

RAPID CONCENTRATION/DETECTION OF *ESCHERICHIA COLI* O157:H7 AND  
*LISTERIA MONOCYTOGENES* FROM LETTUCE WASH WATER

By

Ryann Elizabeth Gustafson

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## ABSTRACT

### RAPID CONCENTRATION/DETECTION OF *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* FROM LETTUCE WASH WATER

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Dead-end ultrafiltration concentration (DEUF-C) prior to qPCR or standard enrichment was used to determine presence/absence of *Escherichia coli* O157:H7 (*Ec*) and *Listeria monocytogenes* (*Lm*) in various lettuce wash waters generated using both a pilot- and commercial scale processing line. After inoculating and pilot-scale processing of the ice berg lettuce, 8 of 24 (33%) of *Ec* and *Lm* samples were positive by DEUF-C, but negative by commercial FDA BAM. In addition, populations of both pathogens were significantly higher in DEUF-C samples compared to unconcentrated samples after 7 h of enrichment ( $P < 0.05$ ). Using the commercial-scale processing line, 14 of 14 (100%) samples yielded *Ec* and *Lm* using DEUF-C compared to 1 of 15 (6.7%) and 3 of 15 (20%) wash water grab samples, respectively. Higher total filterable volumes were achieved for flume ( $30.6 \pm 9.9$  L) compared to centrifugation water ( $9.9 \pm 2.0$  L) ( $P < 0.05$ ). *Ec* and *Lm* were also detected in 16 of 16 (100%) DEUF-C samples compared to 2 of 18 (11.1%) and 3 of 18 (16.7%) grab samples, respectively, from a commercial-scale test facility. These studies indicate that DEUF-C can detect lower levels of *Ec* and *Lm* in lettuce wash water compared to standard culture methods. However, the filtration times and total filterable volumes will need to be further improved for commercial produce wash and centrifugation water containing high organic loads.

To my parents, Jim and Michelle Gustafson

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## KEY TO ABBREVIATIONS

ANOVA	Analysis of Variance
AgMRC	Agriculture Marketing Resource Center
AOAC	Association of Analytical Communities
BLEB	Buffered Listeria Enrichment Broth
CCW	Commercial centrifuge wash
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit(s)
CFW	Commercial flume wash
cm	centimeter
CSPI	Center for Science in the Public Interest
DNA	deoxyribonucleic acid
DEUF	dead-end ultrafiltration
DEUF-C	dead-end ultrafiltration concentration
EHEC	Enterohemorrhagic <i>E. coli</i>
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FDA BAM	Food and Drug Administration Bacteriological Analytical Manual
FSA	Food Safety Agency
FSMA	Food Safety Modernization Act
ft	feet
g	grams

GFP	Green fluorescent protein
h	hour(s)
HACCP	Hazard Analysis and Critical Control Point
HUF	Hollow-fiber ultrafiltration
HUS	Hemolytic uremic syndrome
kg	kilogram
kPa	kilopascal
L	liter(s)
lbs	pounds
m	meter
mBPWp	modified buffered peptone water with pyruvate
min	minute(s)
mL	milliliter(s)
MFV	maximum filterable volume
MOX	Modified Oxford Agar
MSU	Michigan State University
mV	millivolt(s)
MWCO	molecular weight cut off
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
nm	nanometer
ORP	Oxidation/reduction potential
PBS	Phosphate Buffered Saline
PCC	PMACS Contamination Check

PCR	polymerase chain reaction
PL	processing line (commercial)
PMACS	Portable Multi-use Automated Concentration System
ppm	Parts per million
qPCR	quantitative polymerase chain reaction
RPM	revolutions per minute
s	second(s)
SAS	Statistical Analysis Systems
SMAC-CT	Sorbitol MacConkey Agar with Cefixime and Tellurite
SSM	standard sampling method
Stx	shiga toxin
TAP	tap after processing
TBP	tap before processing
TS	total solids
TSB-YE	Trypticase Soy Broth with 0.6% Yeast Extract
UF	ultrafiltration
µm	micron(s)
US	United States of America
USDA	United States Department of Agriculture
USF	University of South Florida

# INTRODUCTION

Consumption of fresh produce and leafy greens has been increasing in the US since 1960, due to Americans moving towards healthier and more convenient eating habits especially in the form of minimally processed, packaged salads (AgMRC, 2015; Butt and Sultan, 2011; ERS, 2001). However, minimal processing of fresh-cut produce affords little opportunity for pathogen inactivation. This lack of an inactivation step, along with increased production and improved strain tracking techniques, has led to increased outbreak detection with at least 88 fresh produce-related outbreaks documented from 1996 to 2016 (CDC, 2016c).

Since 2006, at least 16 lettuce associated outbreaks due to *Escherichia coli* O157:H7 and at least 1 due to *Listeria monocytogenes* have been identified (Foodborne Illness Outbreak Database, 2017). These outbreaks, with their high hospitalization rates, have decreased consumer confidence in the safety of fresh and fresh-cut produce (Arnade et al., 2009; Lynch et al., 2009). Foodborne outbreaks associated with produce have also negatively impacted the market with an economic cost of almost \$39 billion attributed to produce (Scharff, 2010).

Contamination of fresh produce can occur at any point from farm to fork. Once contaminated, fresh produce can cross-contaminate other previously uncontaminated product during harvesting. Transport and further processing can consequently spread contamination. This spread of contamination is expedited during the shredding and washing of leafy greens with about 90% of *E. coli* O157:H7 populations previously demonstrated to transfer from leafy greens to sanitizer-free water during pilot-scale processing (CDC, 2013; Davidson, 2013; Davidson et al., 2014; NACMCF, 2010). The addition of sanitizers to produce wash water is now common practice to maintain water quality and reduce the spread pathogens during flume washing. When used properly, sanitizers may reduce pathogen populations up to 3 log CFU/g on lettuce, but this

does not guarantee absolute pathogen removal (Beuchat et al., 2004; Buchholz et al., 2012; Foley et al., 2002; Gil et al., 2009; Parish et al., 2003; Sapers, 2011).

Given the relatively low infectious dose for many foodborne pathogens, there is a “zero tolerance policy” for these pathogens on commercially processed ready-to-eat fresh-cut produce (Simpson-Stroot et al., 2008; NACMCF, 2010). Current industry testing standards result in a small sample size in comparison to a large flume tank of recirculating water, leading to unrepresentative samples and thus the potential for not detecting pathogen contamination and receiving false negative results. This has led to a call for additional rapid and sensitive methods that can more reliably detect contaminants (Koster et al., 2003; Meadema et al., 2003; Reynolds, 2003; Straub and Chandler, 2003).

One technique that has been recently studied for its incorporation into commercial-scale leafy green processing facilities is dead-end ultrafiltration (DEUF). This type of membrane filtration essentially allows water to pass through pores of the membrane while retaining microbes within the filters. After collecting particulates on the fibers of the membrane, the membrane is back-flushed with either water or a buffer to collect the retentate. Several pilot-scale studies have demonstrated the ability of DEUF to concentrate pathogens and generate samples that are more representative. DEUF can also be combined with standard enrichment or rapid detection methods for enhanced foodborne pathogen screening when monitoring produce wash water (Magaña et al., 2014).

Despite the demonstrated success of DEUF, several additional hurdles remain in making this technology amenable for use in the fresh-cut produce industry. Thus, this study focused on gaining a deeper understanding of this DEUF technology and its abilities. The overall goal of this study was to demonstrate improved detection of *E. coli* O157:H7 and *L. monocytogenes*

using DEUF sampling to rapidly collect, concentrate and recover pathogens from large volumes of produce wash water in combination with an AOAC-approved qPCR rapid detection method. This study evaluated both pilot-scale and commercial-scale generated lettuce wash water.

## **CHAPTER 1:**

### **Review of Pertinent Literature**

## **1.1 Marketing and consumption of leafy greens**

In response to Americans moving towards healthier eating habits, per capita consumption of all lettuce varieties has been increasing since 1960 (AgMRC, 2015; Butt and Sultan, 2011). In recent years, per capita consumption of romaine and loose-leaf lettuce has surpassed iceberg lettuce (AgMRC, 2015). This increased consumption of both romaine and leaf lettuce is partly due to the introduction of convenient, yet still minimally processed, packaged salads (ERS, 2001).

Various processing methods, including canning, drying, and freezing, can be used in the produce industry to improve product shelf life and quality, enhance palatability, and inactivate nutritional inhibitors (Butt and Sultan, 2011). Since leafy greens are consumed fresh, none of these processes are appropriate. Instead, head lettuce is either field-packed for bulk sale or transported to fresh-cut facilities for minimal processing (AgMRC, 2015). Minimal processing of fresh-cut leafy greens includes cleaning, shredding, flume washing, dewatering, drying, mixing and packaging under a modified atmosphere to maintain product quality during distribution and sale (Siroli et al., 2015).

Lettuce production in the United States occurs year-round with California and Arizona, accounting for 71 and 29 percent of U.S. production in 2013, respectively (AgMRC, 2015). The peak production months for these states are May and June for California and December through February for Arizona (Turini et al., 2011). In 2014, lettuce production in the U.S. totaled 3,881 million pounds, with the 2013 value of U.S. lettuce production totaling nearly \$1.5 billion, resulting in lettuce being the leading vegetable crop in terms of value (AgMRC, 2015).

## 1.2 Leafy green recalls, outbreaks, and industry concerns

With minimal processing not containing a pathogen kill step, the product can become a potential source of foodborne pathogen contamination (Castro-Ibanez et al., 2015). Lack of a kill step, increased demand, production, and improved strain tracking techniques have increased the number of identified outbreaks associated with fresh produce, including at least 88 outbreaks traced to consumption of fresh-cut produce from 1996 to 2016 (CDC, 2016c). Consequently, fresh-cut fruits and vegetables are no longer considered low risk in terms of food safety (Bhagwat, 2006; FSA, 2007).

Improved strain tracking techniques have led to foodborne illness outbreaks being detected sooner with the associated media coverage negatively impacting the fresh produce industry. For example, after the 2006 baby spinach outbreak, sales decreased by 70 percent (Todd et al., 2007).

The 2006 *E. coli* O157:H7 lettuce outbreak was initially detected in two restaurants, Taco Bell and Taco John's (Falkenstein, 2011). The Taco Bell and Taco John's restaurants were involved in multi-state outbreaks of *E. coli* O157:H7 causing 78 and 81 illnesses, respectively (CDC, 2006; Foodborne Illness Outbreak Database, 2013). The pathogen was traced back to shredded, iceberg lettuce served in both restaurants which was determined to be the likely vehicle of transmission. Multiple Taco Bell restaurants were involved during this same period, suggesting contamination likely occurred before reaching the restaurant (Falkenstein, 2011). The outbreak strains from Taco John's matched two environmental samples gathered from dairy farms near a lettuce growing area in California's Central Valley, indicating a possible route for initial contamination (Falkenstein, 2011).

Since 2006, there have been at least 16 lettuce associated foodborne outbreaks due to *E. coli* O157:H7 and at least one due to *L. monocytogenes* (Foodborne Illness Outbreak Database,

2017). Select foodborne outbreaks associated to lettuce are depicted in Table 1.1. In January 2016, Dole voluntarily recalled all salad mixes produced in their Springfield, Ohio processing facility due to possible contamination with *Listeria* (CDC, 2016d). This outbreak infected 19 people in the U.S. from nine states; all 19 were hospitalized, with one death as a result of listeriosis (CDC, 2016d). Whole genome sequencing confirmed that the isolates were closely related genetically, indicating a common source of infection (CDC, 2016d). The outbreak strain of *Listeria* was confirmed in the Dole processing facility in Springfield, Ohio; however, there was no mention of where contamination was thought to have originated.

. These foodborne outbreaks have negatively impacted consumer confidence in the safety of fresh and fresh-cut produce (Arnade et al., 2009; Lynch et al., 2009).

Table 1.1: Selected lettuce-associated foodborne outbreaks from 2006 through 2015

Date	Pathogen	States	Reported Illnesses	Product	Source Region
Nov. 06	<i>E. coli</i> O157:H7	NJ, NY, PA, DE	78	Lettuce, Iceberg	CA
Nov./Dec. 06	<i>E. coli</i> O157:H7	MN, IA, WI	81	Lettuce, Iceberg	CA
May 08	<i>E. coli</i> O157:H7	WA	10	Lettuce, Romaine	CA
Sep. 08	<i>E. coli</i> O157:H7	MI, IL, NY, OR, OH, Ontario	74	Lettuce, Iceberg	CA
Apr. 09	<i>E. coli</i> O157:H7	MN	16	Lettuce, prepackaged	Unknown
Sep. 09	<i>E. coli</i> O157:H7	NY, WI, UT, NC, CO, SD	29	Lettuce, Iceberg/Romaine	CA
May 10	<i>E. coli</i> O157:H7	MI, OH, NY, PA, TN	26	Lettuce, Romaine	AZ
Oct./Nov. 11	<i>E. coli</i> O157:H7	10 states	58	Lettuce, Romaine	Unknown
Apr. 12	<i>E. coli</i> O157:H7	CA, Quebec	28	Lettuce, Romaine	CA
Oct./Nov. 12	<i>E. coli</i> O157:H7	CT, MA, NY, PA, VA	33	Spinach and lettuce mix	MA
July 13	<i>E. coli</i> O157:H7	AZ	94	Lettuce	AZ
May 15	<i>L. monocytogenes</i>	9 States	33	Prepackaged salad	OH

Information for November 2006, November/December 2006, and May 2008 outbreaks summarized by Mandrell, 2009; the September 2008 through July 2013 outbreaks are summarized by Foodborne Illness Outbreak database (2013a-f); and May 2015 outbreak is based on Foodborne Illness Outbreak database (2015).

### **1.3 *Escherichia coli* O157:H7**

*Escherichia coli* is classified as a Gram-negative, rod-shaped, facultative anaerobic bacterium (Lim et al., 2010). There are many strains of *E. coli*, most of which are harmless and reside in the gastrointestinal tract of humans and animals as normal gut flora (Lim et al., 2010). The different types of *E. coli* can be categorized according to serotype, pathogenicity mechanisms, clinical symptoms, or virulence factors (Kaper et al., 2004; Nataro and Kaper, 1998). The strains of greatest concern are classified as enterohemorrhagic *E. coli* (EHEC). EHEC produce Shiga toxins that cause hemorrhagic colitis and the life-threatening sequelae hemolytic uremic syndrome (HUS) (Lim et al., 2010). *E. coli* O157:H7 falls into the EHEC category and has been the most frequently isolated serotype of EHEC from ill persons in the U.S., Japan, and United Kingdom (Melton-Celsa et al., 1996; Paton and Paton, 1999; Lim et al., 2010).

In 1982, *E. coli* O157:H7 was first recognized as a human pathogen associated with outbreaks of bloody diarrhea in Oregon and Michigan (Riley et al., 1983; Wells et al., 1983). The severity of these outbreaks has caused *E. coli* O157:H7 to be considered one of the most important foodborne pathogens (Lim et al., 2010). The CDC has estimated that *E. coli* O157:H7 has caused 96,534 illnesses, 3,268 hospitalizations, and 31 deaths annually (CDC 2016a; CDC 2016b). The high number of illnesses has led to additional efforts to control this pathogen and treat those affected, with an estimated annual cost of \$405 million for *E. coli* O157:H7 infections, including lost productivity, medical care, and premature deaths (Frenzen et al., 2005).

*E. coli* O157:H7 is a major public health concern throughout the world with high hospitalization and fatality rates (Mead et al., 1999). This pathogen has an extremely low oral infectious dose of less than 100 cells (Meng et al., 2007). With such a low oral infectious dose,

it becomes even more important to ensure food products offered to consumers are free of this pathogen. If an individual does ingest *E. coli* O157:H7, an incubation of one to ten days ensues before symptoms arise (FoodSafety, 2017). Symptoms typically include severe diarrhea (often bloody), severe abdominal pain, and vomiting lasting between five and ten days, most individuals recovering in six to eight days (FoodSafety, 2017).

HUS can develop as a complication, typically in children, after about one week of incubation of shiga-toxin producing *E. coli* and can lead to kidney failure (Mayo Clinic, 2016). Symptoms include decreased urine production, dark or tea-colored urine, and facial pallor, bloody diarrhea, and abdominal pain (FoodSafety, 2017; Mayo Clinic, 2016). A rare, adult version of HUS may develop as thrombotic thrombocytopenic purpura which results in blood clots in the brain from platelet aggregation producing neurological problems (Merck Manual, 2017).

#### **1.4 *Listeria monocytogenes***

*Listeria monocytogenes* is classified as a Gram-positive bacterium with motility through means of flagella (FDA, 2014). *Listeria monocytogenes* is ubiquitous in the environment, with the pathogen found in soil, silage, and other environmental sources, with estimates of between one and ten percent of humans being intestinal carriers (FDA, 2014). This bacterium is considered hardy as it resists the deleterious effects of freezing, drying, and heat with growth of the organism in refrigerated ready-to-eat foods being of primary concern

Listeriosis, the disease caused by *L. monocytogenes*, includes manifestations of septicemia, meningitis, encephalitis, and intrauterine or cervical infections in pregnant women, which can lead to spontaneous abortion or stillbirth (FDA, 2014). These aforementioned disorders are often preceded by influenza-like and gastrointestinal symptoms, including nausea,

vomiting, and diarrhea, that can occur with an onset time greater than 12 hours for gastrointestinal listeriosis (FDA, 2014).

The infective dose for *L. monocytogenes* is unknown, but is believed to vary with by strain and susceptibility of the victim; however, fewer than 1,000 total cells may cause infection in susceptible individuals (FDA, 2014). Listeriosis is typically only a concern for susceptible individuals (pregnant women/fetus, immunocompromised individuals, cancer patients, and elderly) with a percentage of the population being gastrointestinal carriers of *Listeria* and showing no symptoms (FDA, 2014).

The CDC has estimated that approximately 1,662 illnesses, 1,520 hospitalizations, and 266 deaths from listeriosis occur annually in the U.S. (CDC 2016a; CDC 2016b). The economic burden of *L. monocytogenes* infections is estimated at \$1.7 million per case, with deaths contributing 84% of the economic burden (FoodSafety, 2015). According to one USDA study, the economic burden that *L. monocytogenes* has accrued overall is approximately \$2,834,444,202 (Flynn, 2014). This study did not showcase the entire economic cost as it excluded food industry costs, loss of consumer confidence in brand or business, associated recall expenses, charges from litigation, and cost to taxpayers for local, state, and federal health agencies to respond to outbreaks (Flynn, 2014).

### **1.5 Pre-harvest contamination sources and post-harvest processing**

Contamination of fresh produce can occur at any step from farm to fork. However, from 1996 to 2008 almost half of all fresh produce outbreaks were traced to leafy greens containing *E. coli* O157:H7 with pre-harvest contamination strongly suggested (D'Lima and Suslow, 2009). Sources of pre-harvest contamination typically include manure, manure compost, sewage sludge,

irrigation water, runoff water from livestock operations, and wild and domestic animal interference (Beuchat, L.R., 2006).

The impact of farm management practices and climate change on produce safety have also been studied with both factors influencing the presence and levels of *E. coli* on produce (Liu et al., 2016). Liu et al. reported that temperature had a stronger influence than management practices on *E. coli* contamination with higher field temperatures increasing the presence and numbers of *E. coli* (2016). Infected individuals who work with fresh produce can also transmit the foodborne illness to the produce (FDA, 1998). Farmers and all employees should not only understand, but also follow basic hygiene principals to reduce the possibility of contamination of fresh produce (Liu et al., 2016).

Once lettuce is hand-cut in the field, it is either field packaged or transported to processing facilities to undergo further processing. Field coring and trimming of iceberg lettuce, which involve removing of the core and dirty or damaged outer leaves in order to increase processing plant production yields from the typical 60-70 percent to nearly 100 percent, have become increasingly popular, (McEvoy et al., 2008). In one field study conducted by McEvoy et al., *E. coli* O157:H7 was transferred from an inoculated coring knife to 19 heads of lettuce during harvesting (2008). Pathogen growth significantly increased when the produce was not stored at 30°C as opposed to proper chill temperatures, , indicating that decontamination of coring knives along with prompt chilling is necessary to ensure safety during field coring of iceberg lettuce (McEvoy et al., 2008).

Contamination can also occur during transportation and storage through improper handling, such as inadequate sanitary conditions, temperature abuse, drops, and unsuitable packing of trucks. Temperature abuse effects on iceberg lettuce quality have been extensively studied.

Results indicate that storage at 0°C retains lettuce quality which significantly declines at 20°C (Tian et al., 2014). If ambient temperatures must be used during transport, lettuce quality can be retained with an additional treatment of 1-methylcyclopropene or gibberellic acid, which inhibits browning (Tian et al., 2014).

Post-harvest contamination can also occur through shredding, washing, and packing of fresh-cut leafy greens. Numerous shredding and dicing/slicing studies have been conducted to better understand cross-contamination during processing. These studies indicated that up to 90 percent of *E. coli* O157:H7 populations transferred from leafy greens during pilot-scale washing in sanitizer-free water (CDC, 2013; Davidson, 2013; Davidson et al., 2014; NACMCF, 2010).

Bacteria can transfer from the equipment used to minimally process the product as well as through the wash water (Gil et al., 2009). This is especially true for minimally processed, fresh produce as microorganisms have a greater affinity to adhere to cut rather than uncut surfaces (Gorny et al., 2006). Evidence from various foodborne outbreaks indicates that the quality of the water used for washing and chilling produce after harvest is critical for maintaining quality and safety (FDA, 2008b). In one particular study, *E. coli* O157:H7 was frequently detected on un-inoculated lettuce after washing in the same tank with inoculated baby spinach, suggesting significant pathogen transfer from contaminated to un-contaminated produce during washing (Luo et al., 2012). Similar results were reported by Allende et al. (2008) and Luo et al. (2011) in laboratory-scale studies. The quality of the water becomes even more critical when re-circulated and re-used (Gil et al., 2009; Luo et al., 2011).

### **1.6 Sanitizer treatments for commercial flume washing**

Addition of sanitizer into wash water has become a standard industry practice for reducing the microbial load as supported by numerous pilot-scale studies (Allende et al., 2008; Luo et al.,

2012; Sapers et al., 2001; FDA, 2009). When properly used, chemical sanitizers can reduce microbial populations 1 to 3 CFU/g on lettuce, with tape water alone decreasing *E. coli* O157:H7 approximately 1 log CFU/g during pilot-scale processing (Beuchat et al., 2004; Buchholz et al., 2012; Foley et al., 2002; Gil et al., 2009; Parish et al., 2003; Sapers, 2011). In the new FSMA regulations, FDA recommends, at minimum, monitoring and adjusting the active component of the sanitizer agent and pH of the wash water, especially if sodium hypochlorite is used (FDA, 2013). In so doing, chlorine efficacy can be maintained and the levels of noxious chlorine by-products and chlorine off-gassing can be reduced (Connell, 1996; Luo et al., 2012; Suslow, 2001).

Monitoring the chlorine concentration, pH, and organic load of produce wash water is important for commercial processing facilities to ensure that the sanitizer is working efficiently. When produce is washed, organic matter surges and binds with chlorine, especially when processing high organic load-creating products such as shredded iceberg lettuce. Organic material can include produce tissue, cellular fluids released during cutting, soil, insects, and microbes that accumulate in the recirculating water (Herdt and Feng, 2009). Chlorine binding with organic matter can lead to the chlorine demand exceeding the available concentration, resulting in rapid depletion of free chlorine needed available for microbial inactivation (Luo et al., 2012). Once the sanitizer efficiency drops below this critical level, pathogens present can survive and spread throughout the processing line, resulting in cross-contamination. Decreased chlorine efficacy in produce wash water has been reported in several studies (Davidson, 2013; Davidson et al., 2014; Gonzalez et al., 2004; Shen et al., 2013; Suslow, 2000; Zhang et al., 2009).

Chemical sanitizers are primarily used to maintain the microbiological quality of the water (Gil et al., 2009). Adding a sanitizer to the washing water aids in disinfection decreases the

extent of cross-contamination, but will not ensure end product safety. Commercial sanitizers used in industry are formulated to enhance soil removal and minimize cross-contamination rather than to surface sanitize the product (Gorny et al., 2006; Sapers, 2001). Microorganisms can adhere to surfaces and crevices in produce where sanitizers cannot penetrate (Gorny et al., 2006). Consumers often falsely assume that fresh produce does not contain any microbes. In one consumer survey, 6% of respondents reported never or seldom washing fresh produce before consumption with many consumers also not separating produce from raw meat, poultry, or fish in their refrigerators (Li-Cohen and Bruhn, 2002).

Despite many laboratory- and pilot-scale studies conducted on produce wash water and sanitizer efficacy, few researchers have used actual commercial flume water where different results would be expected when compared to laboratory-generated water containing an artificial organic load (Sapers, 2001; Beuchat et al., 2004). Many of these pilot-scale studies have not considered the effects of organic load on sanitizer efficacy. Current industry practices allow for the use of up to 200 ppm available chlorine on food contact without a potable water rinse with an average treatment of 1 to 2 minutes in 50 to 200 ppm available chlorine (Parish et al., 2003; Schmidt, 2009). Given the varying amounts of available chlorine, different equipment set-ups, diverse types of produce being processed, and fluctuating organic load levels, it is advised that each commercial processing facility conduct their own in-house assessment to determine what practices have the highest efficacy for their products.

### **1.7 Current industry standards for product sampling**

Sanitizer addition to commercial wash water results in minimal reduction of *E. coli* and other foodborne pathogens, regardless of chlorine treatment or organic load (Davidson et al., 2014). For example, a small-scale laboratory study indicated that *E. coli* transferred from inoculated to

uninoculated lettuce during washing with water containing 30 ppm of mixed peracid and a 10 percent organic load, while no *E. coli* cells were recovered from the processing water (Zhang et al., 2009). Similar observations were made in another pilot-scale study where *E. coli* cross-contaminated spinach in wash water containing chlorine plus T-128 (Luo et al., 2012).

Relatively low infectious doses for many foodborne pathogens have led to the current “zero tolerance policy” for their detection in foods (Simpson-Stroot et al., 2008; NACMCF, 2010). Pathogen detection methods include “gold standard” culture-based testing, along with other emerging methods. Classical procedures for foodborne pathogen isolation and detection include cultural enrichment, selective and differential plating, confirmation, and subtyping (NACMCF, 2010). This process is quite lengthy, requiring a minimum of 18 to 48 h to isolate the target organism with 3 to 4 days needed to confirm negative test results (NACMCF, 2010).

Fresh produce has a relatively short shelf life compared to other foods on the market. Commercial processors cannot hold fresh-cut produce for up to four days for a confirmed negative result; otherwise, the product would not have enough time to reach the market and consumer before the quality deteriorates. The increasing number of fresh produce outbreaks has led to technological advances in detection platforms, including enzyme-linked immunosorbent assays (ELISA) and PCR to shorten the time necessary to obtain confirmed negative test results (NACMCF, 2010).

ELISA and PCR use cultural enrichment which generally requires growth to  $>10^3$  CFU/mL in the enrichment broth for detection of the target organism. Thereafter, selective plating is replaced by a rapid detection method that takes only minutes to a few hours to complete (NACMCF, 2010). This approach, which can give a confirmed negative test result in one to two days, allows the industry to either hold product for re-testing or recall already shipped

product before an outbreak occurs. Validation studies for Real-Time PCR methods to detect pathogens in various food matrixes have also been implemented (Tebbs et al., 2011; Wong et al., 2012).

Current industry practices require facilities to test produce and/or wash water for pathogen contamination. Such testing results in small product samples compared to much larger samples of recirculating flume water. Given the large volumes of water used to wash the product compare to the small volumes tested, the possibility of false negative results is high. If wash water is contaminated, the target organism will be rapidly diluted below the detection limit for most available methods, which calls for more rapid, sensitive methods that can increase the probability of (Koster et al., 2003; Medema et al., 2003; Reynolds, 2003; Straub and Chandler, 2003).

### **1.8 Use of physiochemical parameters to monitor produce wash water**

Large volumes of water are used during postharvest handling of minimally processed fruits and vegetables with this water commonly recirculated in industry (Suslow, 2004). Given these large volumes of recirculating water, water samples must be collected and tested to ensure consumer safety. Since contamination can occur at any point during processing, the industry must be prepared to monitor their water quality to prevent potential foodborne outbreaks.

When using chlorine-based sanitizers, both the chlorine concentration and organic load in the wash water need to be carefully monitored to maintain sanitizer efficacy and to minimize microbial cross-contamination from the water (Davidson et al., 2014). Since it is impractical for commercial processing facilities to halt processing of fresh-cut produce to check and adjust water quality, quick, and reliable methods are needed to determine water quality while the processing line is operating.

One parameter monitored to ensure chlorine efficacy during fresh-cut produce processing is the pH of the recirculating flume water. The most common sanitizers used in industry are chlorine-based, with the active component (hypochlorous acid most predominant at pH 6.5 to 7.0 (Beuchat, L.R., 1998; Herdt and Fent, 2009; Luo et al., 2011; Suslow, 2001; FDA, 2008a). The pH of the wash water, easily monitored using various types of probes that give instant and continuous readings, can be adjusted by adding a weak acid, such as citric acid (Herdt and Fent, 2009). The FDA recommends, as part of the proposed FSMA regulation, at minimum, monitoring and adjusting as necessary the concentration of the active component of the sanitizing agent and the pH of the wash water (FDA, 2013).

Another parameter widely utilized for monitoring quality of recirculating produce wash water is oxidation/reduction potential (ORP) (Suslow, 2004). ORP is the potential (voltage, in mV) at which oxidation occurs at the anode (positive) and reduction occurs at the cathode (negative) of an electrochemical cell (Suslow, 2004). This parameter is a direct measurement of the ability of the water to oxidize microbial contaminants. An advantage of ORP is ease of use and real time results which allow for monitoring and tracking of critical disinfectant levels (Suslow, 2004). No regulations have been established for ORP levels in leafy green wash water; however, a typical ORP of 650 mV has been adapted by some commercial processors as a critical limit in their HACCP program due to commodity-specific guidelines set by the Florida Department of Agriculture (FDA, 2008a). The FDA includes a recommendation to measure and monitor ORP to determine sanitizer efficacy during processing (FDA, 2008b). However, recently, the relationship between ORP and sanitizer strength has shown to be nonlinear, raising questions regarding the ability of ORP to accurately predict sanitizer efficacy (Tomas-Callejas et al., 2012).

Additional methods to quantify organic load and quality of wash water include direct methods (total solids and maximum filterable volume) and indirect methods (turbidity). The pitfalls for these parameters is that they cannot be measured instantaneously during processing; instead samples of wash water need to be collected and further processed to conduct the appropriate test. Total solids is a direct method used to determine the amount of solids in suspension or dissolved in a set volume of liquid. The method involves determining the mass of dried solids remaining in a pre-dried, pre-weighed crucible after evaporation of the liquid in an oven (Baxter and Rexing, 2005). Maximum filterable volume (MFV) is another direct method developed through Dr. Elliot Ryser. In this method, the volume of liquid passing through a Whatman filter membrane is measured after 1 min of vacuum filtration at -80 kPa. While more rapid, this method is not as sensitive as total solids. The use of turbidity as an indirect means to quantify organic load and thus water quality was also developed through Dr. Elliot Ryser. The procedure involves a pre-filtration that removes suspended solids from the sample followed by reading the absorbance of the filtrate at 664 nm, a wavelength previously shown to correlate to chlorophyll content (Watanabe et al., 1987). This test is thought to be more sanitizer-specific since these oxidizing sanitizers degrade chlorophyll (Davidson, 2013). Even using these various methods to determine the quality of produce wash water, challenges remaining in determining what exact physiochemical parameter best correlates to various foodborne pathogen persistence during commercial processing.

### **1.9 Water filtration methods**

Various filtration methods have been developed and studied as alternatives to current industry sampling protocols. Two of the most common water filtration methods studied for

incorporation into water quality testing includes hollow-fiber ultrafiltration (HUF) and dead-end ultrafiltration (DEUF).

Ultrafiltration (UF) involves the use of an ultrafilter with pore sizes rated by molecular weight cutoff (MWCO), allowing the filter to concentrate particulates from the water through sieving, instead of adsorption or sedimentation (Liu et al., 2012). For this reason, UF has emerged as a promising technique for recovering diverse microbes in large volumes of water, typically surface and tap water (Hill et al., 2007; Morales-Morales et al., 2003). However, more UF performance data is needed for different types of water samples with varying organic loads to better assess the feasibility of UF for distinctly different water quality characteristics including ionic composition/strength, total organic carbon levels, and organics profiles (Weinrich et al., 2010; Singh and Song 2005; Susanto and Ulbricht, 2008).

### **1.9.1 Hollow-fiber ultrafiltration**

Hollow-fiber ultrafiltration (HUF) utilizes tangential-flow, which involves the recirculation of retentate across the surface of the membrane. The action of “cross flow” along the filter membrane minimizes membrane fouling, maintains a high filtration rate, and allows for higher product recovery since the sample remains in solution (Hill et al., 2005). HUF utilizes tangential flow across the membrane as the water is cycled through thousands of fibers with sidewalls that are permeable to water, but not to particles greater than approximately 20 nm in diameter (Hill et al., 2005). Therefore, larger colloids (viruses and bacteria) remain suspended in the retentate water after volumes of hundreds of liters have been concentrated to a few hundred milliliters (Knappett et al., 2011). This water filtration technique can recover a diverse array of microbes from water, and holds the potential for microbial monitoring of various water sources (Liu et al., 2012). However, since limited data is available on studies incorporating HUF technology into

water monitoring, the potential limitations and efficacy of HUF for various water sources remain unknown.

In a study conducted by Liu et al. (2012), HUF was used to concentrate 10- and 100-L samples of reclaimed water samples. This study demonstrated the ability of HUF to recover a diverse group of microbes from reclaimed water and thus monitor and improve effluent water quality in wastewater treatments (Liu et al., 2012). HUF had recovery efficiencies greater than 50 percent for both 10- and 100-L samples and was sufficiently sensitive to recover viruses, bacteria, and parasites (Liu et al., 2012). Another study conducted by Knappett et al (2011) concluded that HUF increased the concentration of microbial markers in 99 percent of the samples tested, thus shows a high probability of success as a monitoring system for other types of water.

When incorporated into tangential-flow filtration systems, hollow fibers are theorized to be less susceptible to filter fouling due to shear-induced particle diffusion and inertial lift that is associated with cross-flow membrane filtration. In such a system, the water flows across the hollow-fiber surface, keeping particulates from plugging the filter pores (Nguyen et al., 2010; Sethi and Wiesner, 1997). Since these particulates remain suspended in solution, back flushing is unnecessary, with the concentrated water samples suitable for cultural methods or PCR for potentially faster, more reliable detection of the target microorganism.

### **1.9.2 Dead-end ultrafiltration**

The major difference between dead-end ultrafiltration (DEUF) and HUF is the flow of water through the membrane filter. In HUF, water is recirculating and flows tangentially over the filter, whereas DEUF relies on a single-pass through the filter, or dead-end with no recirculation (Leskinen et al., 2009; Smith and Hill, 2009). However, some DEUF methods still incorporate

the use of a hollow-fiber membrane, retaining the ability of the membrane to allow water to pass through the pores, retaining microbes within the filter. In this method, particulates collected on the fibers of the membrane are back-flushed with either water or a buffer with the retentate collected as the concentrated water sample.

Various studies have demonstrated the ability of DEUF to produce more representative samples than those obtained by currently existing grab sample approaches. For example, in work conducted by Liu et al (2012), *E. coli* O157:H7 was concentrated from 40-L aliquots of sieved lettuce and spinach water using DEUF (2012). In another study conducted using the DEUF-based Portable Multi-Use Automated Concentration System (PMACS), *E. coli* O157:H7 concentrations (CFU/mL) increased by 2 logs in retentate compared lettuce wash samples after commercial lettuce wash water containing a 5% organic load was processed (Magaña et al., 2014).

DEUF concentration holds potential for reducing the enrichment time necessary to detect foodborne pathogens in produce wash water. Magaña et al. (2014) demonstrated this ability using simulated commercial lettuce wash water where cell populations in the retentate were greater than those in unconcentrated samples after 4, 5 and 6 h of enrichment. This study also reported similar populations in high- and low-inoculum lettuce after 24 h of enrichment; demonstrating that the concentrated matrix does not hinder growth of *E. coli* O157:H7 in mBPWp (Magaña et al., 2014). Therefore, DEUF shows promise as an innovative sampling procedure that can be combined with standard enrichment or rapid detection methods for enhanced foodborne pathogen screening when monitoring produce wash water (Magaña et al., 2014).

DEUF was incorporated into the study which will now be presented, with this method having potential commercial applications for the produce industry. Commercial facilities do not have extra time during processing, excess funds to spend on technology, or additional time to train multiple employees to operate a complex filtration system. DEUF methods are generally faster than HUF, the PMACS is semi-automated requiring minimal training to operate, no pre-treatment of the water is required (HUF methods require filters to be blocked before use by incubation periods ranging from 1 to 16 h) and most importantly, has been demonstrated to be able to concentrate target pathogens from water even at low levels (Kearns et al., 2008).

### **1.9 Overall project goals and objectives**

The safety of fresh-cut leafy greens is receiving increasing scrutiny due to on-going outbreaks of illness. Consequently, various novel technologies that can potentially reduce these outbreaks and raise consumer confidence in fresh produce are now being explored. Current areas of interest include improved sampling methods and the use of various filtration techniques to obtain more representative samples that will yield a higher probability of detection.

This study focused on the use of DEUF technology, through the use of the PMACS, to gain a deeper understanding of this technology and its abilities. The overall goal of this study was to demonstrate improved detection of *E. coli* O157:H7 and *L. monocytogenes* using the DEUF sampling procedure to rapidly collect, concentrate and recover pathogens from large volumes of produce wash water in combination with an AOAC-approved qPCR rapid detection method.

This goal consisted of two objectives, with two tasks per objective:

Objective 1. Compare the DEUF concentration method with the standard FDA BAM sampling method for detection of *E. coli* O157:H7 and *L. monocytogenes* in flume water during

production of fresh-cut iceberg lettuce at the MSU fresh-cut leafy green pilot-scale production facility.

Task A. Compare the detection of *E. coli* O157:H7 and *L. monocytogenes* in flume water during production of fresh-cut iceberg lettuce at the MSU fresh-cut leafy green pilot-scale production facility using the FDA-BAM and DEUF concentration methods.

Task B. Evaluate the reductions in time of detection for enriched samples containing very low levels of *E. coli* O157:H7 and *L. monocytogenes*.

Objective 2. Assess the ability of the DEUF sampling method to rapidly concentrate and detect *E. coli* O157:H7 and *L. monocytogenes* in lettuce wash waters generated in commercial scale leafy-green processing facilities.

Task A. Compare the pathogen detection probabilities using DEUF concentration versus standard grab sampling in lettuce wash waters generated from a commercial-scale processing line.

Task B. Assess the ability of DEUF to concentrate pathogens from inoculated commercially generated flume and centrifugation lettuce wash water.

## **CHAPTER 2:**

### **Rapid Concentration/Detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in Lettuce Wash Water using a University Pilot-Scale Leafy Green Processing Facility**

## 2.1 ABSTRACT

A novel dead-end ultrafiltration (DEUF) sampling method was used prior to qPCR or standard enrichment to determine presence/absence of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in wash water after pilot-scale processing of inoculated iceberg lettuce. This study evaluated the use of DEUF in the MSU fresh-cut leafy green pilot-scale processing facility for 1) detecting of pathogens in flume water using the FDA BAM and DEUF concentration methods, and 2) reducing the time to detection for enriched samples containing very low levels of pathogens. In the pilot-scale processing facility, 22.7 kg of iceberg lettuce was processed with a single inoculated head containing ~4.9 or 7.2 log CFU/head of *E. coli* O157:H7 and *Listeria* in 890 L of wash water containing either 0, 2.5, or 5% lettuce solids with or without a chlorine-based sanitizer, respectively. After processing, duplicate 400 mL wash water samples were collected, along with duplicate 40 L samples and processed using DEUF. All samples were enriched and examined for presence/absence of the target organism using standard culture and qPCR techniques after 24 and 48 h of enrichment. In objective 2, additional samples were collected at various time points during enrichment and examined by both standard culture and qPCR. In objective 1, 16 of 24 samples (67%) of *E. coli* and *Listeria* samples had the same results from DEUF qPCR and/or enrichment culture as from conventional FDA BAM sample qPCR and/or enrichment culture in trials containing 0, 2.5 and 5% organic loads with or without the use of a chlorine-based sanitizer. Additionally, 8 of 24 samples (33%) of *E. coli* and *Listeria* samples were positive by DEUF qPCR and/or enrichment culture, but negative by conventional FDA BAM sample qPCR and/or enrichment culture. In objective 2, both pathogens had significantly higher populations in DEUF concentrated samples compared to standard sampling method by hr 7,  $2.06 \pm 0.6$  and  $0.0 \pm 0$ , and  $1.25 \pm 1.4$  and  $0.58 \pm 0.9$ , for *E. coli* and *L.*

*monocytogenes*, respectively ( $P < 0.05$ ). These results suggest that the DEUF method with its larger, more representative sample is better able to detect low levels of *E. coli* and *Listeria* in leafy green processing water.

## 2.2 OBJECTIVE

The overall objective of this study was to compare the DEUF concentration method with the standard FDA BAM sampling method for detection of *E. coli* O157:H7 and *Listeria monocytogenes* in flume water generated during production of fresh-cut iceberg lettuce at the MSU fresh-cut leafy green pilot-scale facility. This study contained the following two tasks – 1) compare the detection of *E. coli* O157:H7 and *L. monocytogenes* in flume water containing either 0, 2.5 or 5% lettuce solids with or without a chlorine-based sanitizer, using the FDA BAM and DEUF concentration methods, and 2) determine the enrichment times needed to detect *E. coli* O157:H7 and *L. monocytogenes* in lettuce wash water using the FDA BAM and DEUF concentration methods.

## **2.3 MATERIALS AND METHODS**

### **2.3.1 Experimental design**

Dead-end ultrafiltration (DEUF) concentration was compared with the standard Food and Drug Administration Bacteriological Analytical Manual (FDA BAM) sampling method to detect *Escherichia coli* O157:H7 and *Listeria monocytogenes* in flume water during production of fresh-cut iceberg lettuce using the Michigan State University (MSU) fresh-cut leafy green pilot-scale processing facility. Three different organic loads, both with and without a chlorine-based sanitizer, were assessed in triplicate by processing one head of iceberg lettuce (4.9 or 7.2 log CFU/head) with 22.7 kg of uninoculated iceberg lettuce using wash water with or without a chlorine-based sanitizer or an organic load. All lettuce was processed by shredding, conveying, fluming, and shaker table dewatering after which flume water samples were collected and quantitatively/qualitatively examined for *E. coli* O157:H7 and *L. monocytogenes* using both DEUF concentration and the standard FDA BAM method.

### **2.3.2 Produce**

Individually wrapped heads of iceberg lettuce (*Lactuca sativa* L.) were obtained from a local wholesaler (Stan Setas Produce Co., Lansing, MI), arriving in cases containing 24 heads each. Product originated from either California or Arizona depending on the growing season. All lettuce was stored in a 4°C walk-in-cooler and used within 3 days of delivery. On the day of processing, lettuce heads were unwrapped and hand-cored to obtain four 5.7 kg batches (~22.7 kg total).

### **2.3.3 *Escherichia coli* O157:H7 strains**

Four non-toxicogenic, GFP-labeled stx1<sup>-</sup>, stx2<sup>-</sup> strains of *Escherichia coli* O157:H7 were obtained from Dr. Michael Doyle at the Center of Food Safety, University of Georgia, Griffin,

GA, in January 2008. *E. coli* O157:H7 strains included – ATCC 43888 (human feces isolate), CV2b7 (environmental isolate), 6980-2 (environmental isolate), and 6982-2 (environmental isolate). Based on a previously conducted bacterial attachment study, these avirulent *E. coli* O157:H7 strains behaved similarly to virulent strains linked to outbreaks involving similar produce commodities. All strains had previously been transformed with a pGFPuv plasmid containing a GFP gene and ampicillin-resistance gene. All four strains were stored at -80°C in tryptic soy broth (Difco, BD, Sparks, MD) containing 0.6% (w/v) yeast extract (TSBYE; Difco, BD), and 10% (v/v) glycerol (Sigma Chemical Co., St. Louis, MO) until needed. Working cultures were prepared by two successive transfers in 9 mL of TSBYE and incubation at 35°C for 24 h before use.

#### **2.3.4 *Listeria monocytogenes* strains**

Three avirulent strains of *Listeria monocytogenes* were obtained from Dr. Sophia Kathariou at North Carolina State University, Raleigh, NC. *L. monocytogenes* strains included – J22F and J29H (avirulent derivatives of H7550-Cd<sup>s</sup>) and M3 (avirulent derivative of 1/2a3). All three strains were stored at -80°C in TSBYE until needed. Working cultures were prepared by subculturing the frozen stock cultures twice in 9 mL of TSBYE and incubating at 35°C for 24 h before use.

#### **2.3.5 Lettuce inoculation**

The four *E. coli* O157:H7 and three *L. monocytogenes* strains were combined in equal volumes to obtain separate 36- and 27-mL cocktails. Each cocktail was then separately diluted in sterile-distilled water to achieve a level of either  $\sim 10^4$  CFU/mL or  $\sim 10^7$  CFU/mL for trials without and with the chlorine sanitizer, respectively. A single, hand-cored head of iceberg lettuce was spot-inoculated in center of core with 1 mL using a pipette and appropriate *E. coli*

O157:H7 and *L. monocytogenes* suspension to obtain inoculated levels of 4.9 or 7.2 log CFU/head. The inoculated lettuce head was stored in a covered container for ~30 min before processing. The inoculum suspension was also appropriately diluted and plated on either MacConkey Agar with Sorbitol, Cefixime and Tellurite (SMAC-CT) or Modified Oxford Agar (MOX) to determine the initial inoculation level for *E.coli* O157:H7 and *L. monocytogenes* at the time of processing.

### **2.3.6 Lettuce wash water**

Simulated lettuce wash water was prepared by homogenizing 0, 22.2, and 44.5 kg of iceberg lettuce in filtered tap water using a Rotostat blender model XP-02 (Admix, Manchester, NH) with this lettuce homogenate then appropriately diluted in filtered tap water to obtain 890 L of water with organic loads of 0, 2.5% and 5.0% (w/v lettuce solids). These three organic loads were assessed in triplicate both without and with a sanitizer. A chlorine-based sanitizer (XY-12, Ecolab, St. Paul, MN) was added to the water to achieve 50 ppm total chlorine and then acidified to pH 6.5 using citric acid (Sigma-Aldrich, St. Louis, MO) as measured with a pH probe (pHTestr 30, Oakton, Vernon Hills, IL). The total chlorine level was confirmed using chlorine test kit 321 (Ecolab) before processing.

### **2.3.7 University pilot-scale lettuce processing facility**

A pilot-scale leafy green processing line was used, consisting of a lettuce shredder, step conveyer, flume tank, and shaker table. The commercial lettuce shredder (TRS 2500 Urschel TranSlicer, Valparaiso, IN) was operated at a feed belt speed of 198 m/min and a slicing wheel speed of 905 RPM to obtain a lettuce shred size of approximately 5 x 5 cm. The commercial motorized step conveyer (Domer model 736018 mc series, Domer Manufacturing, Hartland, WI) was mounted with a smooth polyurethane belt (ThermoDrive, Mol Industries, Grand Rapids, MI)

and was operated at 0.11 m/s (Figure 2.1). The stainless steel recirculation tank (~1,000 L capacity) contained 890 L of tap water (~15°C) and was connected to a 3.6 m long stainless steel flume tank (Heinzen Manufacturing, Inc., Gilroy, CA) by a 4.14 m long, 10 cm-diameter plastic discharge hose and centrifugal pump (Model XB754FHA, Sterling Electric, Inc., Irvine, VA) that recirculated the water at a rate of ~10 L/s (Figure 2.2). The flume tank was also equipped with two overhead spray jets. At the end of the flume tank was a custom-made stainless steel screen containing 1.25 cm-diameter holes spaced 0.65 cm apart (Heinzen Manufacturing, Inc.) which retained the product during washing (Figure 2.3). A 1 HP Baldor washdown duty motor (Baldor Electric Co., Ft. Smith, AR) operated the stainless steel shaker table at 1760 RPM to partially dewater the lettuce (Figure 2.2). Water removed from the lettuce during mechanical shaking passed through the custom-made stainless steel screen at the end of the flume tank and was fed into the water holding tank through a water recirculation spout underneath the shaker table.

### **2.3.8 Lettuce processing**

Four sequential 5.7 kg batches of uninoculated, cored iceberg lettuce were processed, with single inoculated head processed between the first and second batch. Lettuce was hand-fed into the shredder at a rate of ~0.5 kg per second. Shredded lettuce was then step-conveyed to the gated flume tank and washed for ~90 seconds in 890 L of recirculating wash water either with or without sanitizer and/or an organic load. Lettuce was partially dewatered on the shaker table and collected in a single centrifugation basket.

### **2.3.9 Sample collection**

After lettuce shredding and washing, 100 L of flume water was pre-sieved and pre-filtered into a sterile container containing 5 g of sodium thiosulfate (Fisher Scientific, Waltham,

MA) for neutralization. The pre-sieve assembly contained a PVC sieve and 125  $\mu\text{m}$  polypropylene mesh attached to an inlet tube which connected to a sump containing a 5  $\mu\text{m}$  pre-filter. The sump also had an outlet tube connected to allow the release of pre-sieved and pre-filtered lettuce wash water (Figure 2.4). Two 400 mL pre-sieved and pre-filtered lettuce wash water samples were collected to represent standard sampling methods (SSM). Two additional 40 L aliquots from the remaining 100 L of lettuce pre-sieved and pre-filtered lettuce wash water were concentrated using the DEUF concentration protocol (DEUF-C). The PMACS was prepared for each wash water trial by installing a new Optiflux F200NR polysulfone ultrafilter (2.0  $\text{m}^2$  surface area,  $\sim 30$  kDA pore size; Fresenius Medical Care North American, Waltham, MA) (Figure 2.5). The PMACS automated collection cycle filtered water, followed by PMACS automated recovery cycle initiation to recover material collected on the filter (0.01% sodium polyphosphate in 0.1 M sodium phosphate buffer backflushed filter to generate retentate (DEUF-C sample). After concentration, the samples were analyzed for *E. coli* O157:H7 and *L. monocytogenes* using the FDA BAM enrichment protocol followed by cultural isolation and qPCR (Figure 2.6).



Figure 2.1: Lettuce shredder and step conveyoyr



Figure 2.2: Gated flume tank and shaker table



Figure 2.3: Recirculation tank

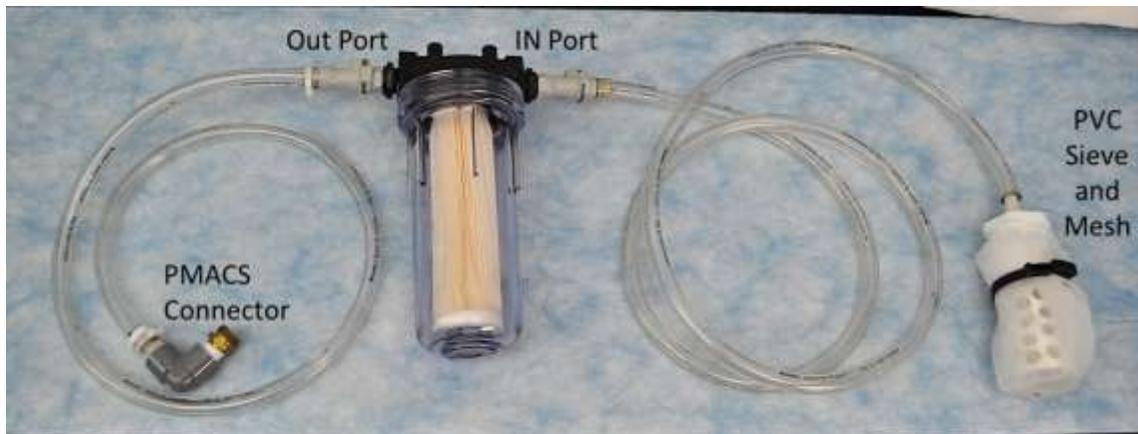


Figure 2.4: Pre-sieve and pre-filter set-up

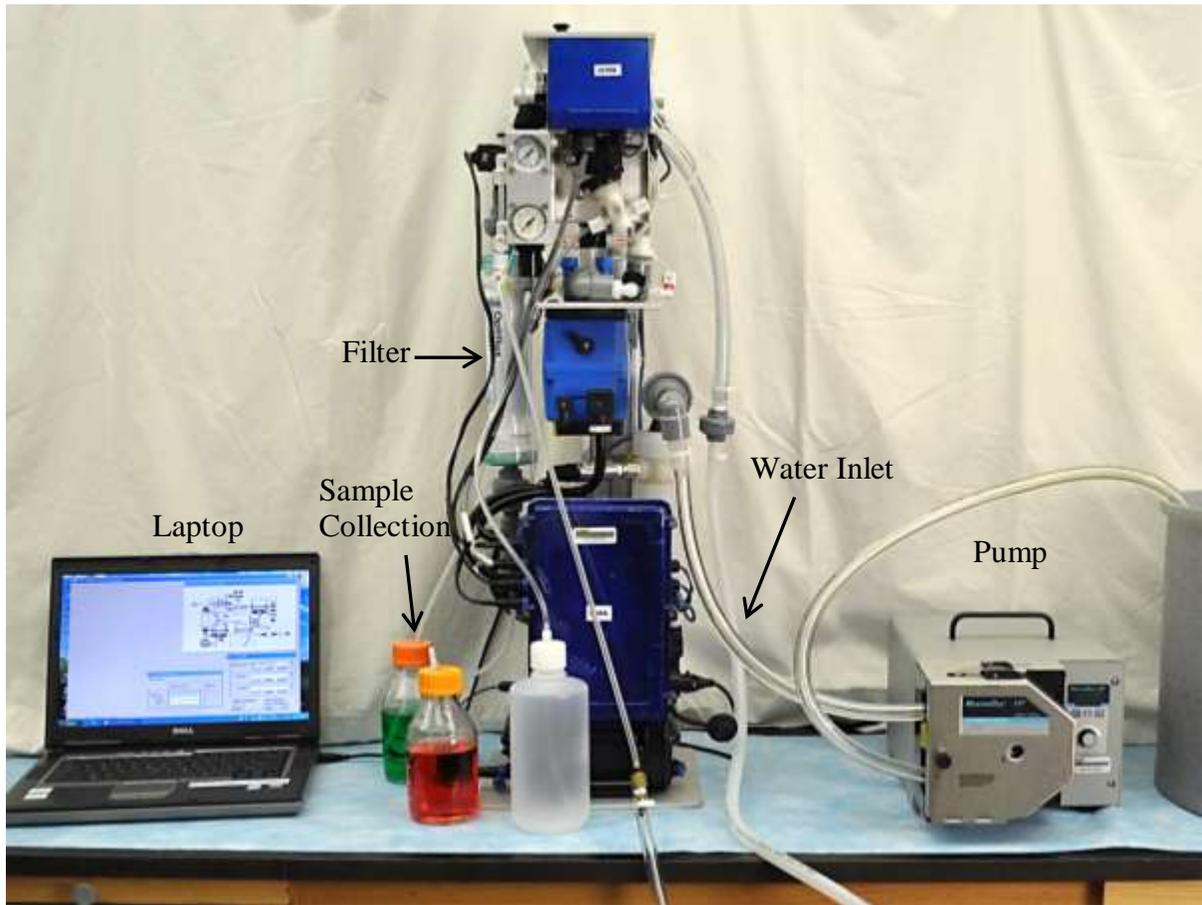


Figure 2.5: PMACS equipment set-up

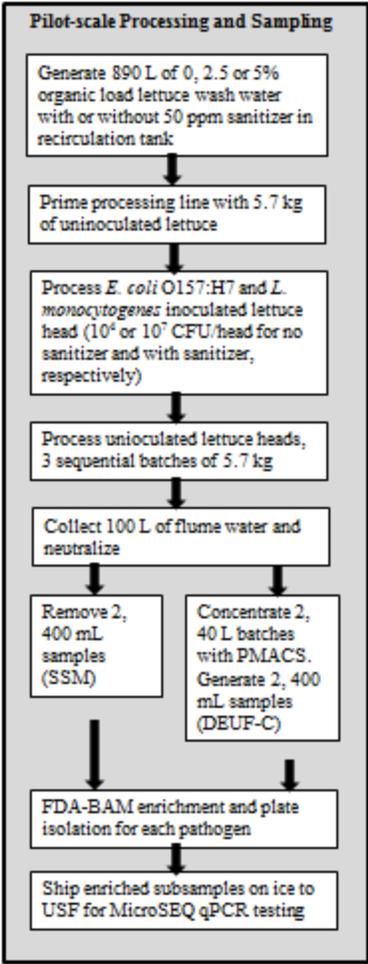


Figure 2.6: Pilot-scale processing and sampling at MSU

### **2.3.10 *Escherichia coli* O157:H7 analyses**

Each trial consisted of six separate samples: one contamination check (negative control), one positive control, two grab samples (SSM) and two concentrated samples (DEUF-C). All six samples were analyzed for *E. coli* O157:H7 using the FDA BAM enrichment protocol followed by cultural isolation and qPCR. For *E. coli* O157:H7 analysis, a 125 mL sample was enriched in 125 mL 2x modified buffered peptone water with pyruvate (2x mBPWp) (Neogen Corp., Lansing, MI) and incubated for 5 h at 37 °C. Thereafter, the samples were supplemented with acriflavin-cefsulodin-vancomycin (Sigma-Aldrich, St. Louis, MO) and incubated an additional 18 – 24 h at 42 °C. After incubation, enriched samples were then appropriately diluted and streaked to plates of sorbitol MacConkey containing cefixime and tellurite (Sigma-Aldrich, St. Louis, MO) and Rainbow O157 agars (Biolog, Hayward, CA). Aliquots of these enriched samples were also collected, frozen at -20°C, and shipped overnight on ice to University of South Florida (USF) for qPCR analysis. Following 18 – 24 h of incubation at 37 °C, all colorless colonies on sorbitol MacConkey and all small black colonies without halos on Rainbow O157 were counted as *E. coli* O157:H7 (Figure 2.7).

### **2.3.11 *Listeria monocytogenes* analyses**

Each of the six samples enriched for *E. coli* O157:H7 was also separately enriched for *L. monocytogenes*. All six samples were analyzed for *L. monocytogenes* using the FDA BAM enrichment protocol followed by cultural isolation and qPCR at USF. For *L. monocytogenes* analysis, 25 mL samples were enriched in 225 mL of Basal Buffered Listeria Enrichment Broth (Neogen Corp., Lansing, MI) for 4 h at 30 °C. After adding the Buffered Listeria Enrichment Broth Supplement (Neogen Corp., Lansing, MI) incubation continued at 30 °C for 44 h. After a total of 48 h of incubation, the enriched samples were appropriately diluted, plated on Modified

Oxford Listeria Agar (Neogen Corp., Lansing, MI) and incubated for 48 h at 35 °C. Following 24 and 48 h of incubation at 35°C, all black colonies were counted as *L. monocytogenes*. In addition, aliquots of enriched samples were also collected after both 24 and 48 h of enrichment, frozen at -20°C, and shipped on ice to USF for qPCR analysis (Figure 2.7).

### **2.3.12 Sample enrichment times**

For processing trials containing 0% and 5% organic load with sanitizer, plating of enriched samples and additional aliquots were removed initially and after 3, 4, 5, 6, and 7 h of enrichment (Figure 2.8). At the same time, samples were diluted as necessary and plated on the appropriate selective media for quantification of *E. coli* O157:H7 and *L. monocytogenes* colonies. In addition, aliquots were removed at each interval, frozen at -20°C and shipped on ice to USF for qPCR analysis.

### **2.3.13 qPCR analysis**

Aliquots taken from each *E. coli* O157:H7 and *L. monocytogenes* enrichment were screened for the target pathogen using AOAC-approved confirmatory qPCR testing. For each sample, bacterial DNA was extracted using the PrepSEQ Rapid Spin Sample Preparation kit (Applied Biosystems, Life Technologies, Inc., Grand Island, NY). Following DNA extraction, amplification was done using the AOAC-approved MicroSEQ *E. coli* O157:H7 and *L. monocytogenes* Detection Kits (Applied Biosystems, Life Technologies, Inc., Grand Island, NY). The MicroSEQ qPCR kits were previously shown to detect the avirulent strains used in this project based on publically-available patents describing the DNA regions to be amplified. Levels of assay inhibition were also evaluated by assessing internal control reactions for each pathogen in the MicroSEQ kits.

### 2.3.14 Statistical analysis

*E. coli* O157:H7 and *L. monocytogenes* results obtained before and during were based on triplicate experiments. Results were converted to log CFU/mL, averaged, and subjected to an analysis of variance (ANOVA) using JMP 13 (SAS Institute Inc., Cary, NC). These results were also subjected to the Tukey-Kramer HSD test with  $P$  values of  $\leq 0.05$  considered significantly different. *E. coli* and *L. monocytogenes* results obtained at 24 and 48 h after enrichment were recorded as either positive or negative for experiments conducted both with and without sanitizer at the various organic loads. Regarding the time needed to filter 40 L of lettuce wash water, experiments were conducted in triplicate with time recorded in minutes. Results were averaged, subjected to ANOVA using JMP 13, and also subjected to the Tukey-Kramer HSD test with  $P$  values of  $\leq 0.05$  considered significantly different.

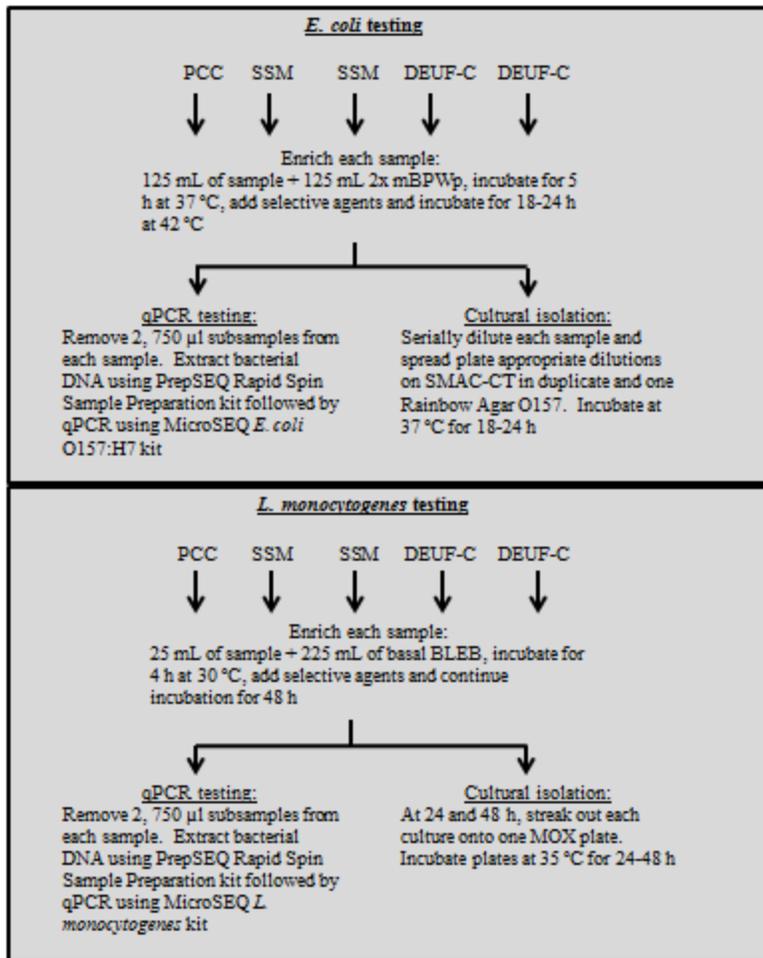


Figure 2.7: *E. coli* O157:H7 and *L. monocytogenes* enrichment process

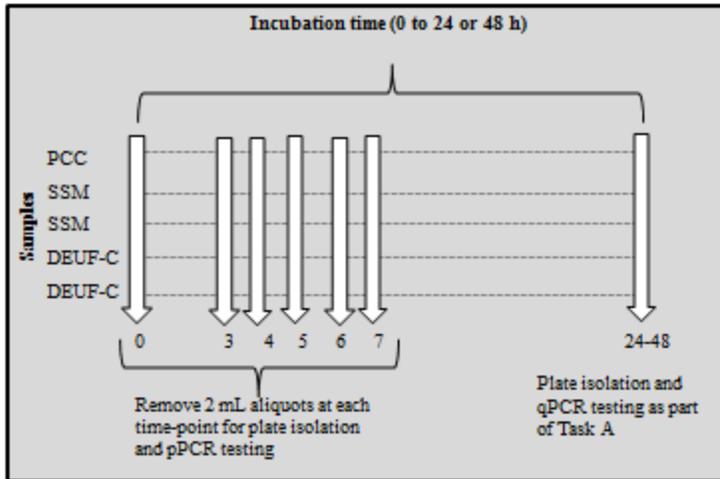


Figure 2.8: Sampling times for *E. coli* O157:H7 and *L. monocytogenes*

## **2.4 RESULTS**

### **2.4.1 Probability of detecting *E. coli* O157:H7 in lettuce wash water without sanitizer at various organic loads**

The probability of detecting *E. coli* was greater for trials with organic loads of 0 and 2.5% using DEUF as compared to the FDA Bam method, with 0 and 83.3, and 83.3 and 100% of the samples positive, respectively at 0 and 2.5% organic load by both culture and PCR results (N=6; N=6) (Figure 2.9). At the 5% organic load, no difference in the probability of detecting *E. coli* was seen using DEUF or the FDA BAM methods, with culture and PCR results being identical (N=6) (Figure 2.9). As organic load increased, the probability of detecting *E. coli* in samples collected using either DEUF or the FDA BAM method also increased.

### **2.4.2 Probability of detecting *L. monocytogenes* in wash water without sanitizer at various organic loads**

In trials without an organic load, the probability of detecting *Listeria* was higher for the DEUF concentrated samples as compared to the standard FDA BAM method, with 9 and 33.3, and 100 and 100% of the samples positive using the FDA BAM culture and PCR samples and the DEUF concentrated culture and PCR methods, respectively (N=6) (Figure 2.10). In trials with a 2.5 and 5% organic load, no difference was seen in the probability of detecting *Listeria* using either the DEUF or FDA BAM method. In trials containing an organic load of 2.5%, the FDA BAM samples yielded detection probabilities of 100 and 67%, respectively for the culture and PCR methods; whereas 100 and 67% of the DEUF samples were positive by culture and PCR respectively (N=6) (Figure 2.10). In trials with a 5% organic load, all FDA BAM and DEUF samples were positive by both culture and PCR (N=6) (Figure 2.10).

### **2.4.3 DEUF concentration filtration times for lettuce wash water without sanitizer at various organic loads**

As expected, filtration times were slower as the percentage of organic load in lettuce wash water increased ( $P < 0.05$ ) with an average of  $22.3 \pm 0.3$ ,  $30.2 \pm 2.5$ , and  $48.9 \pm 2.8$  minutes needed to concentrate 40 L of wash water containing organic loads of 0, 2.5, and 5%, respectively (Table 2.1).

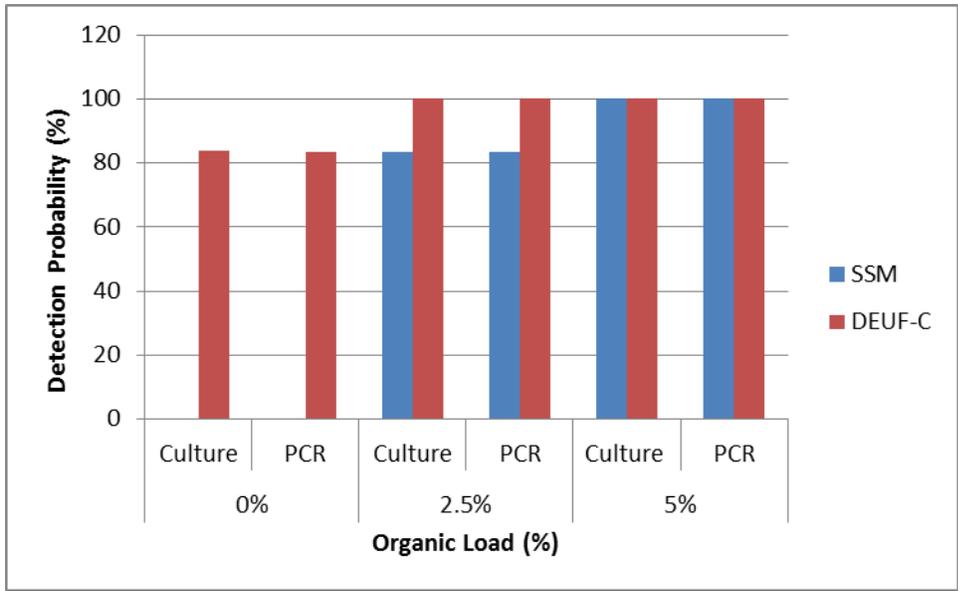


Figure 2.9: Probability of detecting *E. coli* O157:H7 in trials without sanitizer using standard sampling method (SSM) and DEUF-C method (DEUF-C)

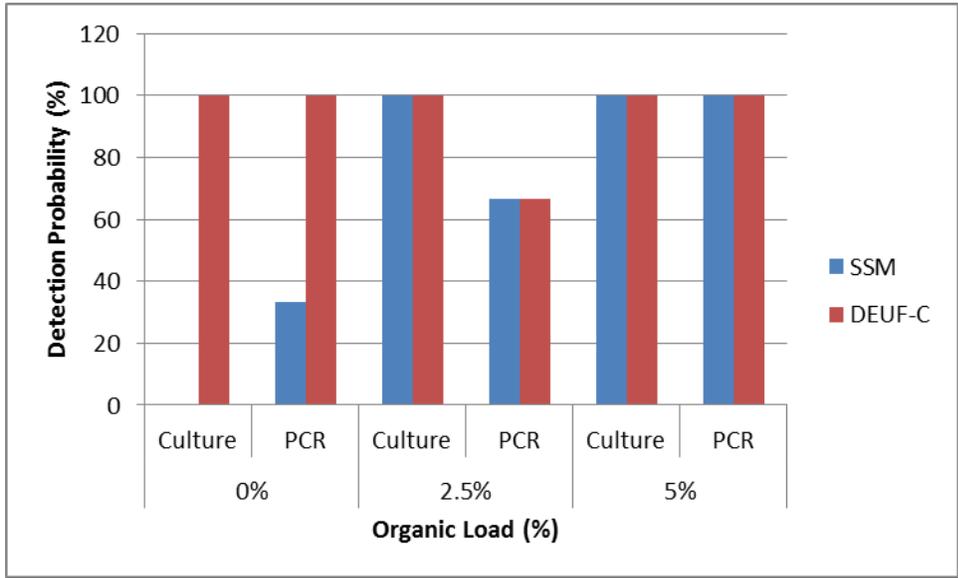


Figure 2.10: Probability of detecting *L. monocytogenes* in trials without sanitizer using standard sampling method (SSM) and DEUF-C method (DEUF-C)

Table 2.1: Filtration times for 40 L of lettuce wash water without sanitizer at various organic loads<sup>a</sup>

Mean $\pm$ SD flume water filtered and amount of time taken to filter		
% Organic load (w/v)	Amount Filtered (L)	Time (min)
0	40.1 $\pm$ 0.0 A	22.3 $\pm$ 0.3 A
2.5	40.1 $\pm$ 0.1 A	30.2 $\pm$ 2.5 B
5	40.0 $\pm$ 0.0 A	48.9 $\pm$ 2.8 C

<sup>a</sup> Means with different capital letters are significantly different ( $P \leq 0.05$ ).

#### **2.4.4 Probability of detecting *E. coli* O157:H7 in lettuce wash water with sanitizer at various organic loads**

In trials without an organic load, *E. coli* was more likely to be detected in samples collected using the standard FDA BAM sampling procedure for both culture and PCR compared to DEUF concentrated samples, with 83.3 and 66.7% for culture SSM and DEUF-C, and 33.3 and 16.7, for PCR SSM and DEUF-C, respectively (N=6) (Figure 2.11). This same unusual trend was observed for the PCR results from trials containing 2.5% organic load, 33.3 and 0% for SSM and DEUF-C, respectively (N=6) (Figure 2.11). However, culture results yielded a higher probability of detection for the sanitizer-free 2.5% organic load samples concentrated by DEUF, 33.3 and 66.7% positive for SSM and DEUF-C, respectively (N=6) (Figure 2.11). Trials containing a 5% organic load were as expected with a higher probability of detection observed for DEUF concentrated samples in comparison to the standard FDA BAM samples (50 and 83.3% for SSM and DEUF-C, respectively (N=6) (Figure 2.11)). However, none of the DEUF concentrated or standard FDA BAM samples containing a 5% organic load were positive by PCR (N=6) (Figure 2.11).

#### **2.4.5 Probability of detecting *L. monocytogenes* in lettuce wash water with sanitizer at various organic loads**

In most trials, a higher probability of detection observed using DEUF compared to the standard FDA BAM method. In trials without organic load, the FDA BAM standard sampling method resulted in an 83.3 and 100% probability of detection, by culture and PCR, respectively, while all DEUF samples were positive by both culture and PCR (N=6; N=6) (Figure 2.12). At a 2.5 percent organic load, culture results were similar for both the standard FDA BAM and DEUF methods, with a 33.3% probability of detection (N=6) (Figure 2.12). However, PCR results

indicated a high probability of detection for the DEUF (50%) as opposed to standard FDA BAM method (16.7%) (N=6) (Figure 2.12). At the 5% organic load, DEUF was superior to FDA BAM with detection probabilities of 66.7 and 33.3%, and 100 and 66.7%, for FDA BAM standard sampling culture and PCR and DEUF concentrated culture and PCR, respectively (N=6; N=6) (Figure 2.12).

#### **2.4.6 DEUF concentration times for trials with sanitizer at various organic loads**

Similar to the sanitizer-free trials, the increase in filtration time paralleled the increase in organic load ( $P < 0.05$ ). Overall,  $21.3 \pm 0.9$ ,  $27.6 \pm 2.4$ , and  $40.7 \pm 2.8$  min was needed to concentrate 40 L of lettuce wash water containing both a sanitizer and an organic load of 0, 2.5 and 5%, respectively (Table 2.2).

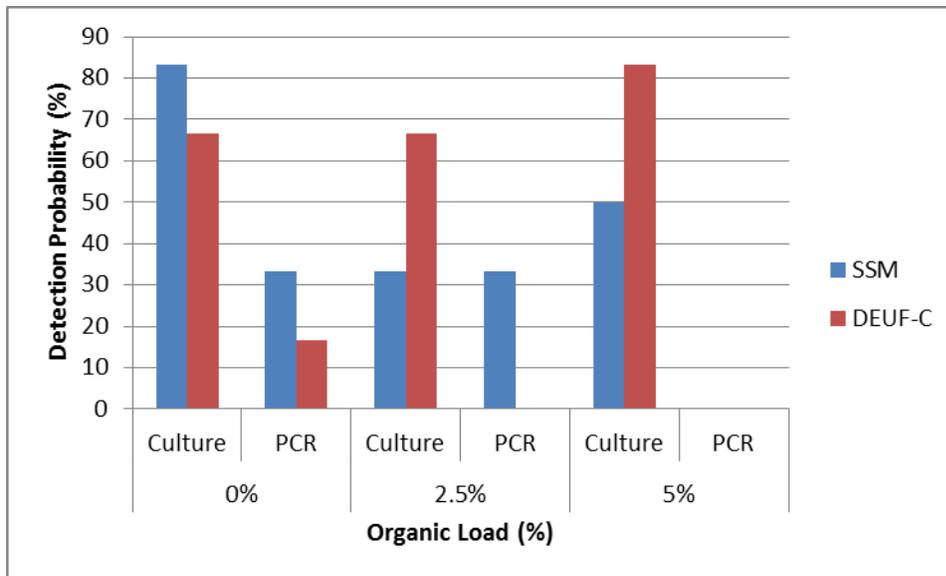


Figure 2.11: Probability of detecting *E. coli* O157:H7 in trials with sanitizer using standard sampling method (SSM) and DEUF-C method (DEUF-C)<sup>a</sup>

<sup>a</sup>PCR results for 5% organic load were 0.

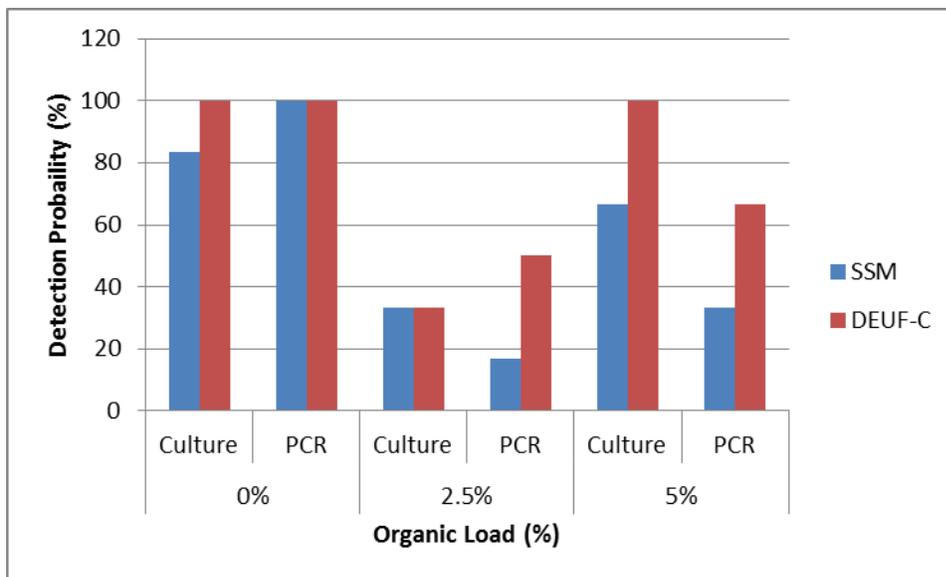


Figure 2.12: Probability of detecting *L. monocytogenes* in trials with sanitizer using standard method (SSM) and DEUF-C method (DEUF-C)

Table 2.2: Filtration times for 40 L of lettuce wash water containing a sanitizer and various organic loads<sup>a</sup>

Mean $\pm$ SD flume water with sanitizer filtered and amount of time taken to filter		
% Organic load (w/v)	Amount Filtered (L)	Time (min)
0	40.0 $\pm$ 0.1 A	21.3 $\pm$ 0.9 A
2.5	40.0 $\pm$ 0.1 A	27.6 $\pm$ 2.4 B
5	40.0 $\pm$ 0.0 A	40.7 $\pm$ 2.8 C

<sup>a</sup> Means with different capital letters are significantly different ( $P \leq 0.05$ ).

#### **2.4.7 Enrichment times for detecting *E. coli* O157:H7 and *L. monocytogenes* in lettuce wash water without an organic load**

As expected, the populations of both target pathogens increased with enrichment time for both the DEUF and FDA BAM samples. However, due to large standard deviations no significant differences in populations were seen between the hours sampled ( $P > 0.05$ ). For samples collected using standard sampling and DEUF, *E. coli* populations increased  $0.94 \pm 1.1$  and  $1.3 \pm 1.5$  log CFU/mL, respectively, after 7 h of enrichment (Table 2.3). When comparing samples using standard sampling method to samples collected using DEUF concentration there is no significant difference in *E. coli* populations at any time point ( $P > 0.05$ ).

For *Listeria monocytogenes* samples collected using the standard sampling method and DEUF, populations increase  $0.45 \pm 0.7$  and  $1.38 \pm 1.1$  log CFU/mL, respectively, after 7 h of enrichment (Table 2.3). As was true for *E. coli*, *Listeria* populations were not significantly different in the DEUF concentrated samples as compared to standard sampling method at any time point ( $P > 0.05$ ).

#### **2.4.8 Enrichment times for detecting *E. coli* O157:H7 and *L. monocytogenes* in lettuce wash water containing a 5% organic load**

Overall, less growth of both *E. coli* O157:H7 and *L. monocytogenes* was observed for enrichments containing a 0 compared to 5% organic load. When samples were collected using the standard sampling and DEUF concentration methods, *E. coli* populations increased only 0.12 and 0.37 log CFU/mL, respectively, after 6 h with these changes ( $P < 0.05$ ) (Table 2.4). However, when comparing the *E. coli* populations from the standard to DEUF concentrated samples, significantly higher populations were observed for the DEUF concentrated samples at every time point ( $P < 0.05$ ).

The same trends were observed for *Listeria* in lettuce wash water samples containing a 5% organic load. Using the standard sampling and DEUF, *Listeria* populations increased  $0.58 \pm 0.9$  and  $0.13 \pm 1.4$  log CFU/mL after 7 h of enrichment, respectively, with these changes again not significant ( $P > 0.05$ ) (Table 2.4).

Table 2.3: *E. coli* O157:H7 and *L. monocytogenes* populations during enrichment of lettuce wash water containing a 0% organic load with sanitizer<sup>a</sup>

		Mean $\pm$ SD log CFU/mL bacteria in lettuce produce wash water with 0% organic load						
		Hour Sampled						
		Before Enrichment	0	3	4	5	6	7
<i>E. coli</i> O157:H7	SSM	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.51 $\pm$ 0.8	0.94 $\pm$ 1.1
	DEUF-C	0 $\pm$ 0	0 $\pm$ 0	0.22 $\pm$ 0.2	0 $\pm$ 0	0.38 $\pm$ 0.6	0.94 $\pm$ 1.5	1.35 $\pm$ 1.5
<i>L. monocytogenes</i>	SSM	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.45 $\pm$ 0.7
	DEUF-C	0 $\pm$ 0	0.43 $\pm$ 1.1	0.44 $\pm$ 1.1	0.46 $\pm$ 1.3	0.83 $\pm$ 1.3	0.83 $\pm$ 1.3	1.38 $\pm$ 1.1

<sup>a</sup> Means are not significantly different ( $P \leq 0.05$ ).

Table 2.4: *E. coli* O157:H7 and *L. monocytogenes* populations during enrichment of lettuce wash water containing a 5% organic load with sanitizer<sup>a</sup>.

		Mean $\pm$ SD log CFU/mL bacteria in lettuce produce wash water with 5% organic load						
		Hour Sampled						
		Before Enrichment	0	3	4	5	6	7
<i>E. coli</i> O157:H7	SSM	0.57 $\pm$ 0.9A	0.25 $\pm$ 0.6A	0 $\pm$ 0A	0.22 $\pm$ 0.5A	0.36 $\pm$ 0.9A	0.33 $\pm$ 0.5A	0 $\pm$ 0A
	DEUF-C	2.13 $\pm$ 0.7B	1.69 $\pm$ 0.9B	1.89 $\pm$ 0.8B	1.89 $\pm$ 0.6B	1.99 $\pm$ 0.6B	1.94 $\pm$ 0.5B	2.06 $\pm$ 0.6B
<i>L.</i> <i>monocytogenes</i>	SSM	0 $\pm$ 0A	0.33 $\pm$ 0.5A	0.17 $\pm$ 0.4A	0.17 $\pm$ 0.4A	0.17 $\pm$ 0.4A	0.17 $\pm$ 0.4A	0.58 $\pm$ 0.9A
	DEUF-C	2.25 $\pm$ 0.7B	1.12 $\pm$ 1.1B	1.22 $\pm$ 1.1B	1.56 $\pm$ 0.7B	1.44 $\pm$ 1.0B	1.13 $\pm$ 1.1B	1.25 $\pm$ 1.4B

<sup>a</sup> Means with different capital letters are significantly different ( $P \leq 0.05$ ).

## 2.5 DISCUSSION

This study had two main objectives. The first objective compared the DEUF concentration sampling method to the standard FDA BAM sampling method for detection of *E. coli* O157:H7 and *L. monocytogenes* in flume water during production of fresh-cut iceberg lettuce in a pilot-scale processing facility. The second objective evaluated the potential reductions in time to detection for enriched samples containing very low levels of *E. coli* O157:H7 and *L. monocytogenes* when collected using DEUF concentration.

DEUF has been used to concentrate microorganisms from a wide range of water types including tap, river, ground, beach, and cooling tower water using both a manual (Hunter et al., 2011; Leskinen et al., 2010; Leskinen et al., 2009) and automated system (Kearns et al., 2008; Leskinen et al., 2012). All of these studies demonstrated the ability of DEUF to concentrate target organisms and reduce sample variability compared to standard protocols that rely on far smaller. Reduced sampling variability was also demonstrated through benchtop experiments with the DEUF concentrated more representative than the unconcentrated grab samples (Magaña et al., 2013; Magaña et al., 2014). In several studies, DEUF concentration allowed pathogen detection when the standard sampling method failed (Kearns et al., 2008; Leskinen et al., 2012; Magaña et al., 2013). Even though DEUF concentration has been used for many different types of water samples, few studies have evaluated DEUF for enhanced detection of pathogens in fresh-cut produce wash water (Hunter et al., 2011; Kim et al., 2009; Magaña et al., 2014; Magaña et al., 2013; McEgan et al., 2009).

In this study, one lettuce head spiked with very low levels of both *E. coli* O157:H7 and *L. monocytogenes* was shredded along with uninoculated lettuce heads which gave lowest detectable limits of 1 and 5 CFU/125 mL for *E. coli* and *L. monocytogenes*, respectively, in

lettuce wash water using the FDA BAM method. Multi-strain cocktails of each pathogen were selected to alleviate concerns regarding variability in genotypic/phenotypic stress tolerance and host adaptation. These avirulent strains were previously shown to possess bacterial attachment characteristics similar to those seen in a set of virulent strains linked to other fresh-cut produce outbreaks (Buchholz et al., 2012).

When a sanitizer was not used, both *E. coli* O157:H7 and *L. monocytogenes* were more likely to be detected in DEUF-concentrated compared to the grab samples as in previous studies (Kearns et al., 2008; Leskinen et al., 2012; Magaña et al., 2013). However, commercial lettuce wash water invariably contains both a sanitizer and lettuce exudates.

As organic load increased in trials without sanitizer, probability of the standard sampling method detecting the target pathogen increased which decreased the advantages of DEUF. In trials with a 5% organic load more closely mimicking commercial lettuce wash water relatively little difference in probability of detection was seen between either *E. coli* or *Listeria* DEUF and grab samples, both of which resulted in nearly 100% detection. One possibility for greater detection at the higher organic load is the increase in chlorine demand. Deterioration of water quality due to accumulation of soil, debris, and plant exudates (organic load in this study) decreases sanitizer efficacy (Yaguang et al., 2012) as shown in several other studies (Gonzalez et al., 2004; Suslow, 2000; Zhang et al., 2009). As more produce is washed, organic matter accumulates and binds with the chlorine, creating a situation where chlorine demand may exceed the available chlorine concentration (Yaguang et al., 2012). This rapid depletion of free chlorine allows microorganisms including bacterial pathogens to survive and spread through the water (Yaguang et al., 2012). Davidson et al. (2014) reported that chlorine efficacy decreased markedly

with increased organic load in flume water, resulting in increased persistence of *E. coli* throughout processing (2014).

In this pilot-scale study, free chlorine levels likely decreased during processing. Since this pilot-scale fresh-cut processing line did not have a continuous monitoring system, the changes in free chlorine could not be continually tracked during processing, which again would account for the higher probability of detecting both pathogens using DEUF and standard sampling methods for trials with higher organic load for both.

However, when the sanitizer was working efficiently and not inhibited by excess organic matter, as in the trials with a 0% organic load, the probability of detection increased for the DEUF -concentrated as compared to standard grab samples. Thus, DEUF yielded a more-representative sample with a greater probability of detection compared to the much smaller standard grab samples.

When evaluating the time needed to filter relatively large volumes (40 L) of lettuce wash water, DEUF was faster than other ultrafiltration methods used in previous studies. Hill et al. (2005) reported filtration times of 12-14 minutes for 10 liters aliquots of dechlorinated tap water or presumably 48-56 min for 40 liters. However, when compared to Hill's study, only half the amount of time was needed to filter our lettuce wash water using DEUF. Thus, it is possible to concentrate 40 liters of produce wash water containing a 5% organic load in the same amount of time that it took Hill et al. to concentrate 10 L of clean tap water. These findings indicate that DEUF using the PMACS designed for this study was not only able to concentrate low levels of *E. coli* and *L. monocytogenes* but also filter water samples more efficiently than ultra-filtration methods used by Hill et al. (2005).

*E. coli* O157:H7 and *L. monocytogenes* are considered hazardous to public health and are not allowed in food at any detectable level. Thus a “zero tolerance” has been established whereby the target organism must be absent by enrichment (NACMCF, 2010). These strict standards have led to time-consuming enrichment methods with 3 to 4 days required to obtain a negative result (NACMCF, 2010). These methods remain impractical for fresh-cut produce companies with quicker methods now a high priority. DEUF concentration yields a more representative sample with a higher probability of detection when normal grab sampling methods fail. Consequently, efforts were taken to reduce the enrichment time to detect pathogens in DEUF-concentrated samples.

As expected, both pathogens grew during enrichment, with significantly greater populations seen in DEUF as compared to standard grab methods. Rapid detection systems, such as antibody- and PCR-based kits, are available commercially; however, these rapid detection methods still require sample enrichment before analysis (D’Lima and Suslow, 2009; NACMCF, 2010). PCR methods have been consistently able to identify presence of pathogens in the shortest period of time and with fewer false-negatives compared to other rapid pathogen detection systems (D’Lima and Suslow, 2009). Using PCR results in a shorter detection time without compromising assay validity, which is an important consideration for analysis of produce wash water samples (Kearns et al., 2008).

This study indicates the potential for combining DEUF and qPCR to decrease both the enrichment time and the test method time to achieve positive qPCR results. However, further optimization is critical to determine at which time point target organisms have exceeded the limit of detection for the rapid detection method chosen.

One question raised in this study revolved around discrepancies between the culture and PCR results for both *E. coli* O157:H7 and *L. monocytogenes* in trials with and without sanitizer. In previous validity studies, results from the MicroSEQ *E. coli* O157:H7 and MicroSEQ *L. monocytogenes* Detection Kits were statistically similar to culture reference methods (Tebbs et al., 2011; Wong et al., 2012). In our study, similar agreement was generally observed using sanitizer-free lettuce wash water; however, trials in which sanitizer was added to the wash water resulted in discrepancies between the culture and PCR results.

One hypothesis as to why some culture and PCR results differed in the sanitizer trials relates to the inability of real-time PCR to distinguish between DNA from dead and live cells (Elizaquivel et al., 2012). Given the widespread commercial use of chlorine-based sanitizers to minimize cross-contamination during washing of produce, some of the pathogens introduced into the flume tank were rendered non-viable, concentrated through DEUF, prepared for real-time qPCR and thus interfered with determining the actual number of viable cells. This hypothesis could help explain those instances where PCR had a higher probability of detection than the culture method.

Even though the real-time qPCR MicroSEQ Detection Kits are approved for detection of both *E. coli* O157:H7 and *L. monocytogenes* in leafy greens, their approval only applies for inoculated produce, not wash water. In this study, samples consisted of highly concentrated lettuce wash water with a sanitizer that were promptly enriched after sample collection, frozen, and shipped to USF for qPCR analysis. These extra steps of freezing, shipping and unknown freezing/thawing cycles during transit could have resulted in cell lysis, DNA leakage, and thus lower pathogen populations available for detection through qPCR, resulting in fewer positive qPCR results compared to the culture methods. It is critical to further investigate how

differences between the validity studies and this study could have negatively impacted the ability of the MicroSEQ Detection Kits to detect the target pathogens.

In conclusion, DEUF has the ability to concentrate pathogens from lettuce wash water when standard grab sampling method cannot. As organic load increased in lettuce wash water, pathogen detectability increased for both the DEUF and standard grab methods, suggesting that additional debris and exudates in the water act as vehicles for cross-contamination of the product during flume washing. When sanitizer is introduced into the wash water, the pathogen was more likely to be recovered using DEUF; however, PCR and culture results showed some discrepancies which indicates the need for further optimization. Finally, this study indicates that DEUF concentration using the PMACS is not only able to concentrate lettuce wash water, but does so more rapidly than other concentration systems utilized in previous studies.

## **CHAPTER 3**

### **Rapid Concentration/Detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in Commercial Lettuce Wash Water Generated at Commercial Facilities**

### 3.1 ABSTRACT

A dead-end ultrafiltration concentration (DEUF-C) sampling method previously demonstrated increased detection of pathogens in lettuce wash water in pilot-scale testing. This study investigated the use of DEUF-C in commercial-scale tests through the following two objectives: 1) comparison of pathogen detection probabilities using DEUF-C versus standard grab sampling in lettuce wash water generated from a commercial test line, and 2) assessment of DEUF-C to concentrate pathogens in spiked commercially generated flume and centrifugation lettuce wash water. In objective 1, one lettuce head was spiked to contain  $2-4 \times 10^4$  CFU of *Escherichia coli* O157:H7 and  $3-9 \times 10^5$  CFU of *Listeria monocytogenes* and then processed along with 907 kg of lettuce using sanitizer-free tap water. Two to four 40-L volumes of lettuce wash water were processed by DEUF-C to generate 400-mL concentrated samples. Detection probabilities for the DEUF-C samples were compared to standard grab samples after 24 h of enrichment followed by qPCR. In objective 2, flume and centrifuge lettuce wash water was collected from a commercial plant, treated with sodium thiosulfate (100 mg/L) to neutralize the chlorine-based sanitizer, spiked with both *E. coli* O157:H7 and *L. monocytogenes* at  $10^2$  CFU/mL and then processed by DEUF-C to determine total filterable volume (TFV). Detection (qPCR) probabilities in non-enriched DEUF-C and grab samples were determined to compare target organism recoveries. Experiments were repeated in quadruplicate. In objective 1, *E. coli* O157:H7 and *L. monocytogenes* were detected in 100% of the DEUF-C samples (N=14) compared to 6.7% and 20%, respectively, in standard grab samples (N=15). In objective 2, TFV was higher for flume (N=4;  $30.6 \pm 9.9$  L) as compared to centrifugation water (N=4;  $9.9 \pm 2.0$  L) ( $P < 0.05$ ). *E. coli* O157:H7 and *L. monocytogenes* were detected in 100% of the DEUF-C samples compared to 11.1% and 16.7% respectively, in standard grab samples. Based on these findings,

DEUF-C offers improved probability of detection for *E. coli* and *Listeria*; however, TFV for commercial produce wash water needs to be further improved.

### **3.2 OBJECTIVE**

This chapter aimed to assess the DEUF-C sampling method to rapidly concentrate and detect *E. coli* O157:H7 and *L. monocytogenes* in lettuce wash water generated in commercial-scale leafy-green processing facilities and included two tasks - 1) compare pathogen detection probabilities using DEUF-C versus standard grab sampling from lettuce wash water generated from a commercial processing test line, and 2) assess the ability of DEUF-C to concentrate pathogens in spiked commercially generated flume and centrifugation lettuce wash water.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Experimental design

Dead-end ultrafiltration concentration (DEUF-C) was compared to standard FDA BAM grab sampling for detection of *E. coli* O157:H7 and *L. monocytogenes* in three different types of fresh-cut iceberg lettuce wash water - commercial wash water collected from the secondary flume tank (CWF), commercial wash water collected during centrifugal drying (CWC), and water generated after processing 907 kg of iceberg lettuce at a commercial-scale test facility. Commercial wash water was inoculated at  $10^2$  CFU/mL with *E. coli* O157:H7 and *L. monocytogenes* after neutralization and quantitatively examined for *E. coli* and *L. monocytogenes* using both DEUF-C and the standard FDA BAM method. Commercial test line water was generated by processing 907 kg of iceberg lettuce inoculated at  $\sim 1$ - $2$  CFU/125 mL ( $10^4$  CFU/head) with *E. coli* O157:H7 and  $\sim 1$ - $4$  CFU/25 mL ( $10^5$  CFU/head) with *L. monocytogenes*. Processing steps included shredding, conveying, double flume washing, and shaker table dewatering with water samples collected from the second flume tank and quantitatively examined for *E. coli* and *L. monocytogenes* using DEUF-C and standard FDA BAM method. Each commercial test line trial included a contamination check (negative control), a positive control, grab samples and concentrated samples. Each commercial wash trial consisted of a contamination check (negative control), positive control, grab samples, and concentrated samples for both flume water and centrifugation water.

#### 3.3.2 Lettuce

In commercial wash water experiments (CWF and CWC), iceberg lettuce (*Lactucasativa* L.) was obtained from local growers (Salinas, CA) and processed in a commercial processing facility (Taylor Farms, Salinas, CA). Wash water was collected after approximately 3 h of

lettuce processing (processing began about 7:30 am each morning) and transported to the facility with the commercial test line (SmartWash, Salinas, CA) for testing.

In the commercial test line experiments, approximately 907 kg of iceberg lettuce (*Lactucasativa* L.) was delivered to SmartWash 30 min before processing.

### **3.3.3 *E. coli* O157:H7 strains**

For both the commercial wash water and commercial test line experiments, a cocktail of four non-toxicogenic, GFP-labeled stx1<sup>-</sup>, stx2<sup>-</sup> strains of *Escherichia coli* O157:H7 were obtained from Dr. Michael Doyle at the Center of Food Safety, University of Georgia, Griffin, GA, in January 2008. The *E. coli* O157:H7 cocktail included ATCC 43888 (human feces isolate), CV2b7 (environmental isolate), 6980-2 (environmental isolate), and 6982-2 (environmental isolate). In previous bacterial attachment studies, these avirulent *E. coli* O157:H7 strains were shown to behave similarly to virulent strains linked to outbreaks involving similar produce commodities. All four *E. coli* O157:H7 strains were stored at -80°C in tryptic soy broth (Difco, BD, Sparks, MD) containing 0.6% (w/v) yeast extract (TSBYE, Difco, BD), and 10% (v/v) glycerol (Sigma Chemical Co., St. Louis, MO). Working cultures were prepared by first streaking the stock cultures onto TSBYE slants which were incubated at 35°C for 24 h and then stored overnight at 4°C before over-night shipment to Salinas, CA. In Salinas, the cultures were subjected to two successive transfers in 9 mL of TSBYE and incubated at 35°C for 24 h before use.

### **3.3.4 *L. monocytogenes* strains**

For both the commercial wash water and commercial test line experiments, a cocktail of three avirulent strains of *Listeria monocytogenes* obtained from Dr. Sophia Kathariou at North Carolina State University, Raleigh, NC were utilized. The cocktail included J22F and J29H

(derivatives of H7550-Cd<sup>s</sup>) and M3 (avirulent derivative of 1/2a3). All three *L. monocytogenes* strains were stored at -80°C in TSBYE until needed. Working cultures were prepared by first streaking stock cultures onto TSBYE slants which were incubated at 35°C for 24 h and then stored overnight at 4°C before over-night shipment to Salinas, CA. In Salinas, the cultures were subjected to two successive transfers in 9 mL of TSBYE and incubated at 35°C for 24 h before use.

### **3.3.5 Flume water preparation**

For the experiments where water was collected from both the secondary flume tank (CWF) and the centrifugal dryer (CWC), lettuce processing began at the commercial leafy green processing facility (Taylor Farms, Salinas, CA) around 7 am. After 3 h of continuous processing, approximately 100 L of both CWF and CWC was collected in plastic carboys and transported within 5 min to commercial test line facility (SmartWash, Salinas, CA) for testing. In the commercial test line experiments, the flume tanks and wash system at the commercial test line facility were filled with sanitizer-free tap water.

### **3.3.6 Inoculation methods**

Commercial wash water collected from Taylor Farms was first neutralized with sodium thiosulfate (Fisher Scientific, Waltham, MA) and confirmed to have 0 mg/L free chlorine and 0 mg/L total chlorine using a Pocket Colorimeter II, Chlorine (Hach, Loveland, Colorado). Thereafter 100 L aliquots of both CWF and CWC were spiked to contain 10<sup>2</sup> CFU/mL of each *E. coli* O157:H7 and *L. monocytogenes* cocktail. Both commercial water samples were then stirred with a sterile plastic paddle to evenly distribute the inoculum. In the commercial test line trials, two heads of iceberg lettuce were inoculated, one with *E. coli* O157:H7 at approximately 1-2 CFU/125 mL (10<sup>4</sup> CFU/head) of cocktail, and the other with *L. monocytogenes* at approximately

1-4 CFU/25 mL ( $10^5$  CFU/head) and held for 30 min before processing. In addition, the *E. coli* O157:H7 and *L. monocytogenes* cocktail suspensions were appropriately diluted and plated on SMAC-CT and MOX, respectively, to determine the inoculation levels at the time of processing.

### **3.3.7 Commercial lettuce processing test line**

Processing line trials were conducted at the SmartWash Solutions Lettuce Processing Test Facility in Salinas, CA with the aid of James Brennan and his co-workers (Figure 3.1). The 10,000 ft<sup>2</sup> facility included a lettuce shredder (TRS 2500 Urschel TranSlicer, Valparasio, IN), FTNON double flume tank wash, several conveyors and a 50-lb (22.7 kg) capacity centrifugal Spin Dryer (model SD50-LT) in addition to a produce cooling tube and a water treatment plant. Water samples were collected from the second flume wash tank for DEUF-C, standard sampling, and additional physiochemical analyses. Approximately 453.5 kg of iceberg lettuce was processed, followed by single head inoculated with *E. coli* and *L. monocytogenes* and 453.5 kg of uninoculated iceberg lettuce, totaling 907 kg of iceberg lettuce processed in each of the four trials.

### **3.3.8 Sample collection**

After lettuce shredding and washing, 40 L batches of flume water were pre-sieved and pre-filtered into a sterile container with an appropriate amount of sodium thiosulfate (Fisher Scientific, Waltham, MA) added to neutralize the chlorine sanitizer. The pre-sieve assembly contained a PVC sieve and 125 µm polypropylene mesh attached to an inlet tube which connected to sump containing a 5 µm pre-filter. The sump also had an outlet tube connected to allow the release of pre-sieved and pre-filtered lettuce wash water. Two 400 mL grab samples collected from the second flume tank and neutralized represented the standard collection procedures. Additional 300 mL water samples were collected for physiochemical analysis. Pre-

sieved and pre-filtered flume water was then concentrated using the DEUF protocol. Commercial wash centrifugation and flume water arriving at the facility was chlorine-inactivated, pre-sieved, pre-filtered, and concentrated using the PMACS DEUF protocol (Figure 3.2). The PMACS was prepared for each wash water trial by installing a new Optiflux F200NR polysulfone ultrafilter (2.0 m<sup>2</sup> surface area, ~30 kDA pore size; Fresenius Medical Care North American, Waltham, MA). The PMACS automated collection cycle filtered water, followed by PMACS automated recovery cycle initiation to recover material collected on the filter (0.01% sodium polyphosphate in 0.1 M sodium phosphate buffer backflushed filter to generate retentate (DEUF-C sample). All samples were analyzed for *E. coli* O157:H7 and *L. monocytogenes* using the FDA BAM enrichment method followed by cultural isolation and qPCR protocols.

### **3.3.9 *E. coli* O157:H7 analysis**

All samples from both trial types were enriched for *E. coli* O157:H7 and *L. monocytogenes* according to the FDA BAM followed by cultural isolation and qPCR analysis as previously described in Chapter 2.

### **3.3.10 Physiochemical analysis of wash water**

Commercial centrifuge and flume water samples, along with samples of commercial test line flume water before and after processing were collected for physiochemical analyses which included: pH, oxidation/reduction potential (ORP), total solids (TS), turbidity, maximum filterable volume (MFV) and free chlorine. These analyses were conducted for all water types, except for ORP, which was conducted only for commercial flume and centrifugation water and free chlorine which was only conducted for commercial test line flume water before and after processing. Measurements of pH and oxidation/reduction potential were done using YSI Professional Plus (YSI, Yellow Springs, OH). Total solids was determined by drying 10 mL of

wash water in a pre-heated/pre-weighed crucible in an oven (Model 625-A, Precision Scientific Inc, Chicago, IL) at  $103^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for 2 h. Turbidity was measured by pouring a 50 mL sample through a 24 cm-diameter Grade 113 Whatman Filter (Piscataway, NJ) to remove suspended solids and then measuring absorbance at 663 nm in a spectrophotometer. MFV was quantified as the volume of a 50 mL water sample pulled through a  $0.45\ \mu\text{m}$  membrane in 1 min with a -80 kPa vacuum drawn using a Millipore vacuum pump (Model WP6211560, Millipore, Billerica, MA). Turbidity, MFV, pH, ORP, and free chlorine were analyzed immediately after processing in the test facility, whereas samples for total solids were stored at  $\sim 4^{\circ}\text{C}$  and shipped to Michigan State University for analysis.

### **3.3.11 qPCR analysis**

Aliquots taken from each *E. coli* O157:H7 and *L. monocytogenes* enrichment were screened for presence/absence of the target pathogen using the same AOAC-approved confirmatory qPCR assays detailed in Chapter 2.

### **3.3.12 Statistical analysis**

All *E. coli* O157:H7, *L. monocytogenes*, and physiochemical analyses were based on results from 4 experiments. All *E. coli* and *Listeria* counts were recorded as CFU/mL with the positive/negative enrichment results used to calculate the percentage of positive samples. The physiochemical results were recorded in the appropriate units for each test, averaged, and subjected to an analysis of variance using JMP 13 (SAS Institute Inc., Cary, NC). The physiochemical analysis results were also subjected to the Tukey-Kramer HSD test with *P* values of  $\leq 0.05$  considered significantly different. The TFV results were averaged, subjected to both ANOVA using JMP 13, and the Tukey-Kramer HSD test with *P* values of  $\leq 0.05$  considered significantly different.

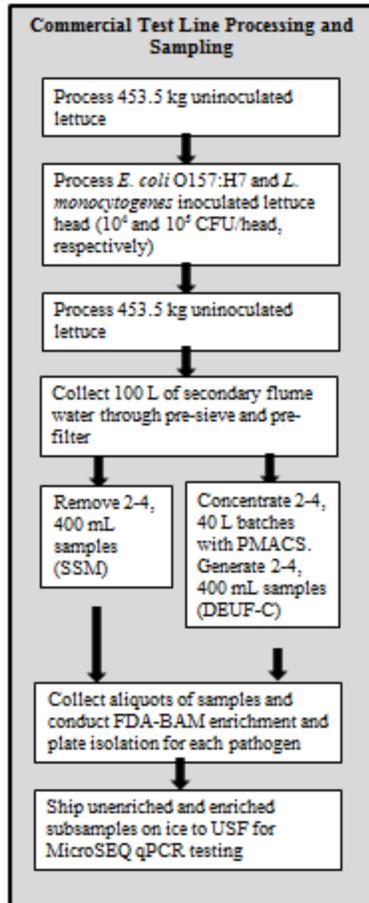


Figure 3.1: Commercial test line processing and sampling

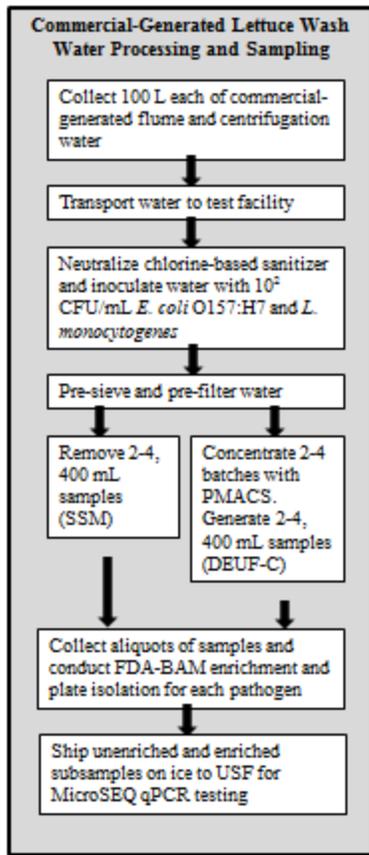


Figure 3.2: Commercial facility generated lettuce wash water processing and sampling

## 3.4 RESULTS

### 3.4.1 Probability of detecting *E. coli* O157:H7 and *L. monocytogenes* in lettuce wash water from a commercial-scale pilot plant facility

A detection probability of 100% was achieved for both *E. coli* O157:H7 and *L. monocytogenes* in DEUF concentrated samples (N=14) using lettuce wash water spiked to contain  $2-4 \times 10^4$  CFU of *Escherichia coli* O157:H7 and  $3-9 \times 10^5$  CFU of *L. monocytogenes*. However, for samples collected using the standard grab method, detection of *E. coli* and *Listeria* decreased to 6.7 and 20%, respectively (N=15) (Figure 3.3).

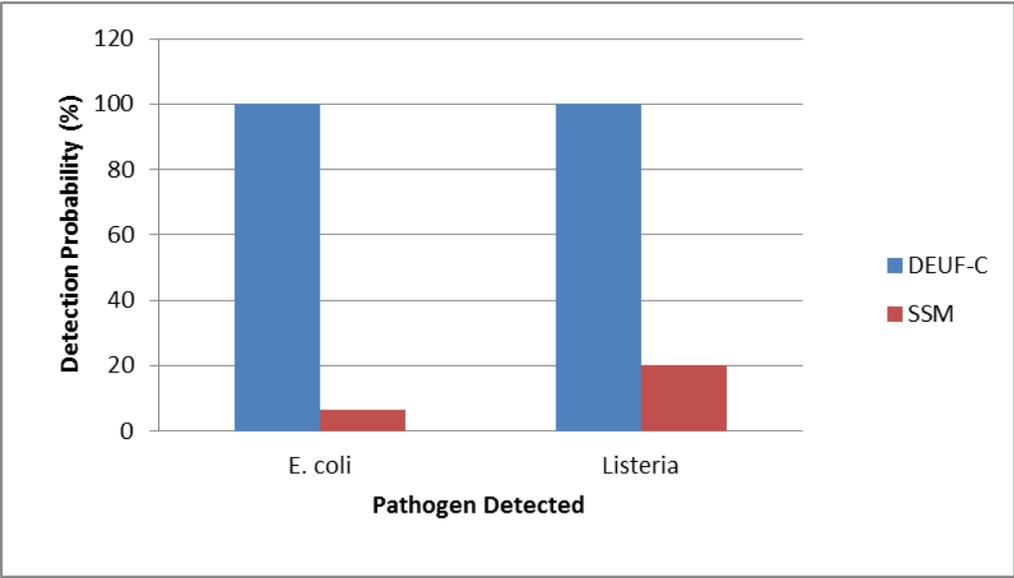


Figure 3.3: Probability of detecting *E. coli* O157:H7 and *L. monocytogenes* in DEUF and standard lettuce wash water samples from the commercial-scale test facility

### **3.4.2 Probability of detecting *E. coli* O157:H7 and *L. monocytogenes* in inoculated lettuce centrifugation and flume water from a commercial facility**

Similar to wash water generated at the test facility, 100% detection was achieved for both *E. coli* O157:H7 and *Listeria* in spiked lettuce wash water from using DEUF (N=16). Grab sampling decreased detection of *E. coli* and *Listeria* to 11.1 and 16.7%, respectively (N=18) (Figure 3.4).

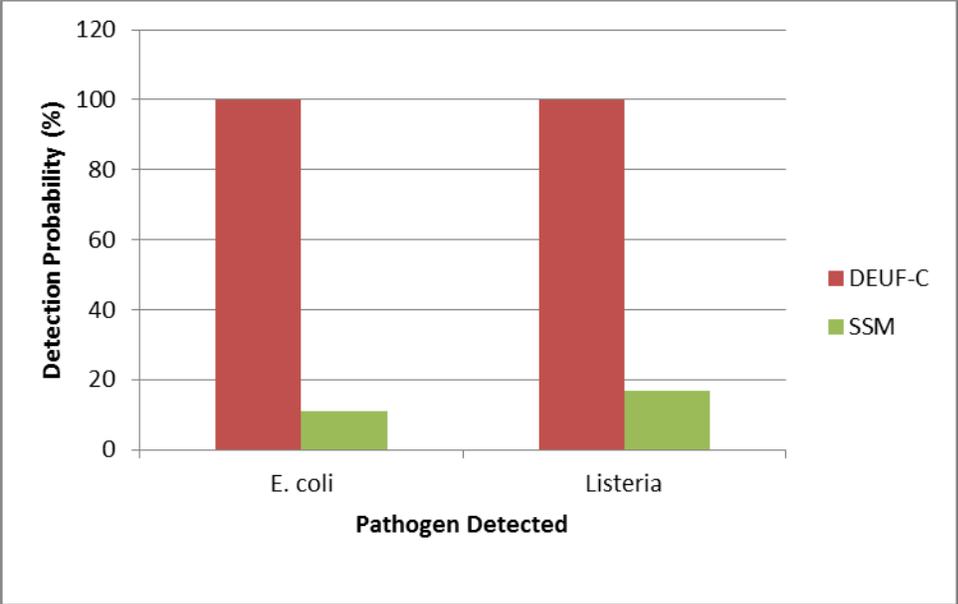


Figure 3.4: Probability of detecting *E. coli* O157:H7 and *L. monocytogenes* in spiked DEUF and standard lettuce wash water from a commercial facility

### 3.4.3 DEUF filtration efficiency

For all types of wash water generated, it was anticipated that 40 L of wash water would be filtered to generate DEUF concentrated samples. For wash water generated at the commercial-scale test facility,  $40.0 \pm 0$  L could be filtered in  $17.0 \pm 1.9$  min (N=14) (Table 3.1). However, water from the commercial facility proved more difficult to concentrate using DEUF with maximum filterable volumes of  $30.6 \pm 9.9$  L ( $39 \pm 16.6$  minutes (N=4)) and  $9.9 \pm 2.0$  L ( $13.4 \pm 2.2$  minutes (N=4)) for the flume and centrifugation water, respectively (Table 3.1).

Table 3.1: Maximum filterable volumes and filtration times for commercial lettuce wash water<sup>a</sup>

Mean $\pm$ SD amount and time taken to filter various commercial wash waters		
Water Type	Amount Filtered (L)	Time for Filtering (min)
Commercial Test Facility Water	40.0 $\pm$ 0.0 A	17.0 $\pm$ 1.9 B
Commercial Flume	30.6 $\pm$ 9.9 B	39 $\pm$ 16.6 A
Commercial Centrifuge	9.9 $\pm$ 2.0 C	13.4 $\pm$ 2.2 B

<sup>a</sup>Means with different capital letters are significantly different ( $P \leq 0.05$ ).

#### 3.4.4 Physiochemical analysis of lettuce wash water

Six different physiochemical analyses were conducted on four different types of commercial wash water: commercial test facility water before processing (TBP), commercial test facility wash water after processing (TAP), commercial flume wash water (CFW) and commercial centrifuge wash water (CCW); except for ORP which was only determined for CCW and CWF and free chlorine which was only determined for TBP and TAP. Total solids ranged from  $46.4 \pm 3.5$  to  $50.0 \pm 6.5\%$  which these values are not significantly different ( $P > 0.05$ ). However, commercial facility-generated water was significantly more turbid ( $0.09 \pm 0.03$  and  $0.12 \pm 0.02$  absorbance at 663 nm for CFW and CCW, respectively) compared to test facility-generated water ( $0.0 \pm 0.0$  and  $0.004 \pm 0.0$  for TBP and TAP, respectively). Differences in MFV were also observed between commercial and test facility-generated water with latter far more difficult to filter ( $P < 0.05$ ). Similarly, the test facility-generated water had a significantly higher pH ( $8.2 \pm 0.1$  and  $8.2 \pm 0.1$  for TBP and TAP, respectively) compared to the commercial facility-generated water ( $6.6 \pm 0.2$  and  $6.7 \pm 0.3$  for CFW and CCW, respectively). The ORP values for commercial facility-generated water were statistically similar as were the free chlorine levels for the test facility-generated water ( $P > 0.05$ ) (Table 3.2).

Table 3.2: Physiochemical analyses for test facility water before processing (TBP), test facility wash water after processing (TAP), commercial flume wash water (CFW) and commercial centrifuge wash water (CCW)<sup>a</sup>

Mean $\pm$ SD of various physiochemical analyses on various commercial wash waters						
Water Type	Total Solids (%)	Turbidity (abs @ 663 nm)	MFV (mL)	pH	ORP (mV)	Free Chlorine (mg/L)
TBP	46.4 $\pm$ 3.5 A	0.0 $\pm$ 0.0 B	50.0 $\pm$ 0.0 A	8.2 $\pm$ 0.1 A	N/A	0.5 $\pm$ 0.6 A
TAP	47.4 $\pm$ 2.1 A	0.004 $\pm$ 0.0 B	50.0 $\pm$ 0.0 A	8.2 $\pm$ 0.1 A	N/A	0.04 $\pm$ 0.03 A
CFW	50.0 $\pm$ 6.5 A	0.09 $\pm$ 0.03 A	3.3 $\pm$ 3.9 B	6.6 $\pm$ 0.2 B	218.5 $\pm$ 143.8 A	N/A
CCW	48.8 $\pm$ 1.4 A	0.12 $\pm$ 0.02 A	4.0 $\pm$ 5.2 B	6.7 $\pm$ 0.3 B	127.3 $\pm$ 150.1 A	N/A

<sup>a</sup>Means with different capital letters are significantly different ( $P \leq 0.05$ ).

### 3.5 DISCUSSION

In this study, lettuce wash water samples were spiked with both *E. coli* O157:H7 and *L. monocytogenes*, filtered and then analyzed for the presence/absence of both pathogens. Given several previous pilot-scale studies demonstrating the success of incorporating DEUF into pilot-scale processing (Hunter et al., 2011; Kearns et al., 2008; Leskinen et al., 2012; Magaña et al., 2013; Magaña et al., 2014), it was anticipated that this technology could prove useful in commercial settings. Percent detection for the DEUF concentrated samples was 100% for both pathogens in both flume and centrifugation water produced at commercial-processing facility. This study proves the ability of DEUF to concentrate pathogens from commercial-scale generated wash water for improved detection over the FDA BAM method. Thus, DEUF concentration yields a more representative sample and the potential to reduce false negatives.

Current FDA BAM methods rely on enrichment for determining the presence/absence of *E. coli* and *L. monocytogenes* in produce wash water (FDA, 2016a; FDA, 2016b) with these enrichments taking several days to complete. However, when DEUF is coupled with qPCR, results can be obtained the same day and in as little as 8 h with only a short enrichment needed. Therefore, DEUF can greatly reduce the time necessary to detect target pathogens in commercial wash water. This conclusion is also supported by previous studies indicating that shorter enrichment times are possible for DEUF concentrated samples (Magaña et al., 2013; 2014).

DEUF achieved a filtration rate of  $17.0 \pm 1.9$  min for 40-L of dechlorinated tap water which was about 3 and 10 to 15 times faster than previously reported Hill et al., (2005) and Juliano and Sobsey (1997). Thus, DEUF concentration is not only faster than methods previously reported, but can create more representative samples and detect pathogen contamination when other sampling methods failed.

When wash water generated from a commercial leafy green processing facility was de-chlorinated and spiked the DEUF filtration rate decreased from the original 40 L to  $30.6 \pm 9.9$  and  $9.9 \pm 2.0$  L for flume and centrifuge wash water, respectively. This decreased flow rate suggests filter fouling, most likely from the accumulation of organic material, with the polysaccharides from the lettuce walls likely being partially responsible (Kearns et al., 2008; Magaña et al., 2014).

Polysaccharide fouling of ultrafiltration membranes was first observed for river water. A study conducted by Zularisam et al. (2007) found that river water containing polysaccharides and protein-rich organic matter fouled polysulfone ultrafiltration membranes to a greater extent compared to river water containing an organic load high in humic material. More recent ultrafiltration membrane studies also reported greater filter fouling and flux reductions for solutions containing polysaccharides, either alone or in combination with humic compounds (Katsoufidou et al., 2010).

Even though filtration volumes decreased during filtration of commercial-generated lettuce wash water, DEUF was still able to concentrate the target pathogens from difficult-to-filter commercial water to detectable levels when the standard sampling method failed. However, the filtration of 40 L volumes may not be necessary to detect target pathogens using DEUF-C coupled with qPCR. DEUF-C filtration of only 10-L of centrifugation water yielded 100% detection of both pathogens. In a study by Buchholz et al. (2012), after 1.5 min of washing, significantly higher numbers of *E. coli* O157:H7 ( $P < 0.05$ ) were seen in the spent centrifugation water as opposed to flume water indicating that centrifugation water is most appropriate for pathogen testing. Given the greater likelihood for pathogen detection in centrifugation water, DEUF concentration of volumes less than 40 L may well be sufficient, with

future studies needed to optimize the volume of the water that must be filtered for pathogen detection.

No two commercial processing facilities are exactly alike in terms of product processed, system set ups or time between batches. The widely varying processing conditions between laboratory, pilot-scale and commercial studies (Sapers, 2001; Beuchat et al., 2004) will impact the DEUF filtration rate for lettuce wash water as demonstrated in our work, which was one of the first commercial applications of DEUF-C in leafy-green processing. Consequently, if DEUF is to be used commercially for pathogen screening, each processor will need to monitor their wash water for organic load and other physical characteristics to maintain the desired DEUF filtration rate and reduce filter fouling (Wilhelmsen, 2013).

Incorporating DEUF technology into a commercial testing program will force processors to carefully monitor organic load and sanitizer level with most commercial wash water containing 50 to 200 ppm free chlorine (Haute et al., 2013; Parish et al., 2003; Schmidt, 2009). Despite the 1 to 2 min of contact time between leafy greens and the water during flume washing (Tirpanalan et al., 2011; Parish et al., 2003) continued processing leads to build-up of lettuce exudates in the water that will decrease the filtration rate and potentially lead to filter fouling (Allende et al., 2008).

Wash water generated at the commercial processing facility was visually more turbid compared to that generated at the commercial-scale test facility. Turbidity – a parameter associated with increased organic load in a previous study (Davidson et al., 2014), was also significantly higher in commercial generated wash water. Significantly lower ( $P < 0.05$ ) MFV and pH values were observed in commercial generated wash water as in the study by Davidson et

al. (2014). Therefore, commercial processors are encouraged to minimize the build-up of organic load in flume water during processing.

Commercial lettuce processing facilities rapidly generate high organic loads in flume water during processing which leads to fouling of the DEUF membrane, limiting the potential of this technique (Shi et al., 2014). However, enzymatic pre-treatment or chemical modification of the wash water may be useful in minimizing membrane fouling during filtration (Shi et al., 2014). Additional options could include chemical pre-treatments to break down polysaccharides in the water or the use of several pre-sieves in succession to physically remove organic matter from the wash water before DUEF.

In summary, DEUF concentration improved the probability of detection for both *E. coli* O157:H7 and *L. monocytogenes* in lettuce wash water generated from a processing test line and commercial-generated wash water. However, when filtering commercial-generated flume and centrifugation water, membrane fouling became an issue as evidenced from decreased filtration rates, indicating that further improvements are needed before this technology can be fully integrated into commercial facilities. This study also demonstrated the potentials of DEUF-C to detect target pathogens in smaller volumes of centrifugation water, indicating future optimization studies are necessary to determine the lowest filtration volumes necessary to detect the targeted pathogens.

## CONCLUSIONS

The ability to detect pathogens in fresh produce remains an ongoing food safety issue. Contamination of fresh produce can occur at any point from farm to fork, and with no kill-step during production of fresh-cut produce, even a small amount of contamination can lead to foodborne illness. Given the large amounts of product being processed in industry, flume water must be recirculated to reduce waste and operational costs. In an attempt to maintain water quality and minimize pathogen presence during washing, chemical sanitizers are added to commercial flume water. However, the efficacy of commonly used chlorine-based sanitizers is decreased by organic material that accumulates in the water during processing. Thus, it is extremely important that wash water be monitored for both sanitizer efficacy and presence/absence of pathogens. Current industry sampling plans are based on small grab samples collected from large recirculating tanks that may not adequately represent the microbial population present. Consequently, various filtration systems such as DEUF have been examined.

In the first objective using a pilot-scale processing line, DEUF concentration (DEUF-C) and standard grab sampling were compared for recovery of *E. coli* O157:H7 and *L. monocytogenes* from lettuce wash water along with the time needed for enrichment. Enhanced pathogen detection was clearly demonstrated using DEUF-C with 33% of the *E. coli* and *Listeria* samples positive by DEUF qPCR and/or enrichment method, but negative by the conventional FDA BAM sample qPCR and/or enrichment method. Therefore, 33% of the time current industry methods would have resulted in a false negative and processing would have continued as normal without the knowledge that the product being processed was contaminated. DEUF-C also demonstrated the ability to potentially reduce the enrichment time prior to presence/absence testing. Significantly higher pathogen populations were seen in the DEUF-C samples after 7 h of enrichment compared to the standard sampling method ( $P < 0.05$ ). These results suggest that the

DEUF method yields a more representative sample with enhanced detection of *E. coli* and *Listeria* in leafy green processing water.

Other pilot-scale studies have evaluated the efficacy of DEUF-C for leafy green processing; however, limited knowledge is available regarding the effectiveness of DEUF-C at the commercial level. The second objective compared DEUF-C to standard grab sampling for pathogen detection in lettuce wash water generated from a commercial processing test line and also assessed the ability of DEUF-C to concentrate pathogens in spiked commercially generated flume and centrifugation lettuce wash water. *E. coli* and *Listeria* were detected in 100% of the DEUF-C samples (N=14) compared to 6.7% and 20%, respectively, of the standard grab samples (N=15). However, the DEUF-C filtration volumes were significantly lower for commercially-generated spiked flume and centrifugation water ( $P < 0.05$ ) compared to water from the commercial-scale test line and earlier pilot-scale studies. Total filterable volumes in the pilot-scale study were  $40.0 \pm 0.0$  L compared to  $30.6 \pm 9.9$  and  $9.9 \pm 2.0$  L for commercially-generated flume and centrifugation water, respectively. However, 100% detection of *E. coli* and *Listeria* was achieved for the DEUF-C samples compared to only 11.1% and 16.7% respectively, for standard grab samples. Thus, even though DEUF-C technology appears promising for commercial-scale facilities, additional studies are needed to optimize the filtration rate for maximum pathogen recovery and determine smallest filtration volume necessary for pathogen detection.

Overall, both of these studies have successfully shown the ability of DEUF to concentrate pathogens in lettuce wash water with detection at low levels possible when standard grab sampling methods fail. Thus, DEUF-C holds the potential to not only generate more representative samples for quality control, but to also potentially reduce the number of false

negatives being recorded in industry, detect produce contamination earlier, and reduce the number of foodborne illness outbreaks occurring from fresh-cut fruits and vegetables.

## **FUTURE RESEARCH RECOMMENDATIONS**

Even though these studies indicated the potential for DEUF to concentrate microorganisms in lettuce wash water with increased probability of pathogen detection at low levels, the filtration rates for commercially generated wash water were significantly lower than those previously observed during pilot-scale processing. The slow filtration rate may become an issue upon commercial implementation of DEUF-C. DEUF-C is designed to concentrate large volumes of water samples into smaller, more representative samples, but if filterable volume is decreased, then the samples generated are not as representative of the whole flume tank as they could be, and could ultimately still conclude false negative results. However, even with decreased filtration volumes, DEUF-C was still able to detect target pathogens when the standard sampling method fails. Additional studies are needed to determine what optimum filtration volume is necessary to detect target pathogens.

Additional studies need to be conducted in commercial-scale settings and with water generated at commercial-scale facilities. One suggestion would be to collect flume and centrifugation water from various commercial-scale leafy green processing facilities and determine which waters can be filtered and which ones cannot to get a better understanding of the quality of water needed for DEUF-C filtration. Physiochemical analyses should also be conducted on all commercial generated water collected.

Another suggestion would be an additional pre-treatment step to treat commercial wash water before filtration that could break down the organic load or otherwise create a quality of water that alleviates membrane fouling. This pre-treatment could include a chemical added to the wash water before filtration or a different type of pre-filter than what was used in this study to further decrease the organic load without significantly decreasing pathogen populations.

## **APPENDIX**

**Thermal Inactivation and Growth of *Listeria monocytogenes* during Production and Storage of Caramel Apples**

## AI.1 ABSTRACT

During the fall of 2014, commercially produced pre-packaged caramel apples were linked to 35 cases of listeriosis in 12 states. In response, this study aimed to assess 1) the reduction of different outbreak and non-outbreak strains of *Listeria monocytogenes* during caramel dipping of apples, and 2) subsequent growth of the apple outbreak strains within caramel apples during storage at 22 and 4°C. In aim 1, three unwaxed Jonathan apples were dip-inoculated with three different 4-strain *L. monocytogenes* cocktails (apple outbreak, unrelated outbreak or unrelated environmental) at ~8 log CFU/apple, dried for 1 h, dipped for 5 sec in caramel at 82, 88, 93 or 99°C, cooled for 1 h at room temperature and assessed for survivors. In aim 2, Jonathan apples were spot-inoculated with the apple outbreak cocktail (~3 log CFU/apple) at the stem juncture, dried for 1 h, pushed onto wooden sticks, and dipped in caramel at 82°C. During storage at 4 and 22°C for 28 and 14 days, respectively, four different apple sections (top, middle, bottom and core) were cut from three apples, homogenized and plated for *Listeria*. After dipping apples in caramel at 82 and 99°C, the apple outbreak, unrelated outbreak and environmental *Listeria* strains decreased  $2.0 \pm 0.6$  and  $2.7 \pm 0.1$ ,  $1.8 \pm 0.3$  and  $2.6 + \underline{0.1}$ , and  $1.7 \pm 0.1$  and  $2.9 \pm 0.2$  logs, respectively, with the environmental cocktail significantly less heat resistant ( $P < 0.05$ ) at 99°C compared to the other two cocktails. After 14 days of storage at 22°C, *Listeria* populations were significantly higher ( $P < 0.05$ ) in the core ( $7.4 \pm 0.6$  log CFU/g) compared to the other three sections (4.9 – 5.4 log CFU/g). The same trend was seen for the core ( $7.7 \pm 0.6$  log CFU/g) and the other three sections (5.0 – 5.4 log CFU/g) after 28 days of storage at 4°C. Since dipping in hot caramel cannot ensure pathogen elimination, producers of caramel apples should implement good agricultural practices, post-harvest preventive controls and refrigeration of the final product to minimize the risks from *Listeria*.

## AI.2 INTRODUCTION

Since 2008, at least eight listeriosis outbreaks accounting for 216 illnesses, 207 hospitalizations, and 47 deaths have been linked to fresh produce including lettuce, sprouts, celery, cantaloupe, stone fruit, and more recently apples (Garner & Kathariou 2016; Danisha & Sophia 2016; Beach 2016; CDC 2016d). In addition, six *L. monocytogenes* recalls were issued for *Listeria*-contaminated fresh-cut apples from 2012 to 2015 (Waller 2015; FDA 2013) with only one outbreak of unknown cause traced to consumption of intact apples prior to the fall of 2014 (Keller 2014; Sivapalasingam et al. 2004; CDC 2015). However, this situation changed on December 30, 2014 when the CDC reported a link between the consumption of caramel apples and 35 cases of listeriosis in 12 states that included 34 hospitalizations and seven deaths (CDC 2015). The outbreak strain was subsequently identified at a commercial apple-packing facility in California (CDC 2015; FDA 2015, Flynn 2015) and confirmed by pulsed-field gel electrophoresis. Garner and Kathariou (2016) later reported that since at least three of the individuals affected by the caramel apple outbreak had consumed sliced or whole apples, *Listeria* originated with the apple – not the caramel.

Prior to this outbreak, caramel apples were presumed to pose minimal risk due their inherent high acidity ( $\text{pH} < 4.0$ ) and the low water activity of caramel ( $a_w < 0.80$ ). However, growth of *L. monocytogenes*, along with several other pathogens has been previously reported on fresh-cut apple slices at 15 to 20°C (Conway et al. 2000). Inside puncture wounds and on the surface of fresh-cut apples, *Escherichia coli* O157:H7 began growing within 2 h (Fatemi et al. 2006) with this pathogen also penetrating and growing in calyces, regardless of the inoculation method or size of the calyx opening. In regard to caramel apples, Glass et al. (2015) also hypothesized that stick insertion could lead to a film of apple juice between the caramel and

apple surface, creating a microenvironment that may then become more favorable for *Listeria* growth than either the apple or caramel alone..

During commercial processing, various sanitizers such as chlorine, peroxyacetic acid or ozone are typically added to the dump tank water to minimize cross-contamination and uptake of pathogens during washing of fresh produce (Gurtler et al. 2015) with careful monitoring needed to maintain effective sanitizer levels in the water during washing (Gurtler et al. 2015; Ukuku et al. 2015; Annous et al. 2013).

When Buchanan et al. (1999) immersed apples in a heavy suspension of *E. coli* O157:H7, greatest pathogen uptake occurred from the blossom end into the inner core at  $\sim 10^5$  CFU/g. Once filled with fluid, the stem and calyx cavities of apples can become microbial growth niches (Buchanan et al. 1999; Baskaran et al. 2013; Doores 1983) with these internalized bacteria no longer susceptible to chemical sanitizers. Apple packers also need to ensure that the temperature of the apples being processed is no more than 10°F above that of the dump tank water, which will in turn minimize internalization due to the pressure differential.

Given the 2014 multistate outbreak of listeriosis involving caramel apples, this study aimed to 1) assess the thermal tolerance of three *L. monocytogenes* cocktails consisting of caramel apple outbreak, unrelated outbreak and non-outbreak strains on apples at typical caramel dipping temperatures, and 2) quantify growth of the *L. monocytogenes* caramel apple outbreak strains in caramel apples as a result of stick insertion during prolonged storage.

## **AI.3 MATERIALS AND METHODS**

### **AI.3.1 Produce**

Unwaxed Red Johnathan Apples (*Malus domestica* ‘Jonathan’) having a circumference of ~ 21 cm were provided by Happy Apple Co. (Washington, MO) and also purchased from a local supplier (Stan Seta’s Produce Co, Lansing, MI). Upon arrival, the apples were stored in a 4°C walk-in cooler. Apples for the caramel dipping and storage studies were stored no longer than 3 weeks and 1 week, respectively, before use.

### **AI.3.2 Bacterial strains**

*L. monocytogenes* strains used in this study included 6707 (serotype 4b, caramel apple outbreak, NM, 2015), 6714 (serotype 4b, caramel apple outbreak, AZ, 2015), 6716 (serotype 4b, caramel apple outbreak, TX, 2015) and 6724 (serotype 4b, caramel apple outbreak, AZ, 2015) obtained from Dr. Cheryl Tarr, Centers for Disease Control and Prevention, Atlanta, GA; FSL J1-225 (serotype 4b, human epidemic, MA, 1983), FSL J1-119 (serotype 4b, human epidemic, LA, 1985), FSL N1-225 (serotype 4b, human epidemic, US 1988-89) and FSL R2-499 (serotype 1/2a, human epidemic, sliced turkey, 2000) obtained from Dr. Martin Wiedmann, Cornell University, Ithaca, NY; and CWD 271 (riboprint 19161, dairy plant environment, 2004), CWD 338 (riboprint 19092, dairy plant environment, 2004), CWD 561 (riboprint 19071, dairy plant environment, 2004), and CWD 580 (riboprint 54081, dairy plant environment, 2004) obtained from Dr. Catherine Donnelly, University of Vermont, Burlington, VT. All *L. monocytogenes* strains were stored at -80°C in Trypticase soy broth containing 0.6% (w/v) yeast extract (TSBYE, Becton, Dickinson and Co., Sparks, MD) and 10% (v/v) glycerol.

### **AI.3.3 Inoculation**

Each strain was streaked from the frozen stock culture onto a plate of Trypticase soy agar (Becton, Dickinson and Co.) containing 0.6% (w/v) yeast extract (TSAYE) and incubated at 37°C for 24 h. Thereafter, an isolated colony of each strain was sub-cultured twice in 9 mL of TSBYE at 37°C for 24 h, after which the strains were combined in equal volumes (9 ml each) to obtain the following four-strain cocktails: caramel apple outbreak (strains 6707, 6714, 6716, and 6724); unrelated outbreak (strains J1-225, J1-119, FSL N1-225, and FSL R2-499); and dairy environmental (strains CWD 271, CWD 338, CWD 561, and CWD 580). The inoculum was prepared by adding 9 mL of each cocktail to ~500 mL of sterile distilled water. Apples for subsequent hot caramel dipping were immersed in this suspension for 10 min, removed, air dried for 30 min at 22°C in a biosafety cabinet and then individually stored in sterile Whirl-Pak™ bags (Nasco, Fort Atkinson, WI) at 4°C for 24 h to ensure bacterial attachment with this procedure achieving a population of  $8.2 \pm 0.2$  log CFU/apple. In contrast, apples used to assess *L. monocytogenes* growth during storage were spot-inoculated with 100 µl of the caramel apple outbreak cocktail at the stem end to achieve a population of ~3 log CFU/apple. After inoculation and drying, a wooden caramel apple stick (Daffy Apples, Denver, CO) was inserted into the stem-end to within 2.5 cm from the bottom of the apple.

### **AI.3.4 Apple dipping**

Daffy Apple Dipping Caramel (Daffy Apple, Denver, CO) containing granulated beet sugar, corn syrup, nonfat milk, hydrogenated soybean oil, vegetable shortening, flavor, salt, flour, mono & diglycerides, baking soda, and soy lecithin was stored at 22°C until use. Approximately 300 mL of caramel was heated in a water bath in separate 500 mL glass beakers to either 82, 88, 93, or 99°C as suggested by the manufacturer. After inserting the wooden apple

sticks, the apples to be dip-inoculated were dipped in caramel at 82, 88, 93, or 99°C for 5 sec, removed, and then placed on aluminum foil to cool for 1 h. Spot-inoculated apples were dipped in caramel at 82°C for 5 sec, placed on aluminum foil and allowed to cool for 1 h before being stored at 4 or 22°C for subsequent analysis.

### **AI.3.5 Measurement of pH**

A flat surface gel-filled electrode (Thermo Scientific, Chelmsford, MA) was used to measure the pH of undipped apple flesh at four different locations per apple upon delivery and after 14 and 28 days of storage at 22 and 4°C, respectively (Figure 1).

### **AI.3.6 Microbiological analysis**

Dip-inoculated caramel apples were added to separate Whirl-Pak filter bags™ containing 100 mL of sterile 0.1% phosphate-buffered solution (PBS) and massaged by hand for 2 min. For spot-inoculated caramel apples, four different sections – top, middle, bottom and core, were periodically assessed for numbers of *Listeria* as shown in Figure 1. These four different apple sections for analysis were obtained by slicing the apple into three equal-sized pieces to obtain the top, middle and bottom sections after which a 2-cm diameter sterilized metal cork borer was used to remove the core material from the top, middle and bottom sections. The top, middle and bottom sections included both the skin/caramel portion of apple along with the apple flesh. All spot-inoculated caramel apple samples were weighed, giving average weights of  $24.3 \pm 6.1$ ,  $50.5 \pm 8.8$ ,  $37.6 \pm 4.7$ , and  $4.5 \pm 1.3$  g for the top, middle, bottom and core sections, respectively. Total apple weights averaged  $125.8 \pm 12.4$  g. Samples were placed in a sterile mechanical blender jar (Oster BlendNGo, Sunbeam Products, Inc., Boca Raton, FL) containing 50 mL of sterile PBS and homogenized at the highest speed for 1 min. All sample homogenates were then appropriately diluted in sterile PBS and surface plated on TSAYE containing 0.1% (w/v) esculin

(Sigma-Aldrich, St. Louis, MO) and 0.05% (w/v) ferric ammonium citrate (Sigma-Aldrich, St. Louis, MO). All black colonies resembling *L. monocytogenes* on this non-selective/differential plating medium were counted after 48 h of incubation at 35°C.

### **AI.3.7 Statistical Analysis**

All experiments were performed in triplicate with three dip-inoculated apples and one spot-inoculated apple sampled at each time point. *L. monocytogenes* populations were reported as log CFU/apple for dip-inoculated apples and log CFU/g for spot-inoculated apples. Data were subjected to an Analysis of Variance using JMP 12.0 (SAS Institute Inc., Cary, NC). For all tests, a *P* value of < 0.05 was considered statistically significant. The Tukey-Kramer HSD test was used to identify significant differences in *L. monocytogenes* populations after caramel dipping at various temperatures and during storage of the spot-inoculated apples.

## AI.4 RESULTS AND DISCUSSION

### AI.4.1 *L. monocytogenes* inactivation on dip-inoculated caramel apples. *L. monocytogenes* inactivation on dip-inoculated caramel apples

As expected, greater inactivation of *Listeria* occurred as the caramel dipping temperature increased. Similar reductions of  $2.0 \pm 0.6$ ,  $2.0 \pm 0.1$  and  $2.2 \pm 0.1$ , and  $1.8 \pm 0.3$ ,  $1.8 \pm 0.6$  and  $2.0 \pm 0.5$  log ( $P > 0.05$ ) were seen for the outbreak and unrelated outbreak cocktails after dipping in caramel at 82, 88 and 93°C, respectively, with significantly greater reductions ( $P < 0.05$ ) of  $2.7 \pm 0.1$  and  $2.6 \pm 0.1$  log observed for the apple outbreak and unrelated outbreak cocktails at 99°C (Figure 2). The non-outbreak environmental strains of *L. monocytogenes* were similarly inactivated at 82 and 88°C ( $P > 0.05$ ) with populations decreasing  $1.7 \pm 0.1$  and  $1.7 \pm 0.5$  logs, respectively. However, significantly greater reductions were seen for the environmental cocktail strains at both 92 and 99°C with populations decreasing  $2.4 \pm 0.3$  and  $2.9 \pm 0.2$  logs, respectively ( $P < 0.05$ ). Overall, the two outbreak cocktails exhibited significantly greater thermal resistance at both at 92 and 99°C compared to the non-outbreak environmental strains.

Surface thermal treatments can be effective in reducing microbial populations on certain types of produce. While Annous et al. (2013) demonstrated a greater than 5 log reduction for *Salmonella* Poona on cantaloupe after a 90 s immersion in 92°C water, such a treatment is clearly deleterious for apples and will led to a partially cooked product. According to Glass et al. (2015) caramel apples are commercially prepared by dipping apples in caramel at 104 to 116°C, with the temperature decreasing to  $< 100^\circ\text{C}$  during production. However, these high temperatures will again likely result in some undesirable cooking at the apple surface. Recognizing these industry practices and a recommendation not to exceed 82 °C for the caramel used in the present study, dipping temperatures of 82 to 99°C were chosen. At the higher caramel dipping temperatures of

92 and 99°C, some cooking of the apple was evident with the caramel also becoming thinner and less able to coat the apple surface. Thus, while raising the caramel dipping temperature was advantageous for reducing *Listeria* on the apple surface, more work is needed in regard to specific apple cultivars and dipping caramel formulations to optimize both end-product quality and safety.

#### **AI.4.2 *L. monocytogenes* growth in spot-inoculated caramel apples at 4 and 22°C**

Initially, the caramel apple cores from each top, middle and bottom section were individually assessed for growth of *L. monocytogenes*. However, statistically similar populations of *Listeria* were subsequently recovered from all three core sections ( $P > 0.05$ ) with standard deviations of 0.17 to 0.59, and 0.17 to 0.83 log CFU/g for apples stored at 4 and 22°C, respectively. Consequently, the top, middle and bottom core results were averaged thereafter with the results reported as one core region per caramel apple as depicted in Figure 3. All apples spot-inoculated at the stem end supported growth and spread of the *L. monocytogenes* caramel apple outbreak strains after stick insertion with significantly higher numbers of *Listeria* recovered from the core as compared to the apple tissue ( $P < 0.05$ ). *Listeria* increased significantly faster in apples stored at 22 as compared to 4°C ( $P < 0.05$ ) with the pathogen achieving a population of ~5 log CFU/g in the apple flesh after only 4 days at 22°C compared to 14 days at 4°C. Based on these findings, caramel apples should be stored at 4°C and kept refrigerated until the time of consumption.

Even though apples stored at the higher temperature were not sampled beyond day 14 due to spoilage, , no significant difference in *Listeria* populations was evident between apples stored at either 22 or 4°C ( $P < 0.05$ ) after 14 days due to rapid growth of *Listeria* under both storage conditions. After 28 days of storage at 4°C, *Listeria* populations increased from  $2.5 \pm 0.1$  to 5.3

$\pm 0.1$ ,  $2.1 \pm 0.2$  to  $5.0 \pm 0.1$ ,  $2.1 \pm 0.4$  to  $5.4 \pm 0.0$ , and  $4.2 \pm 0.5$  to  $7.7 \pm 0.6$  log CFU/g in the top, middle, bottom and core samples, respectively; whereas at  $22^{\circ}\text{C}$ , *Listeria* populations increased from  $2.5 \pm 0.8$  to  $5.0 \pm 0.1$ ,  $2.1 \pm 0.2$  to  $5.4 \pm 0.1$ ,  $2.1 \pm 0.4$  to  $5.1 \pm 0.1$ , and  $4.2 \pm 0.5$  to  $7.4 \pm 0.6$  log CFU/g in these same samples (Figure 3). Since caramel apples stored at  $22^{\circ}\text{C}$  lasted only 14 days while those stored at  $4^{\circ}\text{C}$  remained acceptable for up to 28 days, these findings indicate that cold storage can be used to extend the shelf-life of caramel apples. The pH of the apples ranged from  $3.42 \pm 0.04$  to  $3.53 \pm 0.03$  and did not significantly change during storage ( $P > 0.05$ ).

When viewed separately, neither an apple pH of  $< 4.0$  nor a caramel water activity value of  $< 0.80$  are conducive for *Listeria* growth (Wu et al. 2007). Nonetheless, this study indicated that *Listeria* grew even under with these extreme conditions, perhaps within the apoplastic space between the apple tissue cells as has been reported for *Listeria* in lettuce (Shenoy et al. 2017) and *Salmonella* in tomatoes (Brandl et al., 2013). Glass et al. (2015) also observed growth of *Listeria* in Granny Smith caramel apples that were more acidic (pH 3.2) than those used in the present study (pH 3.5). Both of the these studies confirming *Listeria* growth under previously assumed non-growth supporting conditions suggest that more work is needed to better understand the impact of potential microenvironments within caramel apples on *Listeria* growth.

*Listeria* grew in caramel apples stored at  $4^{\circ}\text{C}$  with significant differences ( $P < 0.05$ ) seen in the extent of growth and spreading to the various caramel apple sections (Figure 1). After 7 days of storage at  $4^{\circ}\text{C}$ , *Listeria* populations in the apple flesh and core increased from  $2.2 \pm 0.2$  to  $4.6 \pm 0.3$  and  $4.2 \pm 0.5$  to  $5.7 \pm 0.5$  log CFU/g, respectively. These observations are consistent with a previous study by Penteado and Leitao (2004) indicating that refrigeration reduced but did not prevent *L. monocytogenes* from growing in different acidic fruits including melon,

watermelon and papaya. Salazar et al. (2016) also demonstrated the ability of *L. monocytogenes* to not only survive but also grow in caramel apples. When the stem ends of undipped and caramel-dipped Gala and Granny Smith apples were inoculated with *Listeria* at ~3 log CFU/apple, no growth was observed in the undipped apples during 49 days of storage at 25°C; however, *Listeria* populations increased 4 to 6 logs for caramel apples similarly stored for only 7 days, suggesting that as yet unknown changes within the microenvironment of caramel apples play an important role in fostering *Listeria* growth.

*Listeria* both grew in caramel apples and spread from the core to the surrounding apple tissue as a result of stick insertion. These observations are consistent with previous pathogen uptake studies by Buchanan et al. (1999) in which the stem and blossom areas of apples were of greatest concern in regard bacterial infiltration into the core region. Glass et al. (2015) hypothesized that *L. monocytogenes* cells pushed from the stem area into the core during stick insertion are subsequently spread to the flesh of the fruit where they are protected from the hot caramel during apple dipping. This situation was apparent from day 0 with significantly higher ( $P < 0.05$ ) numbers of *Listeria* recovered from the core compared to the surrounding regions. Fatemi et al. (2006) also reported that *E. coli* O157:H7 was able to penetrate vertically through fresh puncture wounds in apples within 2 h of inoculation with the pathogen further spreading and increasing by 3 logs after 48 h.

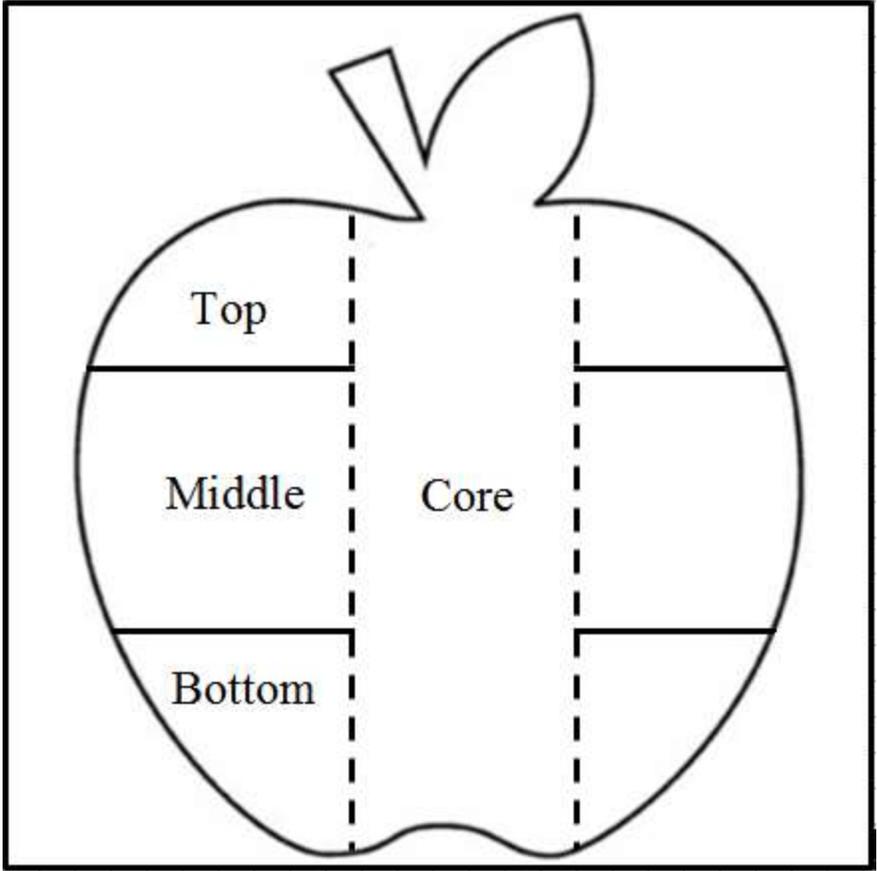


Figure AI.1: Caramel apple sampling sections.

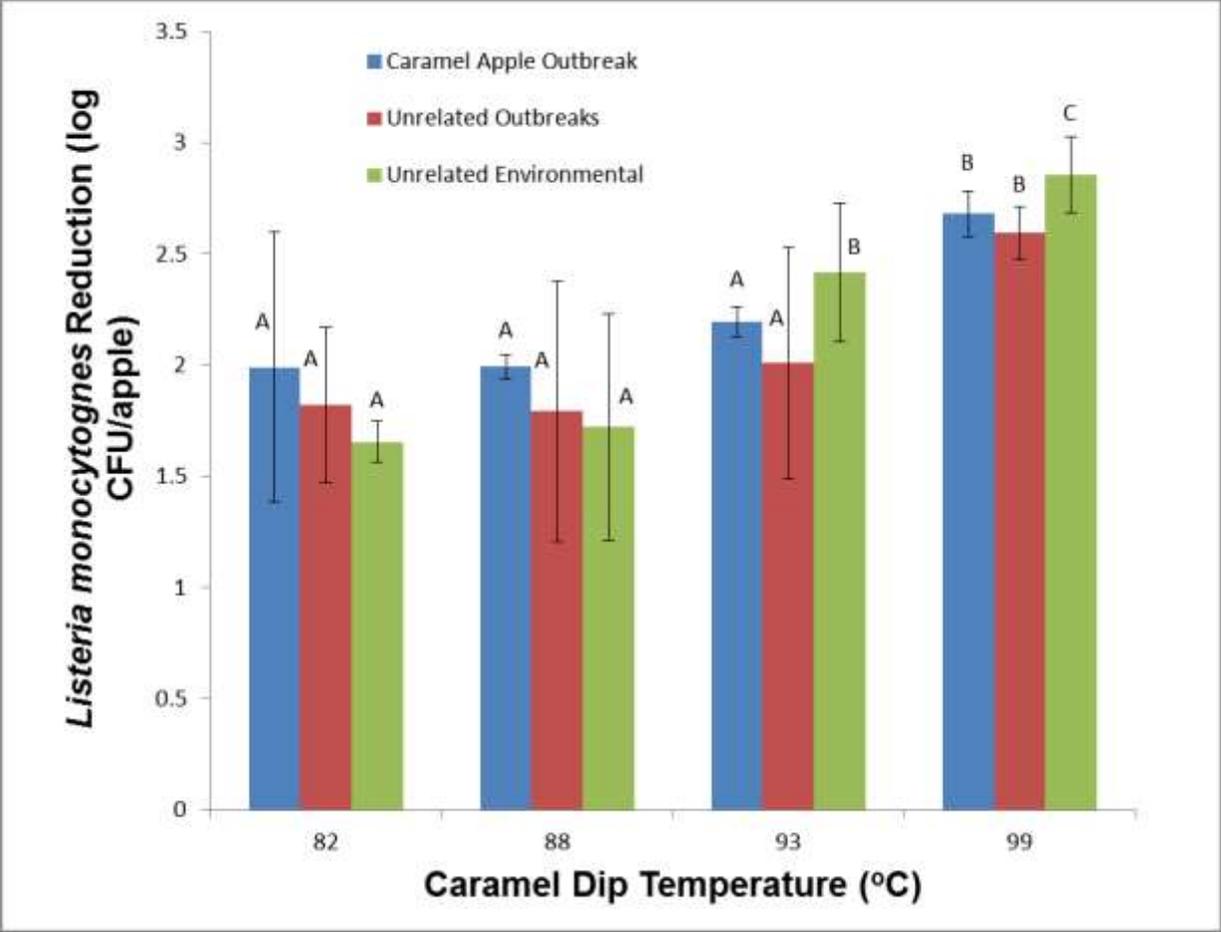


Figure AI.2: *Listeria monocytogenes* reductions (log CFU/apple) in caramel apples after a 5 sec dip in caramel at 82, 88, 93, or 99°C.

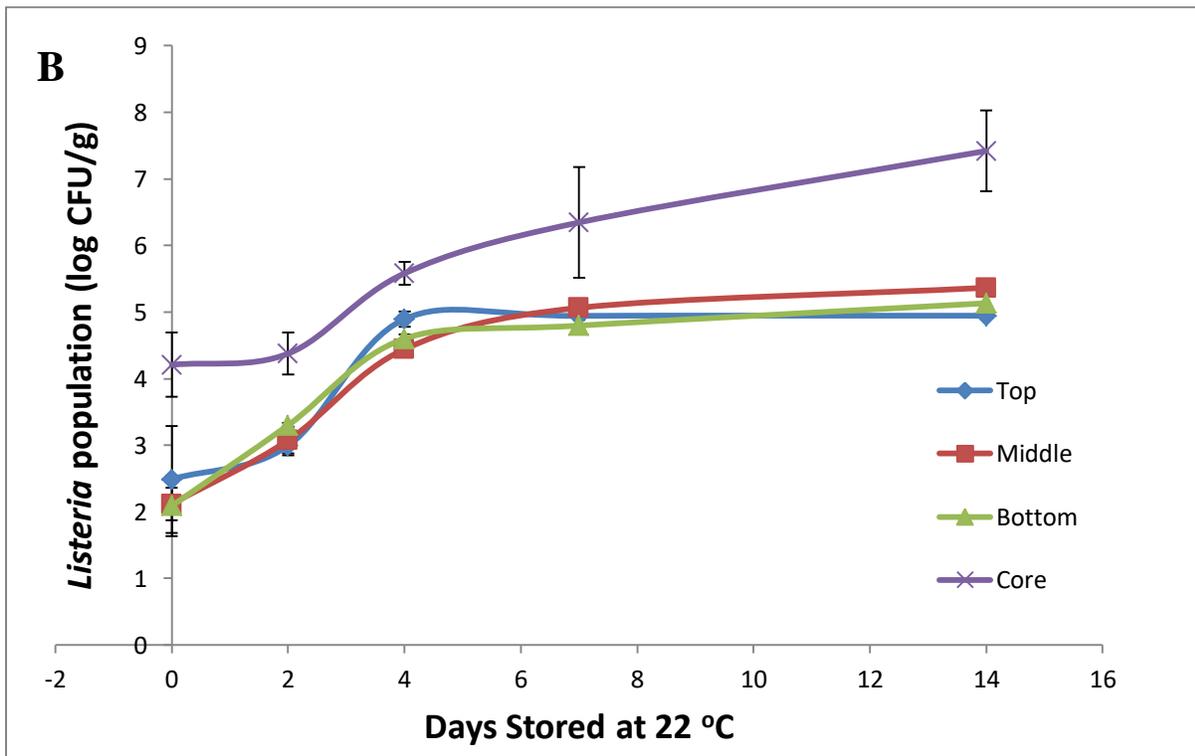
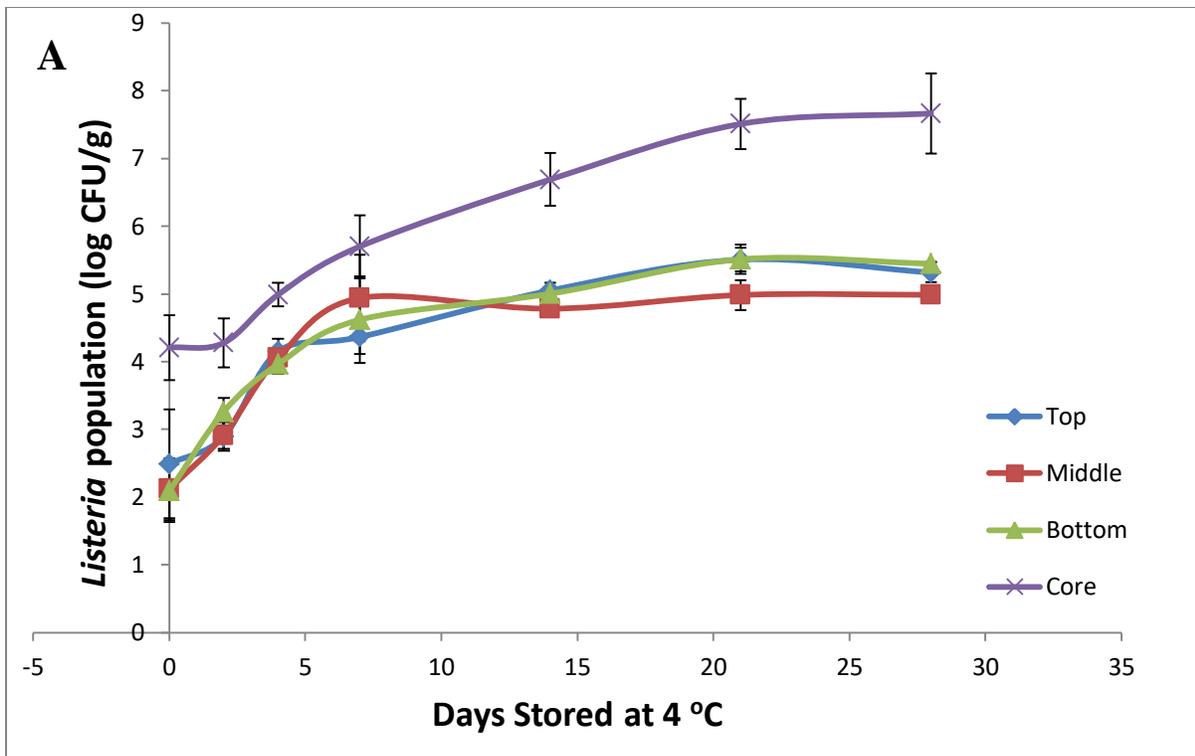


Figure AI.3: Growth of *L. monocytogenes* in caramel apples during storage at 4 (A) and 22°C (B)

## **AI.5 CONCLUSION**

Dipping apples in hot caramel decreased *L. monocytogenes* populations less than 3 logs on the surface of apples with both the caramel apple outbreak and unrelated outbreaks strains of *L. monocytogenes* exhibiting greater thermal resistance compared to the environmental strains.. However, any organisms remaining in the difficult-to-clean stem cavity of the apple may be forcibly transferred into the core as a result of stick insertion and then migrate and potentially grow in the surrounding apple tissue during extended storage. Based on these findings, every attempt should be made to avoid internalization of bacteria through the stem end of apples during washing, which includes maintaining a less than 10°F apple/water temperature differential during washing and effective levels of appropriate chemical sanitizers in the dump tank during washing. In addition, apples for caramel dipping need to be intact and thoroughly dried before stick insertion to minimize the transfer of bacteria from the stem region into the core. After dipping, caramel apples should be also refrigerated and consumed within 14 days to minimize the risk of illness.

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