lettuce (Stafford et al. 2002). Fresh-cut cilantro leaves were also

found to be more susceptible to microbial contamination than whole cilantro leaves (Wang et al. 2004; Allende et al. 2009). In another study, researchers found that *Salmonella* Chester was more difficult to eliminate from cut surfaces of green peppers (Liao and Cooke 2001; Allende et al 2009).

If proper holding temperatures are maintained following shredding, the quality and safety of produce can be maintained. Some studies have shown that certain pathogens, such as *E.coli* O157:H7 present on produce will not grow if held at a low temperature ($\leq 4^{\circ}\text{C}$); however other pathogens, such as *L. monocytogenes* are still capable of growing (Herdt and Fang. 2009; Kaminski et al. 2013). For example, one study found a decline in *E. coli* O157:H7 populations on shredded iceberg lettuce held at 5°C , but a 2 log CFU/g increase occurred when lettuce was held at 15°C for 7 days, and a 3 log CFU/g increase after 14 days (Li et al. 2001). Another study found a plateau in *E. coli* O157:H7 growth on cut iceberg lettuce when held at 4°C and 5°C , but recorded a 1 log CFU/g increase after 2 h with a holding temperature of 10°C (Francis and O’Beirne 2001).

1.9.2 Conveying. Various mechanical conveyor systems are used for transporting fruits and vegetables during post-harvest processing. Two common types of conveyor belt designs are interlocking and continuous (Buchholz et al. 2011). Common materials used to make conveyor belts include high-density polyethylene, and polypropylene (Buchholz et al. 2011). Conveyor belts are highly prone to microbial contamination, proliferation, and sources of cross contamination (Matthews 2014; Buchholz et al. 2011). Due to less vulnerability to contamination and ease of cleaning, continuous belts are most commonly used in the industry (Buchholz et al. 2011).

Studies have been performed to assess cross contamination when produce comes into contact with conveyors. Buchholz and others (2012) quantified the transfer of *E. coli* O157:H7 from artificially contaminated baby spinach, iceberg and romaine lettuce to equipment during small scale processing. *E. coli* O157:H7 transfer was greatest to the shredder and conveyor. The authors explained that the exudates released from leafy greens after shredding increased the amount of liquid and organic load present on these two pieces of equipment due to their close proximity and allowed for the survival of *E. coli* O157:H7 (Buchholz et al 2012).

In other work, Allen and others (2005) showed that *Salmonella* Typhimurium can survive on stainless steel, PVC, and wooden surfaces for up to 28 days at 80% relative humidity and 11 days at 60% relative humidity. Moore and others (2003) reported similar trends of *Salmonella* survival on stainless steel equipment when surfaces were initially contaminated with 10^6 CFU/28mm². *Salmonella* Typhimurium transferred to uncontaminated lettuce that had been placed on the stainless steel surfaces 1-2 h after initial contaminated (Moore et al. 2003).

1.9.3 Flume washing. ‘Fluming’ is a term used to describe the movement of foods by water in troughs and is advantageous because produce is cleaned while simultaneously being transported to the next stage of processing (Fellows 2009). Flume washing is widely used in the fresh produce industry; however other methods of commercial washing include dump tanks, flatbed and U-bed brush washers, reel washers, and pressure washers (Sapers 2014). The aforementioned washing operations can be used alone or in combination. Selection of the proper wash treatment is based on characteristics unique to the particular product such as fragility, size, shape, the type and levels of contaminants anticipated to be present (pesticide residue, field debris, spoilage microorganisms), and the extent of decontamination desired (Sapers 2014; Fellows 2009). An important function of washing fresh produce is to improve overall quality and

appearance by removing dirt, field debris, cooling, and minimize physiological changes (Herd and Fang 2009). However, the quality of the water needs to be adequately maintained in order to ensure the washing step remains effective. A study analyzing the efficacy of sanitizers on shredded lettuce and spinach against *E. coli* O157:H7 found a negligible log reduction in pathogens and attested that high organic load and poor microbial quality of the water were the most significant limiting factors in sanitizer efficacy (Barrera et al. 2012). The researchers also found that the final microbial quality of spinach was most impacted by the initial microbial quality of the incoming produce from the field (Barrera et al. 2012).

Although effective in improving cosmetic appearance and removing planktonic bacteria, washing can be a primary vehicle for the spread of pathogens. Chemical sanitizers are often added to wash water, however their primary purpose is to maintain the microbial quality of the water rather than inactivate pathogens that may be present on the product (Gil et al. 2009). Pathogen persistence on the hydrophobic surface of the plant phylloplane (leaves) limits the ability for chlorinated sanitizers to eliminate pathogens, increasing the chance of an infectious dose remaining on the plant at the time of consumption (Beuchat, 1992, Delaquis et al., 1999 and Heaton and Jones, 2007; Allende et al. 2009). Other process conditions impacting sanitizer efficacy include the ratio of fresh produce to wash water, physical features of the produce such as crevices or cracks, timing between initial contamination and washing, contamination load, concentration of sanitizer used, and contact time between produce and wash water (Gil et al. 2009). The ability of chlorine and other sanitizers to reduce planktonic bacteria and human pathogens in the wash water significantly reduces the risk of foodborne illness, however due to the low infectious dose of certain enteric pathogens (i.e. *Salmonella*, *E. coli* O157:H7), complete safety cannot be assured (Sapers 2014). Maximum reductions of generally no more than 2-3 logs

have been routinely reported when inoculated produce has been subjected to sanitizer washing (Gil et al. 2009; Beuchat et al., 2004; Gonzalez et al., 2004; Inatsu et al., 2005; Ukuku et al., 2005; Allende et al., 2007; Gómez-López et al., 2007; Selma et al., 2008b). Other studies have reported a consistent 2 log maximum reduction of enteric pathogens under conditions that most closely mimicked contamination during commercial production (Brackett, 1987; Zhuang et al., 1995; Beuchat et al., 1998; Garcia et al., 2003). Additional studies have reported a maximum 1-2 log reduction for human foodborne pathogens on inoculated samples that were washed and subsequently held at refrigeration temperatures (Sapers et al., 1999; Wisniewsky et al., 2000; Wright et al., 2000; Lukasik et al., 2003; Caldwell et al., 2003; Nascimento et al., 2003; Beuchat et al., 2004; Oh et al., 2005; Yuk et al., 2005 and Yuk et al., 2006; Shiron et al., 2009; Vandekinderen et al., 2009). The variance in log reductions achieved from past studies using chemical sanitizers in wash water is due to the unique physical characteristics of produce, wash time, initial pathogen load, and time of contamination in relation to wash time, thus making each wash operation highly unique for each produce commodity. Washing fresh produce in sanitizer-free water typically reduces the microbial load ~1 log for tomatoes, baby spinach, iceberg lettuce, and romaine lettuce (Wang and Ryser 2014, Sapers 2006; Buchholz 2012, Chang et al. 2012).

Chlorine-based sanitizers are most commonly used for commercial washing of fresh produce due their low cost, versatility, and capacity to reduce microbial populations (Fellows 2009; Buchholz 2011; Sapers 2014; Suslow 2000). The efficacy of chlorine-based sanitizers relies heavily upon water quality and process conditions. The water must have a low organic load, low turbidity, have an optimal pH range of 6-7, and be at the appropriate temperature for the produce being washed (Sapers 2014). In the industry, produce is typically washed for 1-2

minutes in water containing 50-200 ppm of free chlorine (Sapers 2014). Although highly effective at removing planktonic bacteria, chlorine sanitizers are less effective against bacteria that are attached or internalized in produce (Sapers 2014).

Other popular sanitizers used for commercial washing of fresh produce are chlorine dioxide, ozone, and peroxyacetic acid (Buchholz 2011). While these sanitizers have all proven to be effective in reducing pathogen cross-contamination from the wash water, reduction of pathogens on the produce during washing has remained problematic, regardless of the sanitizer used. Chlorine dioxide and peroxyacetic acid are more expensive than chlorine but have a greater oxidation capacity and thus are more resilient to high organic loads in wash water (Buchholz et al. 2011). Chlorine dioxide can be used at 3 ppm to sanitize fresh produce, provided that the product receives a potable water rinse thereafter (Sapers 2014; 21 CFR 173.300). Studies assessing the efficacy of chlorine dioxide have generally shown pathogen reductions on produce of 1 to 3 logs after treatment with maximum reductions of 3 to 5 logs reported for tomatoes, peaches, and fresh cut cabbage and carrots (Sy et al. 2005; Sapers 2014). Rodgers and others (2004) achieved a ~5.6 log reduction for *L. monocytogenes* and *E. coli* O157:H7 on inoculated apples, lettuce, strawberries, and cantaloupe (2004). Although these reductions are high, the lengthy exposure times used remain problematic for the industry.

Ozone is effective against a broad range of microorganisms in wash and flume water and can be used at low concentrations. However, its ability to reduce bacteria and pathogens on produce is again dependent on the location of the organism. Ozone was ineffective in reducing *E. coli* O157:H7 and *L. monocytogenes* on inoculated alfalfa seeds and the stem and calyx areas of apples due to internalization of the pathogens (Sapers 2014). According to Rodgers and others (2004), ozone (3 ppm) and chlorine dioxide were equally effective in achieving a ~5.6 log

reduction of *E. coli* O157:H7 and *L. monocytogenes* on dip-inoculated fresh produce however, these treatment conditions would not be practical in a commercial setting.

1.9.4 Dewatering and drying. In order to maintain produce quality and shelf life, drying operations are used to remove excess water from plant surfaces. Excess water can sustain the growth of microorganisms and stress the plant, both of which can lead to a shorter shelf life and diminished quality. Dewatering screens are commonly used to remove water following flume washing (Fellows 2009). Other types of drying equipment include shaker tables, air blowers, and centrifugal dryers (Buchholz et al. 2011). Similar to the selection of washing equipment, the drying method must be tailored to the physical sensitivity and limitations of the specific product. For example, due to their fragility, tomatoes are dried using both sponge rollers and air blowers in order to minimize physical damage and prevent skin breakage (Suslow 2004; Buchholz et al. 2011). In contrast, iceberg and romaine lettuce can withstand dewatering and centrifugal dryers. Centrifugal drying can expel up to 30% of the microbial population during drying making this process ideal for microbial testing (Buchholz et al. 2012).

1.9.5 Baby spinach. Spinach can be consumed either fresh, frozen, canned, or dehydrated (Gunes and Dogu 2010). After harvest, spinach is subjected to: pre trimming and packaging, transportation or storage, trimming, sorting, grading, and washing (Gunes and Dogu 2010). The flume washing step can be performed with floating, immersion, rotary or high pressure sprays and must be done with the proper sanitizers. Washing spinach is a crucial step for reducing spoilage and pathogenic microorganisms. Chlorine-based disinfectants are most commonly used for the washing of spinach (Gunes and Dogu 2010). After washing, spinach is then further processing, if needed.

For fresh and fresh-cut RTE spinach, the minimal processing operations include washing with a sanitizer, gentle drying, sorting, packaging, and storage (Gunes and Dogu 2010). The most common forms of minimally processed spinach available on the market are whole leaf and baby spinach. Physical damage will increase the naturally high respiration rate of spinach and lead to a shorter shelf-life. Efforts to optimize spinach quality and control respiration include reducing surface water through dewatering following rinsing, modified atmosphere packaging of fresh cut spinach, and proper temperature control of packaged spinach during storage and transportation (Gunes and Dogu 2010). The recommended conditions for holding spinach during storage and transportation are 0°C with a relative humidity of 95-98% (Gunes and Dogu 2010).

1.9.6 Cilantro. The post-harvest processing steps taken for cilantro vary depending on the capabilities of the growers, handlers, and packing houses. Cilantro can be packed in the field or taken to a processing plant or packaging facility (FDA 2013; Smith et al. 2016). Immediately after arriving at one of these destinations, cilantro must be cooled to maintain quality and shelf-life. Cooling is achieved either by direct ice application or hydrocooling. The packing facility has the option of washing cilantro; however, it is not required (FDA 2013). If washed, approved sanitizers must be used and the water quality must be well maintained in order to minimize bacteria and pathogens. Fresh-cut cilantro is most commonly sanitized with chlorine-based sanitizers (Allende et al. 2009). The water used to make ice for cooling cilantro must meet US-EPA standards and must contain an approved disinfectant in order to reduce microbial loads present (FDA 2013). If cilantro is hydrocooled, the water must also meet US-EPA standards and contain an approved disinfectant in order to maintain the microbial quality of the water (FDA 2013). Once cooled using the ice or hydro method, cilantro must then be held at 4°C during storage and distribution (FDA 2013).

1.10 Food Safety Modernization Act

The FDA Food Safety Modernization Act (FSMA) was signed into law in 2011 and refocuses the way in which the FDA responds to foodborne outbreaks. Under FSMA, the FDA will take a preventative approach to foodborne outbreaks by utilizing scientific data as a foundation of creating new and updating current regulations (FDA 2016b). A paramount mandate for the produce industry was the establishment of science based minimum standards for the growing, harvesting, packing, and holding of produce in order to minimize contamination that may lead to serious health consequences or death (FDA 2016b). FSMA outlines specifications for agriculture water quality and testing. The water intended for hand washing, food contact surfaces, direct contact with produce before and after harvest, and contact with sprouts is subject to water quality standards and consistent testing. There are also new guidelines established for the use of raw manure, growing of sprouts and monitoring of domestic and wild animal activity on farms, as well as new specifications for equipment, tools, buildings, and sanitation practices (FDA 2016b; Gutierrez-Rodriguez 2015).

1.11 Risk assessment tools

The FDA has developed several risk assessment tools in order to estimate risks associated with microbial and chemical contaminants in different foods. The data generated from these tools are being used by the FDA to determine regulations based on scientific evidence. Ultimately, these risk assessment tools will help make the food supply safer and integrate more preventative measures. The two risk assessment tools of greatest value to the fresh produce industry are the Food and Drug Administration's i-RISK and Quantitative Produce Risk Assessment Model (QPRAM). FDA i-RISK is a web-based tool used to compare and rank public health risks

associated with contaminants and different food combinations (FDA 2015c). The FDA uses the data generated from this tool to prioritize high risk foods and allocate necessary funds to reduce the associated risk (FDA 2015c). FDA i-RISK can also be used to assess the efficacy of various preventative measures throughout the farm to fork continuum to mitigate risks (FDA 2015c).

This tool can be used to determine the risk associated with one hazard in multiple foods, multiple hazards in one food, and multiple hazards in multiple foods. QPRAM, a risk assessment tool used specifically for the fresh produce industry, predicts and characterizes risk associated with the handling of fresh produce on the farm, during processing and at the point of consumption (FDA 2015c). The data gathered from this tool can be used to predict and prevent future outbreaks by determining places in the farm to fork continuum that are at highest risk for contamination (FDA 2015c). Once these locations are identified, the proper preventative actions can be implemented accordingly.

1.12 Overall goals

Due to the high numbers of recalls and outbreaks traced back to fresh produce, including leafy greens and culinary herbs, the microbial safety of these products has come into question. Large data gaps currently exist in the ability to accurately characterize produce cross-contamination during processing. A principal step in produce processing is washing, which has the potential to either reduce or concurrently spread contamination to previously uncontaminated produce; however the extent to which this may occur is not well understood.

The primary goals of the present cross contamination study were 1.) to quantify *Salmonella* Typhimurium transfer and redistribution when realistically low initial inoculation levels and weight ratios of inoculated surrogate were introduced to uninoculated baby spinach

and cilantro during post-harvest processing, in sanitizer-free wash water and 2.) assess the efficacy of a chlorine-based sanitizer (60 ppm, acidified to pH 6.5) when the highest inoculation level 10^3 CFU/g and 1:100, 5:100, and 10:100 weight ratios of inoculated surrogate to uninoculated baby spinach and cilantro are used. Information gathered from this study will support related cross contamination studies and help to fill data needs for future FDA risk assessment tools.

CHAPTER 2:
***SALMONELLA* TYPHIMURIUM LT2 TRANSFER AND REDISTRIBUTION ON BABY**
SPINACH AND CILANTRO DURING PILOT-SCALE PROCESSING

2.1 Introduction

Among food categories, leafy green vegetables and fresh herbs present the greatest concern for microbiological hazards (FAO and WHO 2008). Between 2004 and 2013, consumption of fresh produce led to over 643 outbreaks and sickened 20,456 individuals (CSPI 2015). These numbers surpassed all other outbreaks and illnesses occurring during this same time period, including commodities such as meat, poultry and seafood.

Produce can become contaminated at any point in the farm to fork continuum. Specific pre-harvest areas of major concern identified by the Food and Drug Administration are agricultural water, soil amendments (i.e. manure), water runoff from nearby livestock and animal operations, wild and domestic animal fecal contamination, insects, poor field worker health and hygiene, and cleanliness of harvesting equipment, tools and buildings (FDA 2013). Poor field worker hygiene and fecal contamination from wild animals and nearby livestock operations were singled out as possible sources of cilantro and spinach outbreaks, respectively (FDA 2016; Atwill et al. 2015).

Manure and soil are prone to pathogen contamination due to the nutrient rich environment naturally present in these materials (Millner 2014). Pathogens can easily transfer from contaminated manure or soil to produce through direct contact, irrigation water, and harvesting techniques (Millner et al. 2014). Studies assessing the prevalence of pathogens in manure and feces found *Salmonella* spp. in 17/287 sheep, 273/4977 cattle, 44/600 swine, and 12/67 poultry, samples taken on farms where manure originated (Fedorka-Cray et al. 1998; Pao et al. 2005; Callaway et al. 2010; Hutchison et al. 2004). Manure can contain initial loads of *Salmonella* spp. between 10^2 and 10^7 CFU/g (Pell 1997). A risk assessment study reported that 2.0% and 2.6% of soil samples were positive for *Salmonella* spp. from produce-growing regions of California and

New York State, respectively (Strawn et al. 2013). Fecal contamination is further complicated by the ability of enteric pathogens (i.e. *Salmonella*) to attach, internalize, and form biofilms on cilantro and spinach (Berger et al. 2009; Brandl and Mandrel 2002). The FDA advises that growers take proper precautions when treating manure prior to field application and to also be aware of contamination sources beyond the field including cattle and poultry operations. If these guidelines are not followed, likelihood of produce field contamination increases significantly (FDA 2016).

Surveys conducted by the FDA found 9% (n=177) of imported cilantro positive for *Salmonella* or *Shigella* and 1.2% (n=85) of domestic cilantro positive for *Salmonella* (FDA 2003). As a follow up to this study, 3.3% of imported samples (n=33) were *Salmonella*-positive (FDA 2003). From 2002-2009 the FDA randomly tested 2510 cilantro samples for the presence of enterohemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), and *Salmonella* and found 0.64% (n=16) to be positive for the presence of one of these pathogens (FDA 2015a). Of the commodities tested, the 0.64% positive rate for cilantro was the second highest, with spinach producing the highest positive rate with 0.74% (n= 4433). Specific to cilantro, *Salmonella* has caused 8 multi-state outbreaks in the past 20 years and two Class I recalls in 2011 and 2012 after random FDA and USDA samples were *Salmonella*-positive (CDC 2016; US Foods 2017). Random sampling of baby spinach from farms in Spain found 5.2% (n=38) of collected samples to be contaminated with *Salmonella* following harvest (Garcia-Villanova Ruiz et al. 1987). In 2005, *Salmonella* Typhimurium contaminated spinach caused 60 reported illnesses in Finland (Eurosurveillance 2005). In 2007, *Salmonella* java contaminated spinach caused 354 illnesses across Europe in 2007 (Denny et al. 2007). Domestically, spinach has been linked to one multi-

state *Salmonella* outbreak and two Class I recalls in 2011 and 2015 (CDC 2016). Both recalls were due to a random field sample of spinach testing positive for *Salmonella*.

Previous pilot-scale processing studies in our laboratory have shown that a surrogate inoculated with 10^6 , 10^4 , and 10^2 CFU/g *E. coli* O157:H7 can contaminate large quantities of uncontaminated iceberg lettuce as well as various equipment surfaces including a shredder, conveyor, flume tank, shaker table, and dewatering centrifuge during washing in sanitizer-free water (Buchholz et al. 2012; Buchholz et al. 2014). This data demonstrated that even small, localized areas of contamination can spread to contaminate larger quantities of produce and equipment. However, the initial inoculation levels and weight ratios of inoculated to uninoculated product used in the aforementioned study as well as other cross contamination studies were worst case, highly localized levels of contamination. As highly localized contamination becomes disseminated over a wider area, subsequently lower numbers of bacteria can contaminate irrigation water, equipment, or other areas of the field (Beuchat et al. 2010; Ibekwe et al. 2004). In order to more accurately mimic pathogen redistribution in the field, low initial inoculation levels were selected for this study (10^3 , 10^1 , and 10^{-1} CFU/g).

The objectives of the present cross contamination study were to 1.) quantify *Salmonella* Typhimurium transfer and redistribution when realistically low initial inoculation levels and weight ratios of inoculated surrogate were introduced to uninoculated baby spinach and cilantro during post-harvest processing, in sanitizer-free wash water and 2.) assess the efficacy of a chlorine-based sanitizer (60 ppm, acidified to pH 6.5) when the highest inoculation level 10^3 CFU/g and 1:100, 5:100, and 10:100 weight ratios of inoculated surrogate to uninoculated baby spinach and cilantro were used. Information gathered from this study will support related cross contamination studies and help to fill data needs for future FDA risk assessment tools.

2.2 Materials and methods

2.2.1 Overall experimental design. The spread of *Salmonella* during pilot-scale processing of baby spinach and cilantro was assessed using four inoculated:uninoculated product ratios (0.5:100, 1:100, 5:100, 10:100) and three inoculation levels. Red leaf lettuce (10^3 , 10^1 , 10^{-1} CFU/g) served as the colored surrogate and was spot inoculated 18-24 h before processing to reach the targeted inoculation level. In each trial, product was flume-washed in sanitizer-free water for 90 s. In order to better mimic industry practice, 60 ppm available chlorine (acidified to pH 6.5) was used in wash water only at the 10^3 CFU/g inoculation level and 1:100, 5:100, and 10:100 weight ratios. All experiments were conducted in triplicate.

2.2.2 Produce. Baby spinach and cilantro were purchased from a local wholesaler (Stan Setas Produce Co. LLC, Lansing, MI). Red leaf lettuce was purchased from a local retail supermarket. All produce was stored in a walk in cooler at 4°C and produce was used within 3 days of delivery. Prior to use, any damaged leaves were discarded. When preparing the red leaf lettuce, only the red portion from the inner leaves was used for inoculation.

2.2.3 Bacterial strain. To ensure worker safety during pilot-scale processing, avirulent *Salmonella* Typhimurium LT2 was used (acquired from Dr. Michelle Danyluk University of Florida, Gainesville), with this strain previously shown to exhibit similar attachment and growth characteristics to other *Salmonella* strains implicated in fresh produce outbreaks (Wang and Ryser 2014). This strain was maintained at -80°C in Trypticase Soy Broth containing 0.6% (wt/vol) yeast extract (TSBYE; BD, Sparks, MD) and 10% (v/v) glycerol prior to use.

The working culture was prepared by streaking the frozen stock culture onto Trypticase Soy Agar containing 0.6% (w/v) yeast extract (TSAYE; BD, Sparks, MS) and incubating for 24 h at 37°C. A single isolated colony was then subjected to two successive transfers in 9 Ml (24

h/37°C) of TSBYE. Thereafter, the culture containing $\sim 10^9$ CFU/mL was appropriately diluted in sterile phosphate buffer solution (8.5 g of NaCl per liter, 1.44 g of Na_2HPO_4 per liter, and 0.24 g of KH_2PO_4 per liter) for red leaf lettuce inoculation.

2.2.4 Surrogate inoculation. Twenty-four hours before processing, red leaf lettuce was aseptically cut with a knife into $\sim 5 \times 5$ cm pieces (~ 1 g) to obtain the targeted inoculated:uninoculated weight ratios of 0.5:100, 1:100, 5:100, and 10:100. The appropriate number of leaves was placed in a biosafety chamber, spot-inoculated at multiple locations with 50 μL of *S. Typhimurium* LT2 at 10^3 , 10^1 , and 10^{-1} CFU/g, dried for 15 minutes, transferred to sterile plastic containers (PLA NatureWorks NE) and held at 4°C for 18-24 h. Following overnight storage and before processing, a 25 g sample of the inoculated red leaf lettuce was analyzed by direct plating on XLT4 agar (Neogen Corp., Lansing, MI) to determine the starting population of *S. Typhimurium* LT2.

2.2.5 Wash water and sanitizer Sanitizer-free filtered tap water at 10°C was used for washing at the three inoculation levels and four inoculated:uninoculated produce ratios. In addition, efficacy of a chlorine based sanitizer (XY-12, Ecolab, St. Paul, MN) (60 ppm of available chlorine) was also assessed, but only at weight ratios of 1:100, 5:100, and 10:100 at the highest inoculation level (10^3 CFU/g). Water containing the sanitizer was acidified to pH 6.5 with citric acid (Sigma-Aldrich, St. Louis, MO) and measured with a pH probe (pHTestr 30, Oakton, Vernon Hills, IL). The level of available chlorine was measured using a rapid test kit (Ecolab, St. Paul, MN).

Wash water (before and after processing) and expelled centrifuge water samples were collected (~ 50 mL) and direct plated on XLT4 agar to determine *Salmonella* counts for each trial. Water samples collected for the sanitizer trials were treated with 0.5% sodium thiosulfate

(Fisher Science Education, Hanover, IL) immediately following collection in order to neutralize the sanitizer.

2.2.6 Leafy green processing line. The pilot-scale processing line consisted of a 3.6 m-long stainless steel flume tank, shaker table, dewatering centrifuge, and a non-refrigerated recirculation tank (1000 L capacity). The flume tank (Heinzen Manufacturing, Inc., Gilroy, CA) was equipped with two overhead spray jets. A 4.1-m long, 10 cm diameter hard plastic discharge hose and centrifugal pump (model XB754FHA, Sterling Electric, Inc., Irvine, CA) circulated the wash water at 15 L/sec through the flume tank. A stainless steel screen at the end of the flume tank retained the product for the desired washing time. Immediately beyond the screen was a stainless steel shaker table screen operated by a 1 HP Baldor wash-down duty motor (Baldor Electric Co., Ft. Smith, AR) at 1760 RPM which was used for partial dewatering. Water removed from the shaker table flowed back into the recirculation tank. After shaker table dewatering, the produce was centrifugally dried using a 22.7 kg capacity Spin Dryer (model SD50-LT, Heinzen Manufacturing, Inc.) with three internally timed spin cycles totaling 60 s.

2.2.7 Baby spinach and cilantro processing and sample collection. For processing, ~5 kg (± 0.5 kg) of uninoculated baby spinach or cilantro was added to the flume tank containing ~300 L of filtered sanitizer-free tap water (10°C) followed by addition of the required amount inoculated red leaf lettuce. The combined batch of inoculated and uninoculated product was then washed for 90 s in the flume tank. After 90 s, the batch was dewatered on the mechanical shaker for ~15 s and collected in a perforated plastic bin and centrifugally dried for 60 s. After all previously inoculated red leaf lettuce was manually removed, 25 g of washed and processed red leaf lettuce was placed in a Whirl-PakTM bag for quantitative and qualitative analysis. The

remaining 5 kg of baby spinach or cilantro was collected into ~20-24 Whirl-Pak™ bags, each containing 225 g of produce.



Figure 2.1: Flume tank and dewatering shaker table.



Figure 2.2: Collection bin for washed produce following 90 s of flume washing.



Figure 2.3: Centrifugal dryer.



Figure 2.4: Bagged samples containing 225 g baby spinach/cilantro after removal of the inoculated surrogate.

2.2.8 Microbiological analysis. All water and red leaf lettuce samples before and after processing, and bagged baby spinach and cilantro samples were assessed for *Salmonella* Typhimurium LT2 using direct plating or GeneQuence Assay (Neogen Corp., Lansing, MI).

2.2.9 Red leaf lettuce samples. The two 25-g red leaf lettuce samples were collected before and after processing, placed into individual Whirl-Pak™ bags containing 50 mL of phosphate buffer solution, homogenized in a stomacher (Stomacher 400 Circulator, Seward, Worthington, UK) for 2 min at 260 rpm and then plated in duplicate with or without membrane filtration on XLT4 Agar. Another red leaf lettuce sample collected after processing was assessed for presence of *S. Typhimurium* LT2 using the GeneQuence assay analysis.

2.2.10 Water samples. The 50 mL water samples collected before processing, after 90 s of washing, and during centrifugal drying were subjected to membrane filtration and plated on XLT4 agar. The plated samples were then incubated for 24 h at 37°C. All colonies resembling *Salmonella* were counted.

2.2.11 Bagged baby spinach and cilantro. Each 225 g sample of baby spinach and cilantro was enriched in 450 mL of sterile Lactose Broth (LB) and incubated for 24 h at 35°C. Thereafter, 1 mL aliquot and 0.1 mL aliquot of the LB enrichment was transferred to 10 mL of Tetrathionate (TT) Broth and Rappaport-Vassiliadis (RV) Broth, respectively and incubated for 18 h at 42°C. After incubation, 1 mL aliquots from each enrichment were transferred to 10 mL of sterile Gram Negative (GN) broth and incubated for 6 h at 35°C. All GN enrichments were then examined for presence/absence of *Salmonella* Typhimurium using the GeneQuence assay (Neogen Corp., Lansing, MI). The red leaf lettuce sample collected after processing was similarly enriched and examined using the GeneQuence assay.

2.2.12 GeneQuence assay. A 200 μL aliquot of GN enrichment from the previously enriched TT and RV samples was transferred to the same sterile test tube after which 100 μL of the GeneQuence lysis reagent was added. After 5 minutes of heating in a 65°C water bath, 150 μL of the sample was then transferred to a microwell. Thereafter, the GeneQuence testing protocol for *Salmonella* was followed as outlined by the test kit manufacturer, with the results read at 450 nm on a Stat Fax 4200 plate strip reader. Values ≥ 0.10 were recorded as positive, whereas readings ≤ 0.09 were recorded as negative for *Salmonella*.

2.2.13 Statistical analysis. Colony counts obtained from direct plating were used to determine the percentage of *Salmonella* CFUs transferred from the red leaf lettuce to the uninoculated baby spinach or cilantro during processing. GeneQuence results were used to determine the number of *Salmonella*-positive samples for each batch of baby spinach and cilantro processed. Analysis of variance (ANOVA) using JMP 12.2 software (SAS Institute Inc., Cary, NC) was used to analyze data collected from the triplicate experiments. When *P* values were ≤ 0.05 , the Tukey—Kramer HSD test was used to determine statistical significance.

2.3 Results

Both baby spinach and cilantro yielded statistically similar ($P > 0.05$) percentages of *Salmonella*-positive samples. The inoculated:uninoculated weight ratios did not significantly ($P > 0.05$) affect the percentage of positive samples (Figures 2.5 and 2.6). The initial inoculation level significantly ($P < 0.05$) impacted the number of positive samples with the 10^{-1} CFU/g inoculation level yielding significantly fewer *Salmonella*-positive samples compared to the two higher inoculation levels, regardless of weight ratio (Figure 2.5 and 2.6). When the flume water contained 60 ppm available chlorine (acidified to pH 6.5), greater *Salmonella* reductions were seen for baby spinach and cilantro compared to washing without a sanitizer (Figures 2.5 and 2.6).

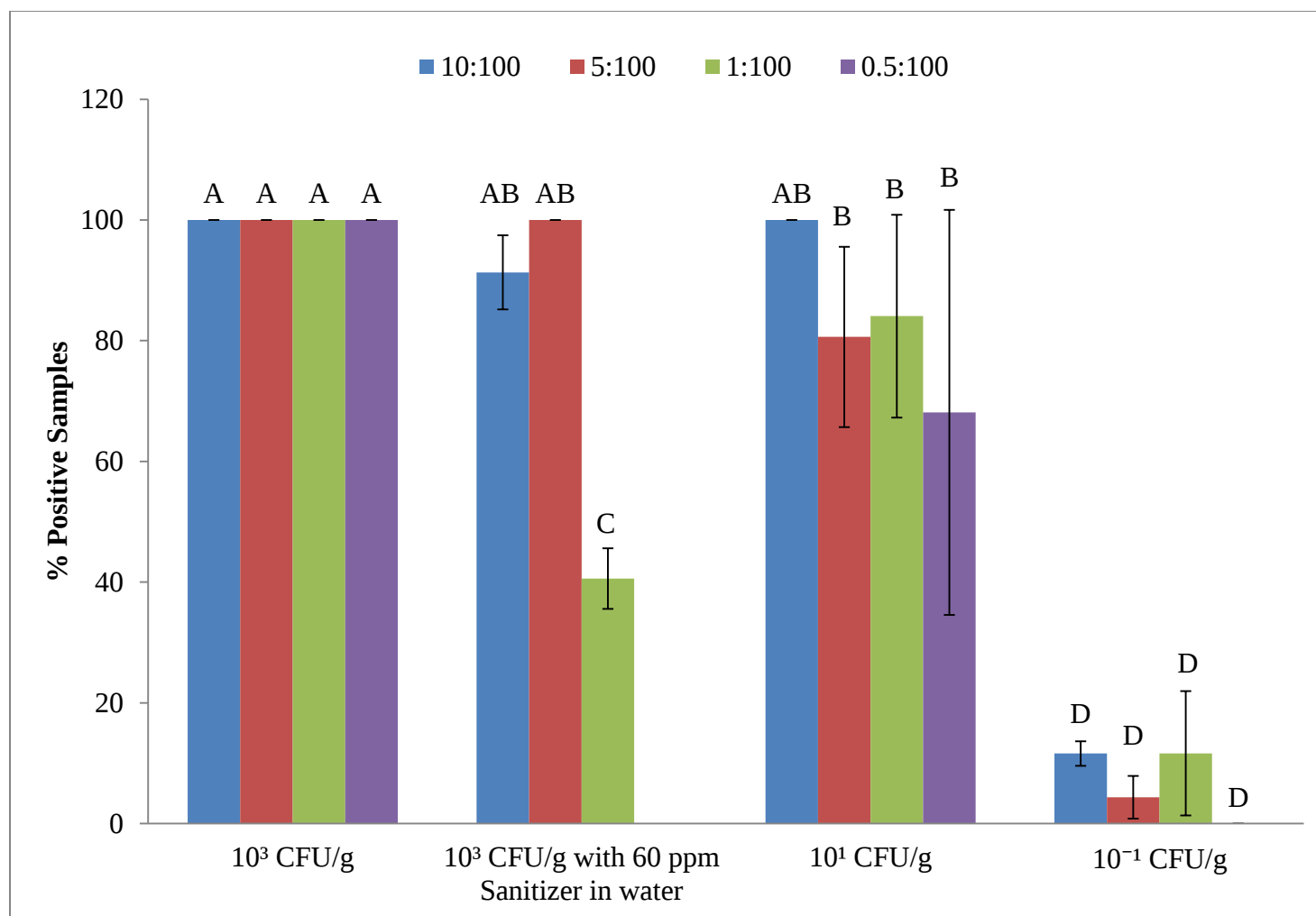


Figure 2.5: Percentage of *Salmonella*-positive baby spinach samples after 90 s of flume washing with and without a chemical sanitizer. Means with different capital letters are significantly different ($P < 0.05$).

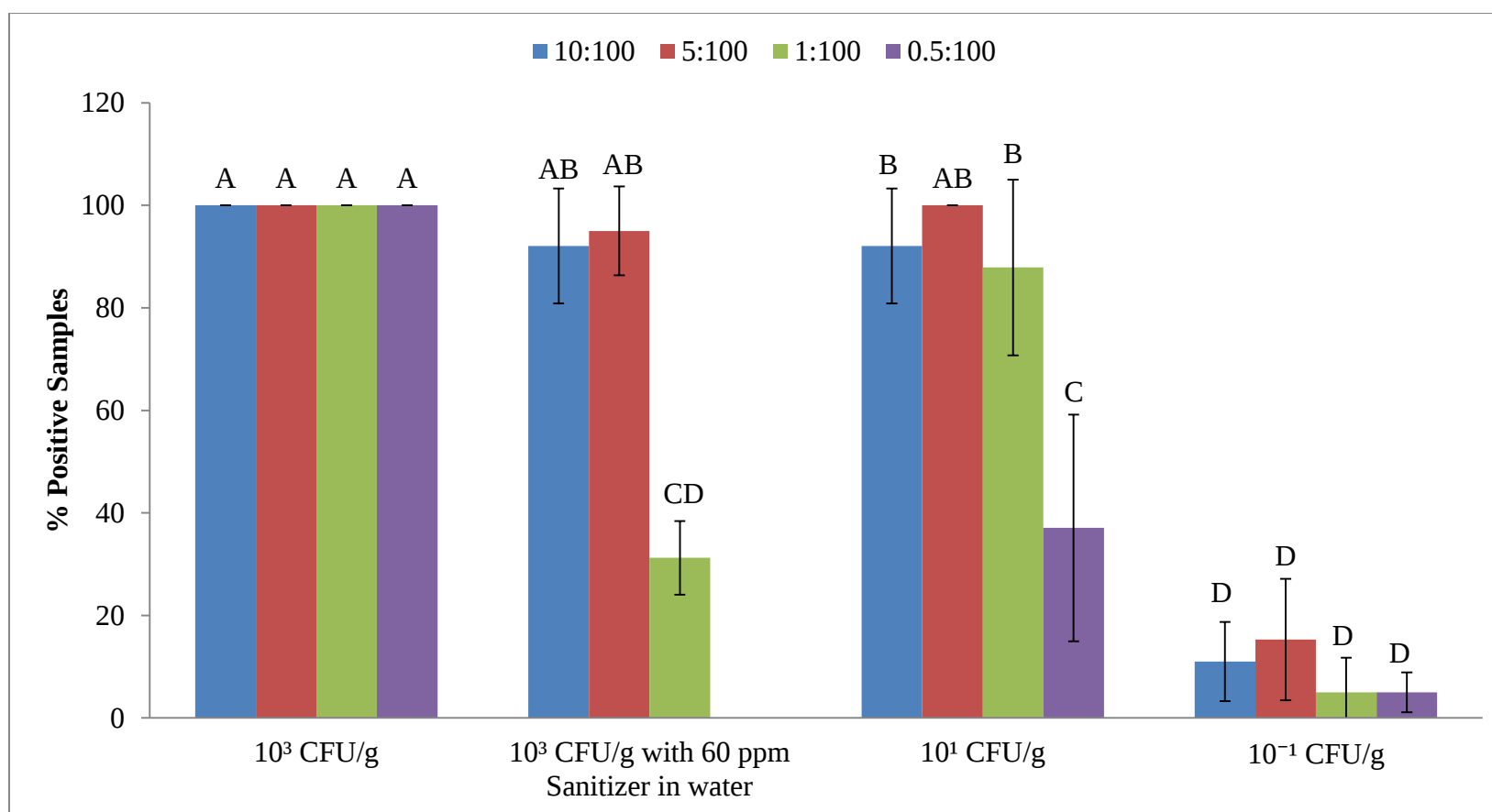


Figure 2.6: Percentage of *Salmonella*-positive cilantro samples after 90 s of flume washing with and without a chemical sanitizer. Means with different capital letters are significantly different ($P < 0.05$).

2.3.1 Baby spinach. As the initial inoculation level decreased, the percentage of positive baby spinach samples also decreased, regardless of the inoculated:uninoculated product ratio. Significantly fewer ($P < 0.05$) positive samples were observed at 10^{-1} CFU/g compared to the 10^1 and 10^3 CFU/g inoculation levels. All samples analyzed at the 10^3 CFU/g inoculation level were positive for *Salmonella* at the four weight ratios tested. At the 10^1 CFU/g inoculation level: 100% ($\pm 0\%$), 80.6% ($\pm 14.9\%$), 84.1% ($\pm 16.8\%$), 68.1% ($\pm 33.6\%$) of the samples yielded *Salmonella* at the 10:100, 5:100, 1:100, and 0.5:100 inoculated:uninoculated ratios, respectively (Table 2.1). For the 10^{-1} CFU/g inoculation level, *Salmonella*-positive samples were: 11.6% ($\pm 2.1\%$), 4.3% ($\pm 3.5\%$), 11.6% ($\pm 10.3\%$), 0% ($\pm 0\%$) for the 10:100, 5:100, 1:100, and 0.5:100 inoculated:uninoculated ratios, respectively (Table 2.1). No significant differences were found between inoculated:uninoculated ratios ($P > 0.05$) at the same inoculation level. Sanitizer helped to reduce the percentage of positive samples to comparable numbers collected in the sanitizer-free 10^1 CFU/g inoculation trials for the 10:100 and 5:100 ratios; however this decrease was not significant (Figure 2.5). When the ratio was reduced to 1:100 during the sanitizer trials; fewer samples were positive ($P < 0.05$) compared to the sanitizer free 10^3 CFU/g trials (Figure 2.5). Spread of *Salmonella* was again generally greater at the 10^3 and 10^1 CFU/g inoculation levels compared to the 10^{-1} CFU/g level at each inoculated:uninoculated product ratio (Figure 2.7).

Table 2.1: Numbers and percentage of *Salmonella*-positive baby spinach samples (23 samples per trial) at different inoculation levels and inoculated:uninoculated product ratios.

Inoculation Level (CFU/g)	Inoculated:uninoculated Ratio	Positive/Total Samples			Avg. Percent Positive Samples
		Rep 1	Rep 2	Rep 3	Mean ($\pm$ SD)
10^3	10:100	23/23	23/23	23/23	100% ($\pm$ 0%)
	5:100	23/23	23/23	23/23	100% ($\pm$ 0%)
	1:100	23/23	23/23	23/23	100% ($\pm$ 0%)
	0.5:100	23/23	23/23	23/23	100% ($\pm$ 0%)
Sanitizer 10^3	10:100	19/23	22/23	22/23	91.3% ($\pm$ 7.5%)
	5:100	23/23	23/23	23/23	100% ($\pm$ 0%)
	1:100	10/23	10/23	8/23	40.6% ($\pm$ 5.0%)
10^1	10:100	23/23	23/23	23/23	100% ($\pm$ 0%)
	5:100	23/23	14/22	18/23	80.6% ($\pm$ 14.9%)
	1:100	21/23	23/23	14/23	84.1% ($\pm$ 16.8%)
	0.5:100	5/23	19/23	23/23	68.1% ($\pm$ 33.5%)
10^{-1}	10:100	3/23	3/23	2/23	11.6% ($\pm$ 2.1%)
	5:100	0/23	2/23	1/23	4.3% ($\pm$ 3.5%)
	1:100	6/23	1/23	1/23	11.6% ($\pm$ 10.3%)
	0.5:100	0/23	0/23	0/23	0% ($\pm$ 0%)

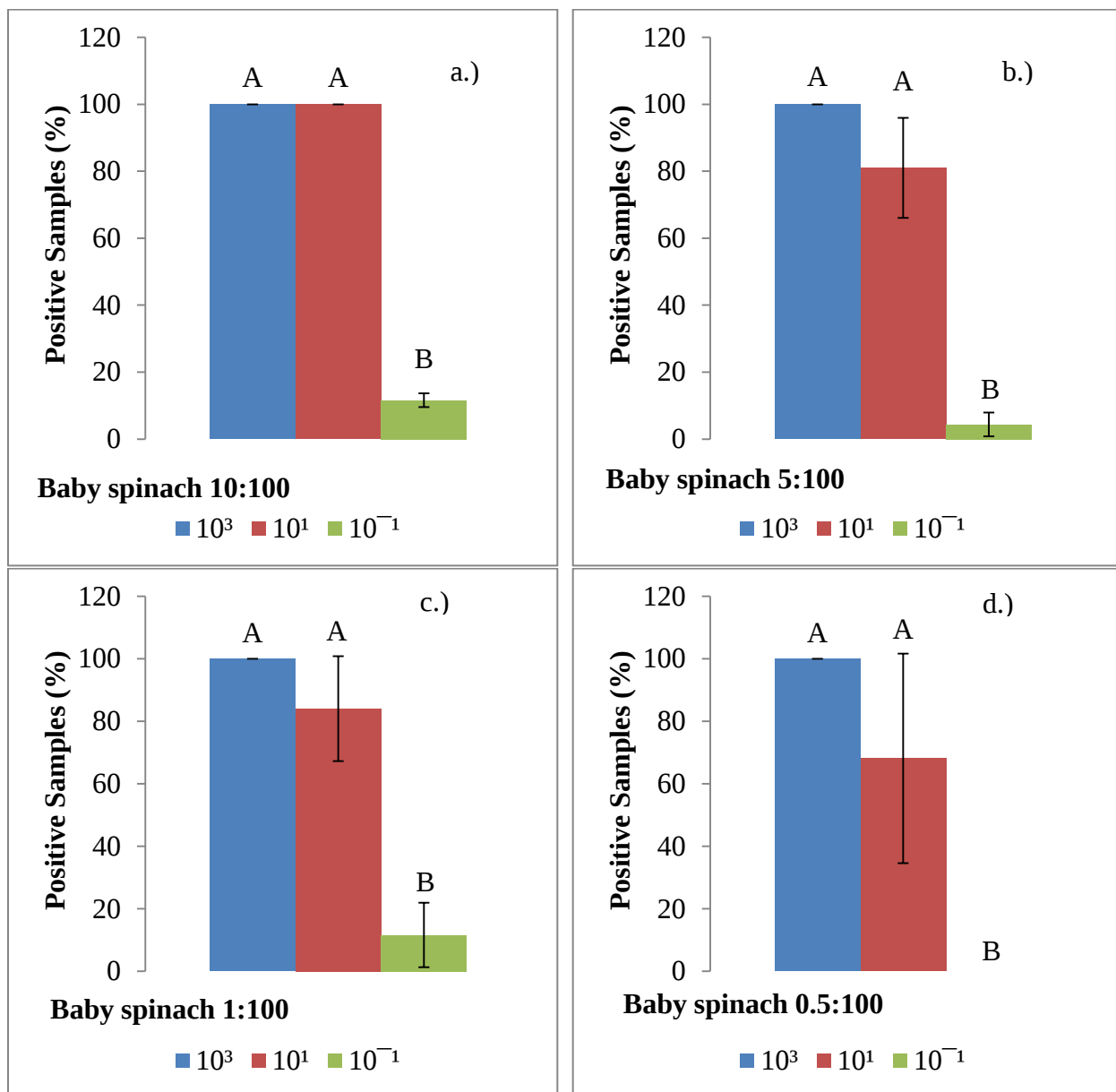


Figure 2.7: Percentage (+/- SD) of *Salmonella*-positive baby spinach samples after processing in sanitizer-free water at inoculated:uninoculated weight ratios of a.) 10:100, b.) 5:100, c.) 1:100, and d.) 0.5:100. Means with different capital letters are significantly different ($P < 0.05$).

2.3.2 Cilantro. Similar to results obtained in the baby spinach trials, the number of *Salmonella*-positive cilantro samples decreased as the initial inoculation level decreased. Significantly fewer ($P < 0.05$) *Salmonella*-positive samples were seen at the 10^{-1} CFU/g inoculation level compared to the two higher inoculation levels (Figure 2.6). Sanitizer use decreased the percentage of positive samples to comparable numbers collected at the 10^1 CFU/g inoculation level for the 10:100 and 5:100 ratios; however this decrease was not significant ($P > 0.05$) (Figure 2.6). Similar reductions ($P < 0.05$) were attained at the 10^1 CFU/g inoculation level at the same weight ratio when no sanitizer was used. These values were comparable to those attained in the baby spinach trials. At 10^3 CFU/g, all samples tested positive for *Salmonella* at each weight ratio. As expected, the number of positive samples decreased as the initial inoculation level decreased. At the 10^1 CFU/g inoculation level, 92.1% ($\pm 11.2\%$), 100% ($\pm 0\%$), 87.9% ($\pm 17.1\%$), and 37.1% ($\pm 22.1\%$) of the samples tested positive for *Salmonella* at the 10:100, 5:100, 1:100, and 0.5:100 inoculated:uninoculated ratios, respectively. At the 10^{-1} CFU/g inoculation level, 10.6% ($\pm 7.7\%$), 15.3% ($\pm 11.8\%$), 4.8% ($\pm 6.7\%$), 4.8% ($\pm 3.9\%$) of the samples tested positive for *Salmonella* at the 10:100, 5:100, 1:100, and 0.5:100 inoculated:uninoculated weight ratios, respectively (Table 2.2). Spread of *Salmonella* was again generally greater at the 10^3 and 10^1 CFU/g inoculation levels compared to the 10^{-1} CFU/g level at each inoculated:uninoculated product ratio (Figure 2.8). However, unlike for baby spinach, significantly less spread of *Salmonella* occurred for the 10^1 and 10^{-1} CFU/g inoculation levels and the 0.5:100 inoculated:uninoculated product ratio compared to the 10^3 CFU/g inoculation level at the same weight ratio.

Table 2.2: Numbers and percentage of *Salmonella*-positive samples for cilantro samples (~ 22 total samples per trial) with different inoculation levels and product ratios.

Inoculation Level (CFU/Leaf)	Inoculated:uninoculated Ratio	Positive/Total Samples			Avg. Percent Positive Samples
		Rep 1	Rep 2	Rep 3	Mean ($\pm$ SD)
10^3	10:100	22/22	22/22	21/21	100% ($\pm$ 0%)
	5:100	22/22	22/22	20/20	100% ($\pm$ 0%)
	1:100	22/22	22/22	21/21	100% ($\pm$ 0%)
	0.5:100	22/22	22/22	21/21	100% ($\pm$ 0%)
Sanitizer 10^3	10:100	21/21	21/21	16/21	92.0% ($\pm$ 13.7%)
	5:100	17/20	20/20	20/20	95.0% ($\pm$ 8.7%)
	1:100	7/22	8/21	5/21	31.2% ($\pm$ 7.2%)
10^1	10:100	22/22	22/22	16/21	92.1% ($\pm$ 11.2%)
	5:100	21/21	22/22	21/21	100% ($\pm$ 0%)
	1:100	22/22	21/22	14/22	87.9% ($\pm$ 17.1%)
	0.5:100	14/22	8/21	2/21	37.1% ($\pm$ 22.1%)
10^{-1}	10:100	4/22	0/22	3/22	10.6% ($\pm$ 7.7%)
	5:100	1/20	7/22	2/22	15.3% ($\pm$ 11.8%)
	1:100	3/21	0/21	0/22	4.8% ($\pm$ 6.7%)
	0.5:100	1/21	2/21	0/21	4.8% ($\pm$ 3.0%)

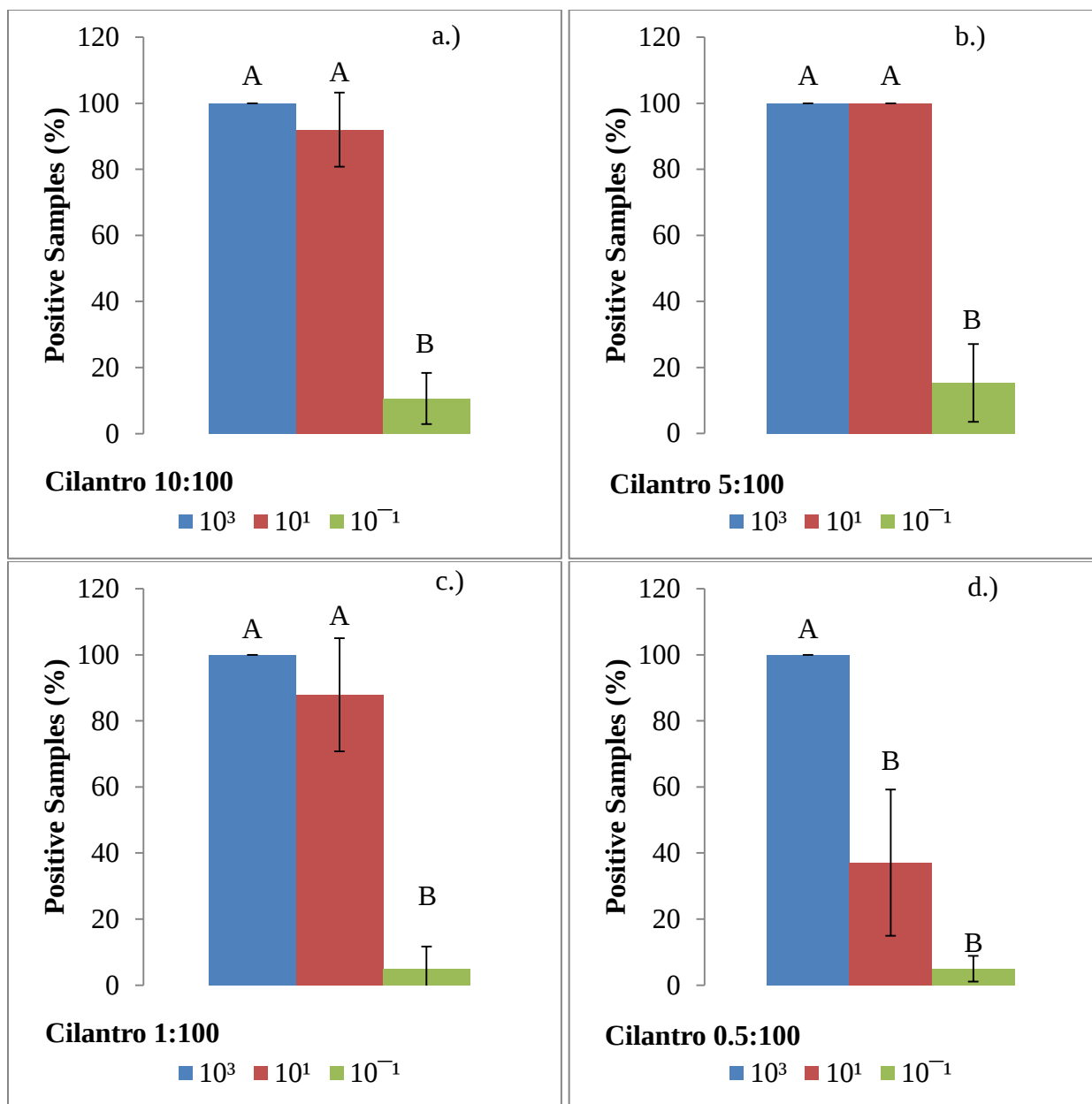


Figure 2.8: Percentage (+/- SD) of *Salmonella*-positive cilantro samples after processing in sanitizer-free water at inoculated:uninoculated weight ratios of a.) 10:100, b.) 5:100, c.) 1:100, d.) 0.5:100. Means with different capital letters are significantly different ($P < 0.05$).

2.3.3 Red leaf lettuce. *Salmonella* populations on red leaf lettuce decreased after 90 s of flume washing. In the baby spinach trials, populations decreased an average of 1.5, 1.0, and 1.8 log CFU/g for the 10^3 , 10^1 , and 10^{-1} CFU/g inoculation levels, respectively. Using the GeneQuence assay, all red leaf lettuce samples were *Salmonella*-positive after washing. Additionally, during the baby spinach trials, red leaf lettuce inoculated with 10^3 CFU/g *Salmonella* experienced population decreases of 3.6 to 1.7, 3.2 to 2.1, 3.2 to 1.4, and 3.3 to 2.3 log CFU/g at the 10:100, 5:100, 1:100, and 0.5:100 weight ratios, respectively. At the 10^1 CFU/g inoculation level, *Salmonella* populations decreased from 1.6 to 0.3, 1.4 to 0.2, 1.5 to 0.4, and 1.4 to 0.3 log CFU/g for the 10:100, 5:100, 1:100, and 0.5:100 weight ratios, respectively. At the 10^{-1} CFU/g inoculation level, *Salmonella* populations decreased from -0.37 to below limit of detection (0.04 CFU/g), -0.4 log CFU/g to below limit of detection (0.04 CFU/g), -0.17 to -0.04 log CFU/g, and -0.4 to below limit of detection for the 10:100, 5:100, 1:100, and 0.5:100 weight ratios, respectively (Tables AI.1 and AI.3). At the 10^3 CFU/g inoculation level, 97.1, 88.5, 94.2, 89.4%, of the *Salmonella* population was shed from the red leaf lettuce during washing at the 10:100, 5:100, 1:100, and 0.5:100 weight ratios, respectively. At the 10^1 CFU/g inoculation level, 94.0, 92.6, 91.6, and 93.4% *Salmonella* was shed, whereas at the 10^{-1} CFU/g inoculation level, 94.8, 100.0, 98.27, and 97.0% was shed at the 10:100, 5:100, 1:100, and 0.5:100 weight ratios, respectively (Table AI.5).

For the cilantro trials, average *Salmonella* reductions on red leaf lettuce were 1.4, 1.4, and 1.6 log CFU/g for the 10^3 , 10^1 , and 10^{-1} CFU/g inoculation levels, respectively. Based on GeneQuence analysis, all red leaf lettuce samples were *Salmonella*-positive after washing. Red leaf lettuce inoculated at 10^3 CFU/g experienced *Salmonella* reductions of 3.4 to 1.7, 3.2 to 1.3, 3.3 to 2.2, and 3.1 to 2.1 log CFU/g at the 10:100, 5:100, 1:100, and 0.5:100 weight ratios,

respectively. At the 10^3 CFU/g inoculation level, *Salmonella* populations decreased from 1.4 to -0.3 log CFU/g, 1.7 to 0.3 log CFU/g, 1.4 to 0.5 CFU/g, and 1.4 to -0.4 log CFU/g for the 10:100, 5:100, 1:100, and 0.5:100 weight ratios, respectively. At the 10^1 CFU/g inoculation level, numbers of *Salmonella* decreased from -0.6 log CFU/g to below limit of detection (0.04 CFU/g), -0.3 to below limit of detection, 0.17 to below limit of detection, and -0.2 log CFU/g -0.04 log CFU/g for the 10:100, 5:100, 1:100, and 0.5:100 weight ratios, respectively (Tables AI.2 and AI.4). The average percentage of the *Salmonella* population shed from red leaf lettuce during 90 s of flume washing for the 10^3 CFU/g level was 94.8, 96.4, 93.1, and 94.6% for the respective weight ratios of 10:100, 5:100, 1:100, and 0.5:100. At the 10^1 CFU/g and 10^{-1} CFU/g inoculation levels, 97.9, 95.1, 95.6, 95.6%, and 100.0, 94.4, 82.2, and 84.0% of the *Salmonella* population was lost for the weight ratios of 10:100, 5:100, 1:100, and 0.5:100, respectively (Table AI.6).

2.3.4 Wash water. At the time of processing, three 50 mL water samples were collected before washing (control), after 90 s of washing, and during centrifugal drying. All water samples taken before processing were negative for *Salmonella*. Based on the experimental design, a total of twelve water samples were taken at each inoculation level studied (4 weight ratios conducted in triplicate =12 trials per level). Overall, 0/12, 1/12, and 0/12 water samples after baby spinach washing and 0/12, 0/12, and 1/12 water samples after cilantro washing yielded detectable counts of *Salmonella* at the 10^3 , 10^1 , and 10^{-1} CFU/g inoculation levels, respectively. *Salmonella* populations detected in the water following 90 s of washing ranged from 1.0 log CFU/100 mL to below limit of detection (0.3 log CFU/100 mL) for both baby spinach and cilantro. When water samples were analyzed following centrifugal drying, a total of 1/12, 2/12, 0/12 baby spinach water samples and 10/12, 3/12, and 1/12 cilantro water samples had detectable counts of *Salmonella* at the 10^3 , 10^1 , and 10^{-1} CFU/g inoculation levels, respectively. *Salmonella*

populations ranged from 0.7 log CFU/mL to 1.0 log CFU/100 mL for baby spinach and 1.0 log CFU/100 mL to 1.6 log CFU/100 mL for cilantro.

2.3.5 Sanitizer implementation. A chlorine-based sanitizer was added to wash water, acidified to pH 6.5 with citric acid, and concentrated to have 60 ppm available chlorine before washing the surrogate inoculated at 10^3 *Salmonella* CFU/g with uninoculated baby spinach or cilantro. Three weight ratios were analyzed: 1:100 5:100, and 10:100. When sanitizer was used, *Salmonella* populations decreased by an additional log on red leaf lettuce compared to sanitizer-free trials (Tables 2.3 and 2.4). None of the 50 mL water samples collected yielded *Salmonella* by direct plating. Among the three weight ratios studied, the 1:00 ratio produced significantly ($P < 0.05$) fewer positive samples compared to the two higher ratios (Figure 2.9). Comparing the number of bagged samples positive for *Salmonella* between the sanitizer-free trials and those where sanitizer was used, significant reductions ($P < 0.05$) were seen at the 1:100 ratio (Figures 2.5 and 2.6).

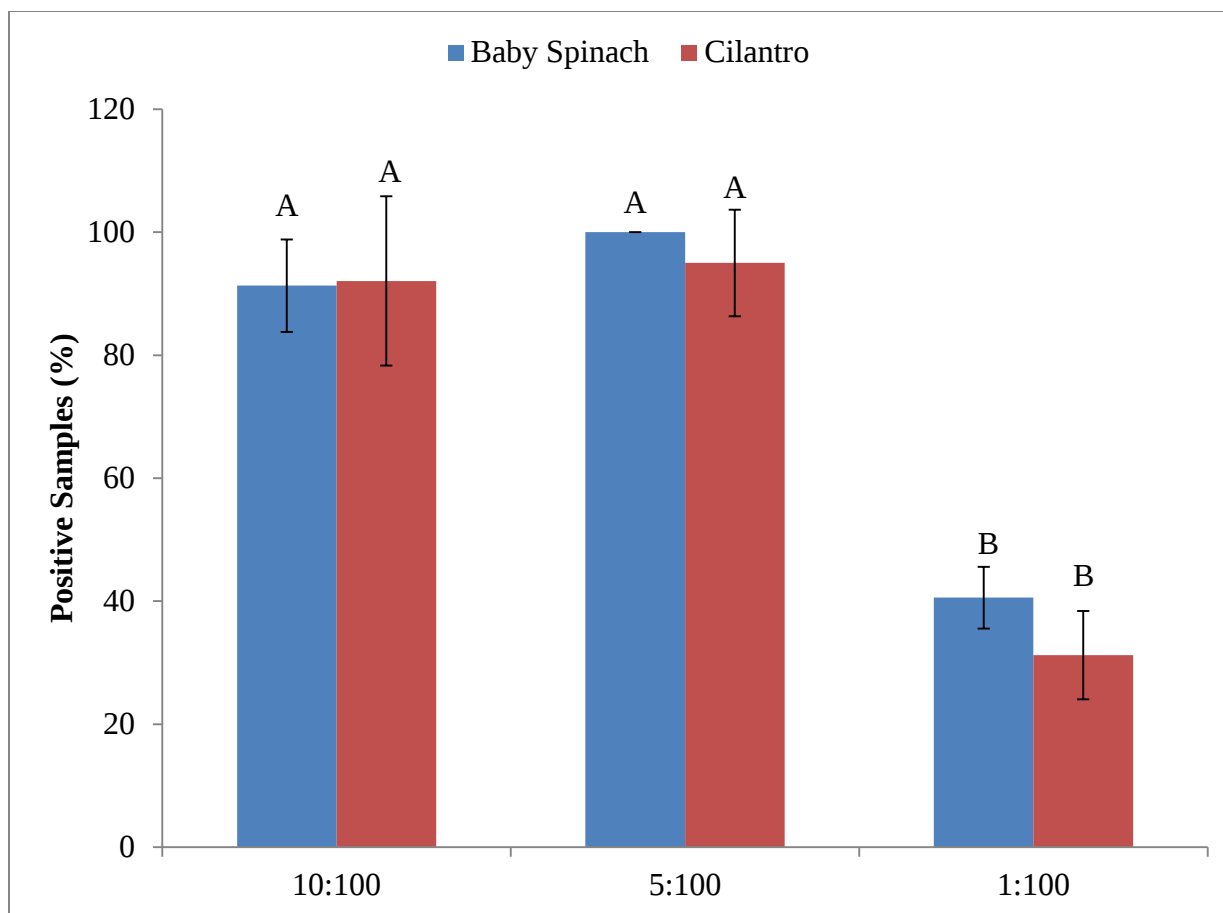


Figure 2.9: Percentage of *Salmonella*-positive samples after 90 s of flume washing in water containing 60 ppm available chlorine. Means with different capital letters are significantly different ($P < 0.05$).

Table 2.3: Comparison of processing trials with and without a chemical sanitizer for baby spinach.

Inoculation Level	Sanitizer Free		60 ppm Sanitizer	
	10 ³			
Inoculated:uninoculated ratio	10:100	10:100	5:100	1:100
% Positive samples	100% (± 0%)	91.3% (± 7.5%)	100% (± 0%)	40.6% (± 5.0%)
% CFU loss on red leaf lettuce after processing	97.1% (± 1.5%)	99.8% (± 0.1%)	91.7% (± 6.7%)	99.3 (± 4.0%)
Average log reduction after processing (log CFU/g)	1.7 (± 0.5)	2.7 (± 0.5)	2.4 (± 0.8)	2.1 (± 0.1)
<i>Salmonella</i> populations in wash water (log CFU/100 mL)	< 0.3	< 0.3	< 0.3	< 0.3
<i>Salmonella</i> populations in centrifugation water (log CFU/100 mL)	< 0.3	< 0.3	< 0.3	< 0.3

Table 2.4: Comparison of processing trials with and without a chemical sanitizer for cilantro.

Inoculation Level	Sanitizer Free		60 ppm Sanitizer	
			10 ³	
Inoculated:uninoculated ratio	10:100	10:100	5:100	1:100
% Positive samples	100% (± 0%)	92.1% (± 13.8%)	95.0% (± 8.7%)	31.2% (± 7.2%)
% CFU loss on red Leaf lettuce after processing	94.8% (± 5.8%)	99.5% (± 0.3%)	97.8% (± 3.7%)	95.3 (± 4.7%)
Average log reduction after processing (log CFU/g)	1.7 (± 0.5)	2.7 (± 0.5)	2.6 (± 0.8)	2.3 (± 0.2)
<i>Salmonella</i> populations in wash water (log CFU/100 mL)	< 0.3	< 0.3	< 0.3	< 0.3
<i>Salmonella</i> populations in centrifugation water (log CFU/100 mL)	1.6	< 0.3	< 0.3	< 0.3

2.4 Discussion

This study assessed the impact of inoculum size and the amount of inoculated product being processed on the spread of *Salmonella* to previously uncontaminated product during pilot-scale washing in sanitizer-free water. Spot, rather than dip or spray inoculation was selected to artificially contaminate red leaf lettuce due to a greater ability to achieve the targeted inoculation level (Lang et al. 2004). This technique closely mimics random fecal contamination from animals or biological soil amendments (e.g. manure) in the field rather than exposure to contaminated irrigation water, where dip inoculation would be more appropriate (Land et al 2004; Gil et al. 2009). Although enteric pathogen contamination from fecal material is hypothesized to be highly localized in the field, bacterial pathogens are capable of spreading to previously uncontaminated produce during and after harvest (Williams et al. 2008). Past cross contamination studies have demonstrated that initial inoculation levels of *E. coli* O157:H7 ranging from 10^7 to 10^2 CFU/g can spread to uncontaminated produce during pilot-scale washing, regardless of sanitizer use (Buchholz et al. 2012; Buchholz et al. 2014; Davidson et al. 2013; Nou and Luo 2010). However, the initial inoculation levels used in these past studies represented worst-case scenario and highly localized field contamination such as direct contact with contaminated manure. Although often highly localized, these same contaminants can be introduced into irrigation water or equipment and can be subsequently spread over wider field areas (Beuchat et al. 2010; Ibekwe et al. 2004). In order to more accurately mimic low level field contamination, which is most commonly presumed, initial inoculation levels of 10^3 , 10^1 , and 10^{-1} CFU/g were selected for this study.

Previous studies by our lab group have shown that an inoculated surrogate containing 10^6 , 10^4 , or 10^2 CFU/g *E. coli* O157:H7 can transfer 1.51 to 2.90 log CFU/g to uninoculated

lettuce during pilot-scale processing without the use of a chemical sanitizer. In similar work by our group using a chemical sanitizer, initial levels of *E. coli* O157:H7 10^6 CFU/g decreased ~1.5 to 2.5 log CFU/g following 90 s washing. The extent of pathogen transfer to uninoculated lettuce and log reductions seen in these previous studies are similar to the initial inoculation levels used in the present study. The inoculation levels of the present study can also represent potential secondary and tertiary contamination occurring during post-harvest washing as bacteria can subsequently spread through flume water in lower numbers following an initial large contaminant exposure.

By necessity, most of the baby spinach and cilantro trials were conducted using sanitizer-free wash water in order to quantify the spread of *Salmonella* to previously uncontaminated product. Other studies have used sanitizer-free water for pilot-scale studies in order to generate baseline data for the number of pathogens transferred during this operation (Buchholz et al. 2012; Buchholz et al. 2014; Davidson 2013; Nuo and Luo 2010). This baseline data can then be extrapolated and applied to predictive models for risk assessments (Pérez-Rodríguez et al. 2011). The present study aimed to generate baseline data that can be applied to future risk assessments for fresh-cut leafy greens.

When red leaf lettuce was inoculated to contain 10^3 , 10^1 10^{-1} CFU/g, significant differences in the number of positive samples were observed between inoculation levels. The different weight ratios used did not produce significant differences in *Salmonella*-positive samples. Therefore, the inoculation level has a greater impact on spread of cross contamination than the amount of contaminated product. A similar trend was seen in a related pilot-scale study from our laboratory by Buchholz and others (2012) where sanitizer-free wash water was used to process lettuce inoculated to contain different levels of *E. coli* O157:H7. In their study, 22.7 kg

of dip inoculated lettuce containing 10^6 , 10^4 , or 10^2 CFU/g *E. coli* O157:H7 was processed without a chemical sanitizer through a shredder, conveyor, flume tank (90 s washing), shaker table, and centrifugal dryer. Immediately thereafter, 90.8 kg uninoculated lettuce was processed in the same manner. Results from the 10^2 CFU/g inoculation level revealed transfer of *E. coli* O157:H7 to 85% of the 90.8 kg uninoculated lettuce (Buchholz et al. 2012). At the two higher inoculation levels, complete transfer of *E. coli* O157:H7 occurred to the entire 90.8 kg uninoculated lettuce batch (Buchholz et al. 2012). The decrease in transfer seen at the lowest inoculation level compared to the increased transfer at the two higher inoculation levels supports the present findings where fewer *Salmonella*-positive samples were obtained as the initial inoculation level decreased. Therefore, the extent of cross contamination is more highly dependent on the level of contamination in the product rather than the amount of product processed

Using sanitizer-free wash water, *Salmonella* populations on red leaf lettuce decreased 1.5, 1.0, and 1.8 log CFU/g for the baby spinach and 1.4, 1.4, and 1.6 log CFU/g for cilantro trials at the 10^3 , 10^1 , 10^{-1} CFU/g inoculation levels, respectively. In other pilot-scale studies, a ~ 1 log CFU/g reduction was achieved after inoculated radicchio, iceberg, and romaine lettuce was washed for 90 s in sanitizer-free water (Buchholz et al. 2012; Buchholz et al. 2014; Davidson et al. 2013; Luo and Nou et al. 2012).

Salmonella was generally not detected in samples of sanitizer-free wash water or spent centrifugation water collected after 90 s of processing. When compared to the aforementioned study by Buchholz et al. (2012), 90% of the *E. coli* O157:H7 population transferred to sanitizer-free wash water after 90 s washing of 22.7 kg inoculated lettuce at 10^6 , 10^4 , and 10^2 CFU/g inoculation levels (Buchholz et al. 2012). Furthermore, *E. coli* O157:H7 was quantifiable in their

50 mL water samples at the following levels: 1.9, 2.2, -0.3 log CFU/g for iceberg lettuce trials and 3.0, 1.2, 0.0 log CFU/g for romaine lettuce trials at the high, medium, and low inoculation levels, respectively. At the lowest inoculation level (10^2 CFU/g), a -0.3 and 0.0 log CFU/g count was recorded for iceberg and romaine, respectively (Buchholz et al. 2012). These low counts at the 10^2 CFU/g inoculation level along with the high ratio of contaminated to uncontaminated product processed (1:4 ratio) help explain the low detection rates for *Salmonella* in wash water samples from the present study. Our weight ratios and inoculation levels were much lower and therefore more difficult to detect due to the small water samples taken (50 mL). Buchholz et al. (2012) also reported that centrifugal drying can expel up to 30% of the microbial population present on the lettuce with such spent centrifugation water best suited for pathogen testing. Although, *Salmonella* counts were limited following centrifugal drying in the present study, these counts were still higher than those seen in the wash water samples after processing.

Sanitizers used in commercial flume water will typically reduce microbial populations on the washed product by 90-99% (Burnett et al. 2004, Keskinen et al. 2009, Sapers 2001; Weissinger et al. 2000; Zhang et al. 2009). However, chlorine-based sanitizers are sensitive to changes in pH, organic load, and temperature, therefore consistent monitoring of wash water is paramount to ensure safety and quality of the produce (Davidson et al. 2013; Gil et al. 2009). If a sanitizer had been used for the lower inoculation levels in the present study, few *Salmonella* cells likely would have remained after processing.

Chlorine-based sanitizers are most commonly used in industry due to their relatively low cost and low negative impact on product quality (Fellows 2009; Buchholz 2011; Sapers 2014; Suslow 2000). They are used at concentrations of 50 - 200 ppm, and exposure time of 1-2 minutes, and are typically treated with an acidifying agent to lower the water pH to 6.0~7.0

(Davidson et al. 2013; Beuchat et al. 1998; Sapers 2014). This same protocol was followed in the present study as wash water containing 60 ppm available chlorine was acidified to pH 6.5 using citric acid for a 90 s exposure time. Although unable to completely remove *Salmonella* from red leaf lettuce when the 10^3 CFU/g inoculation level and 10:100, 5:100, 1:100 weight ratios were used, cross contamination during washing was reduced, indicating marginal success of the sanitizer. The primary purpose of a sanitizer in wash water is to maintain the microbial quality of the water and prevent widespread cross contamination of pathogens that may be present (Gil et al. 2009; Lopez-Galvez et al. 2009; Davidson et al. 2013). In the present study, sanitizer use decreased *Salmonella* levels below the limit of detection (0.3 log CFU/100 mL) in both the wash water and centrifugation water with greater log reductions seen on red leaf lettuce compared to the sanitizer-free trials. In the present study, a 2.7 log reduction in *Salmonella* on red leaf lettuce was achieved. A previous pilot-plant scale study performed by our group reported a 0.88 log CFU/g reduction of *E. coli* O157:H7 when 5.4 kg of iceberg lettuce inoculated with $5.93 \log$ CFU/g was washed for 90 s with chlorine-based sanitizer (50 ppm available chlorine) acidified with citric acid (Davidson et al. 2013). In another study, spinach inoculated with 2×10^5 CFU/g was washed in two different wash treatments, a chlorine-based sanitizer with citric acid and a chlorine-based sanitizer with T-128, both treatments showed log reductions of 0.8-0.9 CFU/g following ~90 s of washing (Luo and Nou et al. 2012). Other pilot-scale and laboratory studies have reported a maximum reductions of 1-3 logs for *E. coli* O157:H7 and *Salmonella* spp. following washing of inoculated produce in the presence of a sanitizer and under practical conditions (Luo and Nou et al. 2012).

The limited ability of sanitizers to achieve complete removal of *Salmonella* can be attributed to attachment of the pathogen to the red leaf lettuce surface. Similar findings were

obtained in a separate study where the use of chemical sanitizers decreased but did not completely eliminate *Salmonella* Typhimurium or *E.coli* O157:H7 from parsley and spinach, respectively (Lapidot et al. 2006; Barrera et al. 2010). The ability of *Salmonella* to form biofilms on plant surfaces after 24 h of storage at 4°C also leads to protection against disinfection (Lapidot et al. 2006). Such pathogens become more difficult to eliminate using chlorine-based sanitizers when internalized in plant tissue with the hydrophobic surface characteristics of fresh produce also decreasing the effectiveness of aqueous hypochlorite solutions (Adams et al. 1989; Pirovani et al. 2004). At best, sanitizers are capable of reducing pathogens a maximum of 3 logs under ideal commercial processing conditions (Gil et al. 2009; Beuchat et al., 2004; Gonzalez et al., 2004; Inatsu et al., 2005; Ukuku et al., 2005; Allende et al., 2007; Gómez-López et al., 2007; Selma et al., 2008b). Therefore, the sanitizer used in this study was limited in preventing *Salmonella* cross contamination during washing simply due to the contaminant load and attachment to red leaf lettuce.

In summary, this study shows that the magnitude of transfer and redistribution of *Salmonella* during pilot-scale washing of leafy greens in sanitizer-free water is influenced by inoculum size, rather than the amount of inoculated product. Such cross contamination can still occur even if the initial contamination level is low, as the 10^{-1} CFU/g inoculation level produced *Salmonella*-positive samples. Use of a chlorine-based sanitizer led to greater *Salmonella* reductions on washed red leaf lettuce and also reduced the number of *Salmonella*-positive samples compared to trials where sanitizer was not used, indicating its presence in wash water made a positive difference. Therefore, proper use of an approved sanitizer is recommended to minimize microbial cross contamination during washing. These results will be essential in future risk assessment strategies as this study is the first of our knowledge to analyze *Salmonella*

transfer to baby spinach and cilantro in sanitizer-free water using realistically low levels of initial contamination.

CHAPTER 3:
CONCLUSIONS AND FUTURE RECOMMENDATIONS

Fresh produce has been implicated in numerous outbreaks, with leafy greens, culinary herbs, and sprouts being particularly problematic. *Salmonella* is the leading cause of multi-state produce outbreaks and has the third highest economic burden to the food industry and consumer. Through the Food Safety Modernization Act, the FDA has taken a more proactive approach to preventing foodborne outbreaks through various risk assessment strategies. Given the threat that pathogen contamination poses to the fresh produce industry, the need for transparency on the degree of cross contamination that may occur during commercial processing of leafy greens is pertinent to risk assessments.

The present study aimed to fill critical data gaps identified by the FDA in regard to the cross contamination and redistribution of *Salmonella* Typhimurium LT2 during pilot-scale processing of baby spinach and cilantro. One objective was to quantify *Salmonella* Typhimurium LT2 transfer and redistribution during production of fresh-cut baby spinach and cilantro in sanitizer-free wash water. Three inoculation levels (10^3 , 10^1 , and 10^{-1} CFU/g) and four different weight ratios of surrogate to uninoculated produce were used to assess the extent of cross contamination that would occur during 90 s of flume washing. Overall, as the initial inoculation level of surrogate decreased, cross contamination decreased. When analyzing the weight ratios, there was no significant difference in cross contamination.

In an effort to best mimic real-world scenarios, low inoculation levels of *Salmonella* Typhimurium and low inoculated:uninoculated product weight ratios were used to represent contamination occurring at pre-harvest. Based on the data collected, decreased contamination of uninoculated produce occurred when the initial contamination load was low following washing and drying, indicating a lower food safety risk. Although contamination occurred at the lowest inoculation level and weight ratio, there was a significant decrease in the percentage of

Salmonella-positive samples revealing that the risk can be minimized by utilizing sanitizers in the water during processing.

The second objective evaluated the efficacy of a chlorine-based sanitizer used at 60 ppm available chlorine, acidified to pH 6.5 under the same conditions as specified above. This was done in order to imitate industry practices, as 50-200 ppm chlorine (acidified to pH 6.0-7.0) can be used in produce wash water. The sanitizer was limited in its ability to completely eliminate *Salmonella* from the system; however it was able to reduce the spread of contamination during washing compared to the sanitizer-free trials.

Future studies should focus on work that adds value to risk assessments and can strengthen food safety at pre-and post-harvest points of fresh produce processing. At pre-harvest, more data is needed on field contamination and subsequent spread of pathogens through the field. This data will provide invaluable information that can be applied to future cross contamination studies where more accurate initial contamination levels can be used. At post-harvest, one potential avenue is to assess the spread of contamination occurring to equipment surfaces such as the shredder, conveyor, flume tank, dewatering shaker, collection bins, and centrifuge during sanitizer-free processing of inoculated product containing initial pathogen levels of 10^3 , 10^1 , and 10^{-1} CFU/g. When applied to the data gathered in the present study, such findings will lead an improved understanding of the spread of cross contamination to surfaces when low initial inoculation levels are used, thus providing added information to further strengthen future risk assessment studies.

APPENDIX

Chapter 10, Volume 1

Microbiology of Fresh and Processed Vegetables

Haley Smolinski and Elliot T. Ryser

Abstract

Produce is vulnerable to being implicated in foodborne outbreaks due its ready to eat nature and lack of cooking step prior to consumption. There are multiple routes during pre- and post-harvest in which produce can become initially contaminated with pathogens. Also, due to the increasingly globalized market of the fresh produce industry, international transport of produce can potentially increase the chances of contamination due to a longer distribution chain to reach the end consumer. Specific strategies have been implemented in order to preserve produce quality and enhance shelf life including hydrocooling, washing, refrigeration, freezing, and utilization of active or modified packaging. Although these techniques are beneficial, they can also directly lead to produce contamination or cross contamination.

Introduction

An increasing number of foodborne outbreaks traced to fresh fruits and vegetables are partially attributed to production, processing, and consumption patterns. In the United States, the progression from locally grown produce to centralized production has led to numerous multistate and nationwide outbreaks of foodborne illnesses. Additionally, consumer demand for year-round availability of fresh produce has made the industry increasingly globalized. In many cases, fruits and vegetables are grown on centralized large-scale farms in locations that specialize in a specific product. A few examples from the United States include the production of baby spinach in California and Arizona, tomatoes in Florida and New Jersey, blueberries in Michigan and New Jersey, and mushrooms in Pennsylvania. Under these conditions, one contamination incident at a large centralized grower or processor could quickly lead to a multistate outbreak with near catastrophic consequences for the industry, as was seen in several recent outbreaks involving baby spinach and tomatoes. By definition, fresh fruits and vegetables do not typically undergo any treatment other than washing for the reduction and/or elimination of potentially hazardous microorganisms. Use of chemical sanitizers in wash water is common practice throughout the industry, however the primary function of these sanitizers is to maintain the microbial quality of the water and improve overall cosmetic appearance. Unfortunately, most commercial sanitizers used for washing fresh produce can only reduce the microbial levels by 99% to 99.9% at best, which still makes these products potential vehicles for the transmission of such microbial pathogens as *Salmonella*, *Escherichia coli* O157:H7, *Cryptosporidium*, Hepatitis A, and Norovirus.

SPREAD OF *ESCHERICHIA COLI* O157:H7 DURING FLUME WASHING AND DRYING OF FRESH-CUT ROMAINE LETTUCE

By

Siyi Wang, Haley Smolinski, Elliot T. Ryser

Abstract

The microbiological safety of leafy greens remains a concern as evidenced from recent outbreaks. This study assessed the spread of *E. coli* O157:H7 during washing and drying of fresh cut romaine lettuce. Radicchio was spot-inoculated at 10^{-1} , 10^1 and 10^3 CFU/g and mixed with uninoculated romaine lettuce to obtain 5 kg batches with inoculated vs uninoculated ratios of 0.5:100, 1:100, 5:100 and 10:100. After 90 s of sanitizer-free flume washing followed by shaker table and centrifugal dryer, the radicchio was removed and the lettuce was divided into 225 g samples to test presence/absence of *E. coli* O157:H7 using GeneQuence assay. Based on triplicate trials, lower inoculation levels led to decreased *E. coli* O157:H7 transfer to romaine lettuce ($P < 0.05$). All lettuce samples yielded *E. coli* O157:H7 when radicchio was inoculated at 10^3 CFU/g. At 10^1 CFU/g, the percentage of positive samples decreased from 96.8% to 93.7%, 81.0% and 63.5% while at 10^{-1} CFU/g, 22.2%, 6.3%, 4.8% and 6.3% were positive at 10:100, 5:100, 1:100 and 0.5:100 ratios. Within each inoculation level, there was no significant difference ($P > 0.05$) among four product ratios. These findings will provide important data for improving exposure assessment in risk assessments for leafy greens.

Table AI.1: *Salmonella* populations (log CFU/g) on red leaf lettuce before washing baby spinach. This data served as the control for the surrogate samples and ensured the targeted initial populations were achieved.

Inoculation Level (CFU/g)	Inoculated:Uninoculated Ratio	<i>Salmonella</i> populations before washing - control (log CFU/g)			Avg. populations
		Rep 1	Rep 2	Rep 3	Mean ($\pm$ SD)
10^3	10:100	3.90	3.30	3.70	3.63 ($\pm$ 0.31)
	5:100	3.20	3.10	3.40	3.23 ($\pm$ 0.15)
	1:100	3.20	3.30	3.20	3.23 ($\pm$ 0.06)
	0.5:100	3.40	3.30	3.20	3.30 ($\pm$ 0.10)
10^3 Sanitizer	10:100	3.30	3.70	3.70	3.57 ($\pm$ 0.23)
	5:100	3.10	3.10	3.10	3.10 ($\pm$ 0.0)
	1:100	3.30	3.30	3.30	3.30 ($\pm$ 0.0)
10^1	10:100	1.90	1.50	1.30	1.57 ($\pm$ 0.31)
	5:100	1.50	1.10	1.60	1.40 ($\pm$ 0.26)
	1:100	1.90	1.20	1.40	1.50 ($\pm$ 0.36)
	0.5:100	1.50	1.50	1.20	1.40 ($\pm$ 0.17)
10^{-1}	10:100	-0.20	-0.30	-0.60	-0.37 ($\pm$ 0.21)
	5:100	-0.30	-0.80	-0.10	-0.4 ($\pm$ 0.36)
	1:100	0.10	-0.80	0.20	-0.17 ($\pm$ 0.55)
	0.5:100	-0.20	-0.40	-0.60	-0.4 ($\pm$ 0.20)

Table AI.2: *Salmonella* populations (log CFU/g) on red leaf lettuce before washing cilantro. This data served as the control for the surrogate samples and ensured the targeted initial populations were achieved.

Inoculation Level (CFU/g)	Inoculated:Uninoculated Ratio	<i>Salmonella</i> populations before washing - control (log CFU/g)			Avg. populations
		Rep 1	Rep 2	Rep 3	Mean ($\pm$ SD)
10^3	10:100	3.20	3.50	3.50	3.40 ($\pm$ 0.17)
	5:100	3.30	3.10	3.20	3.20 ($\pm$ 0.10)
	1:100	3.30	3.20	3.30	3.27 ($\pm$ 0.06)
	0.5:100	3.10	3.20	3.10	3.13 ($\pm$ 0.06)
10^3 Sanitizer	10:100	3.10	3.20	3.10	3.13 ($\pm$ 0.06)
	5:100	3.20	3.30	3.20	3.23 ($\pm$ 0.06)
	1:100	3.30	3.20	3.20	3.23 ($\pm$ 0.06)
10^1	10:100	1.40	1.40	1.30	1.37 ($\pm$ 0.06)
	5:100	1.60	1.60	1.90	1.70 ($\pm$ 0.17)
	1:100	1.10	1.90	1.10	1.37 ($\pm$ 0.46)
	0.5:100	1.10	1.40	1.70	1.40 ($\pm$ 0.3)
10^{-1}	10:100	-0.70	-0.80	-0.20	-0.57 ($\pm$ 0.32)
	5:100	-0.30	-0.20	-0.40	-0.30 ($\pm$ 0.10)
	1:100	0.20	-0.10	0.40	0.17 ($\pm$ 0.25)
	0.5:100	-0.20	-0.10	-0.30	-0.20 ($\pm$ 0.10)

Table AI.3: *Salmonella* populations (log CFU/g) on red leaf lettuce after washing baby spinach.
Note: LOD: < -0.04 log CFU/g.

Inoculation Level (CFU/g)	Inoculated:Uninoculated Ratio	<i>Salmonella</i> populations after washing (log CFU/g)			Avg. populations
		Rep 1	Rep 2	Rep 3	Mean ($\pm$ SD)
10^3	10:100	2.10	1.30	1.80	1.73 ($\pm$ 0.40)
	5:100	1.70	2.30	2.20	2.07 ($\pm$ 0.32)
	1:100	2.20	0.20	1.90	1.43 ($\pm$ 1.08)
	0.5:100	2.40	2.30	2.10	2.27 ($\pm$ 0.15)
10^3 Sanitizer	10:100	0.60	1.60	0.90	1.03 ($\pm$ 0.51)
	5:100	1.20	1.90	2.20	1.77 ($\pm$ 0.51)
	1:100	1.30	1.20	1.10	1.20 ($\pm$ 0.1)
10^1	10:100	0.30	0.40	0.20	0.3 ($\pm$ 0.10)
	5:100	0.30	0.10	0.30	0.23 ($\pm$ 0.11)
	1:100	0.60	0.20	0.40	0.40 ($\pm$ 0.20)
	0.5:100	0.20	0.50	0.10	0.27 ($\pm$ 0.21)
10^{-1}	10:100	<-0.04	-0.04	<-0.04	<-0.04 ($\pm$ 0.01)
	5:100	<-0.04	<-0.04	<-0.04	<-0.04 ($\pm$ 0.0)
	1:100	-0.04	<-0.04	<-0.04	-0.04 ($\pm$ 0.01)
	0.5:100	<-0.04	<-0.04	<-0.04	<-0.04 ($\pm$ 0.0)

Table AI.4: *Salmonella* populations (log CFU/g) on red leaf lettuce after washing cilantro.
Note: LOD: <-0.04 log CFU/g.

Inoculation Level (CFU/g)	Inoculated:Uninoculated Ratio	<i>Salmonella</i> populations after washing (log CFU/g)			Avg. populations
		Rep 1	Rep 2	Rep 3	Mean (± SD)
10^3	10:100	2.20	1.10	1.90	1.73 (± 0.57)
	5:100	1.30	1.50	1.20	1.33 (± 0.15)
	1:100	2.20	2.20	2.20	2.20 (± 0.0)
	0.5:100	2.10	2.10	2.20	2.13 (± 0.06)
10^3 Sanitizer	10:100	0.20	1.20	0.50	0.63 (± 0.51)
	5:100	2.10	0.10	0.30	0.83 (± 1.10)
	1:100	1.50	1.60	2.20	1.77 (± 0.38)
10^1	10:100	-0.60	-0.50	0.10	-0.33 (± 0.38)
	5:100	0.30	0.30	0.40	0.33 (± 0.06)
	1:100	1.10	0.20	0.10	0.47 (± 0.55)
	0.5:100	0.30	-0.70	-0.70	-0.37 (± 0.58)
10^{-1}	10:100	<-0.04	<-0.04	<-0.04	<-0.04 (± 0.0)
	5:100	-0.04	-0.04	<-0.04	<-0.04 (± 0.01)
	1:100	0.04	-0.04	<-0.04	<-0.04 (± 0.01)
	0.5:100	<-0.04	-0.08	<-0.04	-0.04 (± 0.03)

Table AI.5: Percentage of *Salmonella* populations lost from red leaf lettuce surrogate during baby spinach processing.

Inoculation Level (CFU/g)	Inoculated:Uninoculated Ratio	Percent lost from surrogate			Avg. percent lost
		Rep 1	Rep 2	Rep 3	Mean (± SD)
10 ³	10:100	95.61%	96.57%	99.24%	97.14% (± 1.54%)
	5:100	94.86%	74.10%	96.55%	88.50% (± 10.21%)
	1:100	95.65%	95%	92.02%	94.22% (± 1.58%)
	0.5:100	94.74%	86.98%	86.58%	89.43% (± 3.76%)
10 ³ Sanitizer	10:100	99.90%	99.74%	99.67%	99.77% (± 0.10%)
	5:100	85.00%	91.82%	98.31%	91.71% (± 6.65%)
	1:100	98.81%	99.34%	99.60%	99.25% (± 0.40%)
10 ¹	10:100	94.45%	90.87%	96.81%	94.04% (± 2.44%)
	5:100	94.14%	90.77%	92.90%	92.60% (± 1.39%)
	1:100	89.00%	92.08%	93.73%	91.60% (± 1.96%)
	0.5:100	94.17%	90.58%	95.56%	93.44% (± 2.10%)
10 ⁻¹	10:100	100%	84.29%	100%	94.76% (± 7.41%)
	5:100	100.00%	100.00%	100.00%	100.00% (± 0%)
	1:100	98.37%	100.00%	96.43%	98.27% (± 1.46%)
	0.5:100	100.00%	90.90%	100.00%	96.97% (± 4.29%)

Table AI.6: Percentage of *Salmonella* populations lost from red leaf lettuce surrogate during cilantro processing.

Inoculation Level (CFU/g)	Inoculated:Uninoculated Ratio	Percent lost from surrogate			Avg. percent lost
		Rep 1	Rep 2	Rep 3	Mean (± SD)
10^3	10:100	97.98%	99.73%	86.76%	94.82% ± 5.75%)
	5:100	98.62%	95.64%	94.78%	96.35% (± 1.65%)
	1:100	94.52%	92.42%	92.34%	93.09% (± 1.00%)
	0.5:100	99.16%	94.81%	89.79%	94.59% (± 3.83%)
10^3 Sanitizer	10:100	100.00%	100.00%	76.19%	92.06% (± 11.22%)
	5:100	99.88%	99.95%	93.46%	97.76% (± 3.73%)
	1:100	89.83%	97.60%	98.33%	93.25% (± 4.70%)
10^1	10:100	96.40%	98.68%	98.50%	97.86% (± 1.03%)
	5:100	93.43%	95.00%	96.80%	95.08% (± 1.38%)
	1:100	98.60%	97.18%	91.03%	95.60% (± 3.30%)
	0.5:100	93.42%	91.41%	96.00%	95.94% (± 2.04%)
10^{-1}	10:100	100.00%	100.00%	100.00%	100.00% (± 0%)
	5:100	97.23%	100.00%	86.00%	94.41% (± 6.05%)
	1:100	80.00%	100.00%	66.70%	82.23% (± 13.69%)
	0.5:100	100.00%	84.00%	67.00%	83.66% (± 13.47%)

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