QUANTITATIVE TRANSFER OF *ESCHERICHIA COLI* O157:H7 DURING PILOT-PLANT PRODUCTION OF FRESH-CUT LEAFY GREENS

By

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ABSTRACT

QUANTITATIVE TRANSFER OF *ESCHERICHIA COLI* O157:H7 DURING PILOT-PLANT PRODUCTION OF FRESH-CUT LEAFY GREENS

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Cross-contamination/multi-directional transfer of *Escherichia coli* O157:H7 during commercial shredding, conveying, flume-washing and drying of fresh-cut leafy greens has become a major public health concern. However, the extent of bacterial transfer during the various unit operations involved in post-harvest processing of leafy greens is poorly understood. Consequently, a pilot-scale leafy green processing line consisting of a commercial shredder, conveyor, flume tank, shaker table, and centrifugal dryer was assembled to *E. coli* O157:H7 transfer during processing of iceberg lettuce, Romaine lettuce, and baby spinach.

Using 22.7 kg of leafy greens inoculated with a 4-strain avirulent, GFP-labeled, ampicillin-resistant *E. coli* O157:H7 cocktail at 6, 4 and 2 log CFU/g, bacterial transfer coefficients were determined between the product, the water, and the equipment surfaces during processing, with the effect of post-inoculation hold time and shred size on transfer also evaluated. During leafy green processing, ~90% of the *E. coli* O157:H7 inoculum transferred to the wash water. After processing, *E. coli* O157:H7 populations on equipment were highest on the shredder and conveyor. Shred size did not affect the numbers of *E. coli* O157:H7 transferred from contaminated leafy greens during processing. However, extending the time between inoculation and processing decreased the removal and transfer of this pathogen during processing. When 22.7 kg of product containing 10^6 and 10^4 *E. coli* O157:H7 CFU/g were processed followed by 22.7 kg uninoculated product, *E. coli* O157:H7 was quantifiable in all iceberg and romaine lettuce samples. At an inoculation level of 2 log CFU/g, *E. coli* O157:H7

was sporadically detected after processing 81.6 kg of uninoculated iceberg and found throughout the entire batch of uninoculated romaine lettuce.

Using Radicchio as a colored surrogate for iceberg lettuce, this study demonstrated that 9.1 kg of *E. coli* O157:H7-inoculated Radicchio could contaminate 907 kg of subsequently processed uncontaminated iceberg lettuce. After processing, shreds of Radicchio were found in all but one of the ~38 bags of shredded lettuce. Hundreds of contaminated Radicchio shreds remained on the processing line, with the majority found on the conveyor, followed by the shredder, flume tank, and shaker table, showing that contaminated product can be continually spread during leafy green processing long after a contamination event.

The effect bacterial attachment time on cross-contamination was determined by processing 0.5 kg of *E. coli* O157:H7-inoculated Radicchio immediately and after storage for 1 d at 4°C or 5 d at 22°C, followed by 45.5 kg of uninoculated iceberg lettuce. After processing, *E. coli* O157:H7 was found in all uncontaminated iceberg lettuce samples, with mean counts highest for 1 h- followed by 1 d- and 5 d-held product. Scanning electron microscopy images showed greater bacterial attachment for 5 d-held product, with larger cell aggregates enveloped in a dense extracellular matrix.

When transfer between inoculated and uninoculated product was assessed during partial dewatering, faster cross-contamination was seen using an *E. coli* O157:H7-inoculated Radicchio to uninoculated iceberg lettuce ratio of 1:100 than 1:10,000 (w/w) until a maximum population was reached due to the higher number of *E. coli* O157:H7 cells available for transfer. These findings, which demonstrate *E. coli* O157:H7 transfer between product, wash water, and equipment surfaces, and to large quantities of uncontaminated fresh-cut leafy greens, are critical to the development of science-based transfer models for risk analysis.

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KEY TO SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis of Variance
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit(s)
CSPI	Center for Science in the Public Interest
d	days(s)
EHEC	Enterohemorrhagic *E. coli*
FAO	The Food and Agriculture Organization of the United Nations
FSMA	Food Safety Modernization Act
g	grams(s)
GFP	Green fluorescent protein
h	hour(s)
HHS	United State Department of Health and Human Services
HUS	Hemolytic uremic syndrome
min	minute(s)
ml	milliliter(s)
MLVA	Multilocus variable number tandem repeat analysis
mm	millimeter(s)
OD	Optical Density
P3ARRT	Pathogen-Produce Pair Attribution Risk Ranking Tool
PBS	Phosphate Buffered Saline
PFGE	Pulsed-field Gel Electrophoresis
ppm	Parts per million

RTE	Ready-to-eat
s	second(s)
SAS	Statistical Analysis Systems
SEM	Scanning electron microscopy
Stx	Shiga toxin
TSA-YE	Trypticase Soy Agar with 0.6 % Yeast Extract
TSB-YE	Trypticase Soy Broth with 0.6 % Yeast Extract
US	United States of America
USDA	United States Department of Agriculture
USDA	United State Department of Agriculture
VT	Verocytotoxin
WHO	World Health Organization
µl	microliter(s)
µm	micron(s)

INTRODUCTION

As the United States population attempts to move toward healthier eating habits (more fruits and vegetables) outbreaks associated with produce are becoming more frequent (HHS/USDA 2005). In light of recent contamination problems, consumer confidence in the safety of the produce has been declining. The Center for Science in the Public Interest (CSPI 2009) put leafy greens at the very top of the recently published list of "The 10 Riskiest Foods", while the United Nations and World Health Organization's Food and Agriculture Organization report (FAO/WHO 2008), ranked leafy greens a Level 1 Priority for food safety, the highest priority among produce commodities.

An increasing number of foodborne outbreaks traced to fresh fruits and vegetables are partially attributed to production, processing, and consumption patterns. Since 1990, 19 outbreaks were specifically associated with the consumption of leafy greens (CSPI 2008a). In September 2006, contaminated pre-bagged baby spinach was linked to an *E. coli* O157:H7 outbreak in 26 states and Canada that resulted in 205 illnesses and five deaths (Jay and others 2007). A few months later, two additional *E. coli* O157:H7 outbreaks were traced to California grown iceberg lettuce that was shredded and then purchased by two fast-food Mexican restaurant chains in the Midwest and Northeast (FDA 2006, FDA 2007). These two outbreaks sickened a combined total of over 150 individuals (CSPI 2008b) and raised nationwide concerns regarding the safety of leafy greens along with the various routes for contamination.

In the United States, the progression from locally grown produce to centralized production has led to numerous multi-state and nationwide outbreaks of foodborne illness (Calvin 2007). In many cases, fruits and vegetables are grown on centralized large-scale farms in locations that specialize in a specific product. Under these conditions, one contamination

incident at a large centralized grower or processor could quickly lead to a multi-state outbreak with near catastrophic consequences for the industry, as was seen in the recent outbreak involving baby spinach (Calvin 2007, Jay and others 2007). By definition, fresh fruits and vegetables generally do not undergo any treatment, other than washing, for the reduction and/or elimination of potentially hazardous microorganisms. Unfortunately, most commercial sanitizers used for washing fresh produce can only reduce the microbial levels by 90 to 99% ,which still makes these products potential vehicles for the transmission of microbial pathogens such as *Salmonella* and *Escherichia coli* O157:H7 (Beuchat and others 2004, Burnett and others 2004, Keskinen and others 2009, Sapers 2001, Weissinger and others 2000, Zhang and others 2009).

Microbial contamination of fresh produce can occur at any point throughout the farm-to-fork continuum (Beuchat 2002). In the field, spoilage and pathogenic microorganisms that adversely impact product safety and shelf-life can come from many sources, including the soil, manure, irrigation water, and both domestic livestock and wild animals (Beuchat 2006, Beuchat and Ryu 1997). During processing, the microbial load often increases as a result of direct contact with contaminated equipment surfaces (e.g., conveyor belts, slicers, dicers) (Abadias and others 2008, Allende and others 2004, Garg and others 1990, Kaneko and others 1999). Human foodborne pathogens can be readily transferred to much larger quantities during subsequent product handing and further processing, raising even greater food safety concerns. It has been demonstrated that foodborne pathogens can also contaminate the product during processing (Stafford and others 2002).

Even as the number and magnitude of the outbreaks increase, there is still very little known about the transfer of bacteria during various unit operations involved in post-harvest processing (shredding, conveying, washing and dewatering) of leafy greens.

It is hypothesized that *E. coli* O157:H7 is transferred in quantifiable numbers between leafy greens, wash water, and the food contact surface areas of the processing equipment during simulated commercial processing.

The research reported in this dissertation had five primary objectives: 1) quantify *E. coli* O157:H7 transfer to equipment during pilot-scale production of fresh-cut leafy greens; 2) quantify the transfer of *E. coli* O157:H7 from equipment surfaces to iceberg and Romaine lettuce during simulated pilot-scale processing; 3) track an *E. coli* O157:H7 inoculated batch of leafy greens through a pilot-scale processing line; 4) determine the impact of post-inoculation hold time on *E. coli* transfer during leafy greens processing and 5) quantify the *E. coli* O157:H7 transfer from inoculated leafy greens to uninoculated leafy greens during partial dewatering.

CHAPTER 1

Review of Pertinent Literature

1.1 Microorganisms associated with fresh produce

Fresh produce is typically host to a highly diverse group of microorganisms, including bacteria, yeasts, molds, parasites and viruses, as well as insects and other plant pests, that may exist on plants as harmless commensals, plant pathogens or potential spoilage organisms. Aerobic bacterial counts on minimally processed vegetables generally range from 5 to 7 log CFU/g with the majority (80 to 90%) of these organisms being *Pseudomonas*, *Enterobacter* and *Erwinia* species (Francis and others 1999; Garg and others 1990; King and others 1991; Johnston and other 2005; Ruiz and others 1987; Thunberg and others 2002). Lactic acid bacteria are also detected on some leafy greens during temperature abuse (Francis and others 1999; Manvell and Ackland 1978). Bacterial counts on fresh produce can vary greatly depending on growth, harvest, processing, packaging, storage and handling conditions. Based on a few field and retail market surveys, coliform and *Enterococcus* counts ranged from 1 to 4 log CFU/g, with *E. coli* levels of < 1 to 3 log CFU/g (Johnston and other 2005; Mukherjee and others 2004; Ruiz and others 1987). In another survey, 1.6 and 9.7% of conventionally and organically grown produce yielded *E. coli*, respectively. For organically grown produce, *E. coli* populations were 19 times higher using manure or compost aged less than 12 months as compared to older compost (Mukherjee and others 2004).

As part of a diverse microbial community, fresh produce may also harbor potential human foodborne pathogens such as *Salmonella*, *E. coli* O157:H7, *Cryptosporidium* and Hepatitis A virus, all of which are indicators of fecal contamination (Doyle and Erickson 2006). Many reviews have addressed the incidence, growth and survival of foodborne pathogens on fresh and processed produce (Beuchat 1996; Beuchat and Bracket 1990; Beuchat and others 2004; Gleeson and O'Beirne 2005; Harris and others 2003).

1.2 *Escherichia coli* O157:H7

One particular organism, *E. coli* O157:H7, a facultative anaerobic, Gram-negative, rod-shaped bacterium, is an unusually virulent enterohemorrhagic strain (EHEC) of *E. coli*. It is a cause of enteric disease resulting in bloody diarrhea and severe abdominal pain and in some more rare cases, hemolytic uremic syndrome (HUS). Characterized by hemolytic anaemia, thrombocytopenia and hemolytic uremic syndrome (acute kidney failure) (Kaper and others 2004), HUS appears mainly in children, as first described by Karmali and others (1983). *E. coli* O157:H7 is a common inhabitant of the bovine gastrointestinal tract and has historically been a major source of concern in the ground beef industry (Griffin and others 1991). This organism first became recognized as a foodborne pathogen in 1982 after an outbreak tied it to the consumption of undercooked hamburgers, which ultimately sickened 47 individuals (Riley and others 1983). Since its discovery, *E. coli* O157:H7 has been linked to outbreaks associated with ground beef (Bell and others 1994, Riley and others 1983), produce (FDA 2006; FDA 2007; Jay and others 2007), fruit juices (CDC 1996; Cody and others 1999), unpasteurized milk (Griffin and Tauxe 1991; Keene and other 1997), and contaminated drinking water (Swerdlow and others 1992).

E. coli is characterized by a specific combination of the O (lipopolysaccharide), H (flagellar) and sometimes K (capsular) antigens which define the various serotypes (Kaper and others 2004). Strains of pathogenic *E. coli* use multistep schemes of pathogenesis starting with colonization of the mucosal site, evading the hosts immune system, multiplication and damage to the host (Kaper and others 2004). *E. coli* O157:H7 has an extremely low oral infectious dose of less than 100 cells (Meng and others 2007). Enterohemorrhagic strains including *E. coli* O157:H7, destroy the normal microvillar structure of the colon by expressing the virulence factor

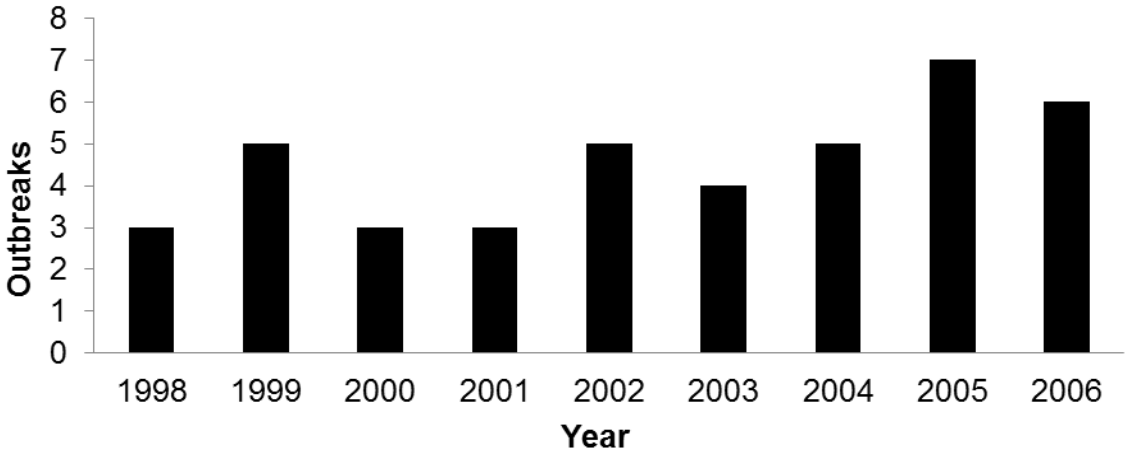
protein, intimin, which allows intimate attachment of the bacteria inducing an attaching and effacing lesion. Shiga toxin (Stx) is the major virulence factor of EHEC and also commonly known as verocytotoxin (VT). Stx is a family of structurally similar cytotoxins, with related biological activity, including two main groups, Stx1 and Stx2, which share about 40% amino acid similarity. Stx is composed of five identical B subunits that bind the holotoxin to the glycolipid globotriaosylceramide (Gb3) on the host cell surface and an A subunit, which cleaves ribosomal RNA to disrupt protein synthesis, ultimately killing the epithelial and endothelial cells. Stx causes local damage to the colon resulting in bloody diarrhea, hemorrhagic colitis and intestinal perforation. HUS results when Stx, produced in the colon, travels through the bloodstream to the renal endothelial cells in the kidney, causing inflammation by obstructing the microvasculature through both direct toxicity and inducing local cytokine and chemokine production (Caprioli and others 2005; Kaper and other 2004; Paton and Paton 1998; Tarr and others 2005).

1.3 Leafy green-associated recalls and outbreaks

As the United States population attempts to move toward healthier eating habits, fresh produce-associated outbreaks are becoming more frequent. Between 1990 and 2006, consumption of fresh fruits and vegetables led to 768 outbreaks that included 35,060 cases of illness (CSPI 2008b), and these numbers are expected to increase. Furthermore, from 1998 to 2006, the average produce outbreak was responsible for 40.1 cases of illness, which is far greater than that seen for beef (23.4), poultry (24.5), and seafood (8.9) (CSPI 2008a). During the 15-year period from 1990 to 2005, *E. coli* O157:H7 and *Salmonella* were respectively responsible for 5 and 23% of these outbreaks (CSPI 2008b). *E. coli* and *Salmonella* associated outbreaks,

although lower in number, draw attention due to the severity of illness and the higher mortality. Produce-linked outbreaks associated with *E. coli* O157:H7 have increased significantly in 2005 and 2006 compared to 1998 - 2004, as can be seen in Figure 1.1 (CSPI 2008a).

Figure 1.1: Produce outbreaks due to *E. coli* O157:H7 between 1998 and 2006 (CSPI 2008a)

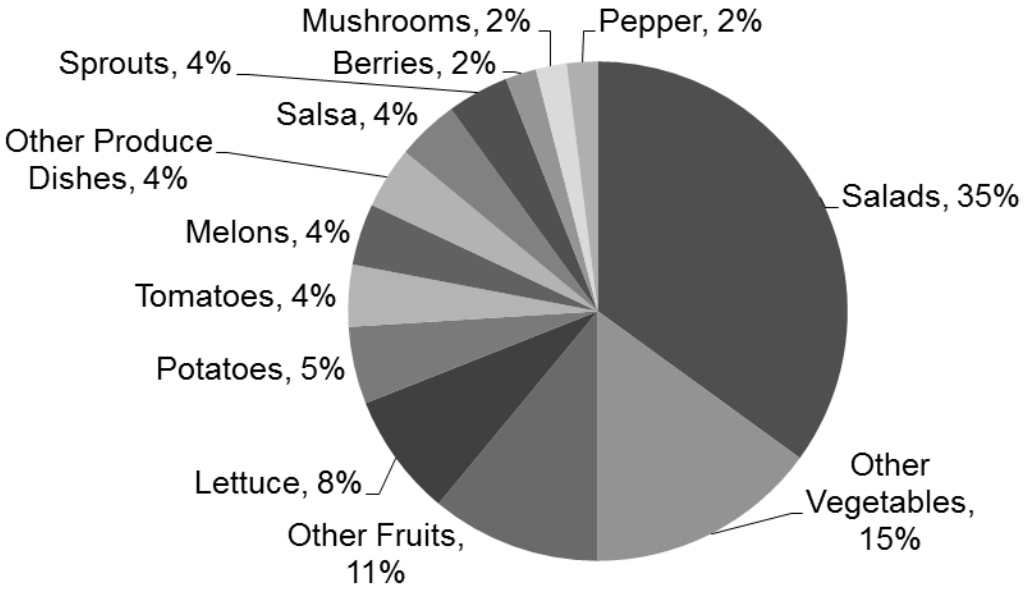


The United Nations and World Health Organization's Food and Agriculture Organization report (FAO/WHO 2008), ranked produce commodities to determine which have the greatest microbiological hazard based on the following criteria: 1) Frequency and severity of disease, 2) Size and scope of production, 3) Diversity and complexity of the production chain and industry, 4) Potential for amplification of foodborne pathogens through the food chain, 5) Potential for control, and 6) Extent of international trade and economic impact. Leafy greens were given the highest ranking because they are frequently exported in large quantities, have been linked to multiple outbreaks resulting in high numbers of illnesses, and because production is quite complex. This assessment is in agreement with recent information from the Pathogen-Produce Pair Attribution Risk Ranking Tool (P3ARRT), a semi-quantitative risk ranking tool for determining which pathogen-commodity causes the greatest public health impact. P3ARRT

showed that enterohemorrhagic *E. coli* in leafy greens was consistently ranked first (Anderson and others 2011).

Based on the data in Figure 1.2, collected by the Center for Science in the Public Interest for specific types of fresh produce, lettuce was most frequently associated with outbreaks of illness, followed by potatoes, tomatoes, melons, sprouts, berries, mushrooms and peppers (CSPI 2008a). Among the broader categories, salads, which would include leafy greens, were responsible for over one-third of all produce-related outbreaks. All of these fresh fruits and vegetables were susceptible to microbial contamination from farm-to-fork. The sources for bacterial pathogens included irrigation water, domestic livestock, wild animals, field workers, processing equipment, consumer cutting boards and home refrigerators (Beuchat 2006).

Figure 1.2: Produce-linked outbreak vehicles 1998 and 2006 (CSPI 2008a)



Although outbreaks associated with leafy greens are most frequently linked to contaminated lettuce, the term “leafy greens” comprises a wide range of salad and other greens

including iceberg lettuce, romaine lettuce, green leaf lettuce, red leaf lettuce, butter lettuce, baby leaf lettuce (i.e. immature lettuce or leafy greens), escarole, endive, spring mix, spinach, cabbage, kale, arugula and chard. Through 2009, 363 outbreaks and 13,568 reported cases of illness in the United States have been associated with the consumption of leafy greens (CSPI 2009b) with many of these outbreaks being traced to *E. coli* O157:H7-contaminated lettuce and spinach grown in the Salinas Valley of California. The number of outbreaks has increased dramatically since 2004 (CSPI 2008a). Table 1.1, modified from Mandrell (2009) and updated to include recent outbreaks, includes a list of select prominent outbreaks associated with leafy green contamination.

In September 2006, contaminated pre-bagged baby spinach triggered an *E. coli* O157:H7 outbreak in 26 states and Canada resulting in five deaths and 205 illnesses (CDC 2006). This outbreak strain was eventually traced to numerous free-roaming feral swine that were spotted on a cattle ranch roughly a mile away from the implicated spinach field (cattle are the primary reservoir of *E. coli* O157) (Jay and others 2007). Droppings from these pigs which had access to the spinach fields could thereby contaminate the crop. Two genetically based typing methods - multilocus variable number tandem repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE), confirmed that the clinical and pig isolates of *E. coli* O157:H7 were identical. This observation was further supported by the discovery of pig tracks, rooting activity and pig feces in the spinach field and adjacent vineyards (Jay and others 2007).

During November and December of 2006, two additional *E. coli* O157:H7 outbreaks were traced to California-grown iceberg lettuce that was shredded and then purchased by two fast food Mexican restaurant chains in the Midwest and Northeast (FDA 2006 and 2007). These two outbreaks sickened a combined total of over 150 individuals (CSPI 2008b). In one of the

outbreaks, state health officials in Minnesota, Iowa, and Wisconsin ultimately matched the strain of *E. coli* O157:H7 to two environmental samples that were collected from dairy farms near the fields where the lettuce was grown (FDA 2007). The source of the other outbreak strain, however, could not be confirmed (FDA 2006).

Table 1.1: *Select leafy green associated outbreak from 1995 through 2011* [a]

Date	**Pathogen**	**Location**	**Illnesses Reported**	**Source**	**Source Region**
Jul. 95	*E. coli* O157:H7	MT	74	Lettuce, Romaine	MT, WA
Sep. 95	*E. coli* O157:H7	ME	30	Lettuce, Iceberg	Unknown
Sep. 95	*E. coli* O157:H7	ID	20	Lettuce, Romaine	Unknown
Oct. 95	*E. coli* O157:H7	OH	11	Lettuce	Unknown
May 96	*E. coli* O157:H7	IL, CT	61	Lettuce, Mesclun mix	CA
Jun. 96	*E. coli* O157:H7	NY	7	Lettuce, Mesclun	Unknown
May 98	*E. coli* O157:H7	CA	2	Lettuce, salad	Unknown
Jul./Aug. 98	*Shigella sonnei*	CA, MA, FL, Canada	> 400	Parsley	Mexico
Sep. 98	*E. coli* O157:H7	MD	4	Lettuce	Unknown
Feb. 99	*E. coli* O157:H7	NE	65	Lettuce, salad	Unknown
Sep. 99	*E. coli* O157:H7	CA	8	Lettuce, Romaine	CA
Sep. 99	*E. coli* O157:H7	WA	6	Lettuce, Romaine	CA
Oct. 99	*E. coli* O157:H7	OH, IN	47	Lettuce, salad	Unknown

Table 1.1 (cont'd)

Oct. 99	*E. coli* O157:H8	OR	3	Lettuce, Romaine hearts	CA
Oct. 99	*E. coli* O157:H7	PA	41	Lettuce, Romaine	CA
Jul. 02	*E. coli* O157:H7	WA	29	Lettuce, Romaine	CA
Nov. 02	*E. coli* O157:H7	IL, WI, MN, SD, UT	24	Lettuce	CA
Sep. 03	*E. coli* O157:H7	CA	57	Lettuce, Iceberg/Romaine	CA
Sep. 03	*E. coli* O157:H7	ND	5	Lettuce, mixed with Romaine	Unknown
Oct. 03	*E. coli* O157:H7	CA	16	Spinach	CA
Nov. 04	*E. coli* O157:H7	NJ	6	Lettuce	CA
Oct.-Dec. 04	*Salmonella* Thompson	Multi, Europe	21	Rucola (arugula)	Italy
Aug./Sep. 05	*E. coli* O157:H7	Sweden	135	Lettuce, Iceberg	Sweden
Aug./Sep. 06	*E. coli* O157:H7	Multi, U.S. (26 states)	> 200	Baby Spinach, bagged	CA
Aug./Sep. 06	*Yersinia pseudotuberculosis*	Finland	> 400	Lettuce, Iceberg	Finland
Nov. 06	*E. coli* O157:H7	NJ, NY, PA, DE	71	Lettuce, Iceberg	CA
Nov./Dec. 06	*E. coli* O157:H7	MN, IA, WI	81	Lettuce, Iceberg	CA

Table 1.1 (cont'd)

May 08	*E. coli* O157:H7	WA	10	Lettuce, Romaine	CA
Jan. 10	*E. coli*	Denmark	260	Lettuce	France
May 10	*E. coli* O145	MI, NY, OH, PA, TN	26	Lettuce, Romaine	AZ
May 10	*Salmonella* Hvittingfoss	IL	90	Lettuce	Unknown
Jul.-Oct. 10	*Salmonella enterica*	U.K.	136	Salad	Unknown
May 11	*Salmonella* Typhimirum	IL	15	Salad	Unknown
Oct./Nov. 11	*E. coli* O157:H7	Multi, U.S. (10 states)	60	Romaine lettuce	Unknown

[a] Information for the January 2010 *E. coli* outbreak is based on Ethelberg and others (2010), the May 2010 *E. coli* O145 outbreak is based on CDC (2010), the May 2010 *Salmonella* Hvittingfoss is based on IDPH (2010), the July – October 2010 *Salmonella enterica* outbreak is based on Gobin and others (2011), the May 2011 *Salmonella* Typhimirum outbreak is based on Sun-Times Media LLC (2011), and the October 2011 *E. coli* O157:H7 outbreak is based on CDC (2011), while all other outbreaks were summarized by Mandrell (2009).

1.4 The leafy green industry

California is the main producer of spinach in the United States, while smaller-scale production takes place in Texas, Oklahoma, New Jersey, Colorado, Maryland, New York and Ohio (Koike and others 2011). Spinach prices vary throughout the season from between $238/ton to $351/ton (Koike and others 2011). California and Arizona are the main producers of Iceberg lettuce, with California's peak production season in May and June, while Arizona's is in December through February (Turini and others 2011). Prior to the series of leafy green outbreaks in the fall of 2006, the pre-washed salad market brought in $2.6 billion annually, with the spinach industry carving out $286 million (Todd and others 2007). Earthbound Farms, under its parent company Natural Selection Farms, had grown from selling spinach at a roadside stand in 1986 into a $360 million industry (Todd and others 2007).

The leafy green industry as a whole was hit particularly hard after the outbreaks, with sales plummeting due to the media frenzy that swept the nation during the outbreaks and slow rate of the outbreak investigation. By mid-October of 2006, Natural Selection's sales of conventional salads were down by 70% and down by 10% for products sold under the Earthbound Farms name (Todd and others 2007). The company was forced to lay off 164 employees and it has been estimated that Earthbound Farms and Dole may have to pay up to $110 million to settle cases with the victims of the outbreaks (Todd and others 2007).

Changes to regulations for the produce industry are in the works. With the FDA Food Safety Modernization Act (FSMA) signed into law in 2011, the FDA is required to issue minimum standards for produce safety. It is likely that fresh-cut produce processors will be required to validate that their washing process, i.e. their preventative control, is effective at preventing cross-contamination of pathogenic microorganisms (FDA 2011).

1.5 Post-harvest processing sources and spread of contamination

The increasing frequency of reported foodborne outbreaks associated with fresh fruits and vegetables is of major concern in the United States. These outbreaks are the driving force for changes needed by the produce industry in the way products are grown, harvested, and processed. Contamination can occur at any point between the farm and the fork. Produce can become contaminated on the farm through fecal contamination from wild and domestic animals, water runoff from livestock operations, green or improperly composted manure, air (dust), insects, contaminated irrigation water, or poor handling practices by the workers during the harvesting process (Beuchat and Ryu 1997). Even when contamination occurs at the growing or harvesting stages, conditions during post-harvest processing can intensify and spread what would normally be just a small, contained contamination event, resulting in a widespread outbreak (Beuchat and Ryu 1997 and Burnett and others 2001).

After harvesting, microbial contamination can come from many different sources such as the water used for cooling and washing, equipment surfaces and workers. The equipment used in leafy green processing facilities varies, but most processors use the same general types of equipment. Figure 1.3 depicts some typical equipment pieces used during leafy green post-harvest processing including a shredder, conveyer belt, flume tank, shaker table, and dewatering centrifugal dryer. Microbial contaminants can spread to multiple batches of product during processing (e.g., washing, peeling, shredding, slicing, drying and sorting) which can lead to a potential outbreak of illness if the contaminant is a human foodborne pathogen. Table 1.2 shows sources of post-harvest processing contamination for fresh produce.

Figure 1.3: *Leafy green processing equipment (clockwise from top left): lettuce shredder, conveyer belt, flume tank, shaker table, plastic collection basket and dewatering centrifuge*

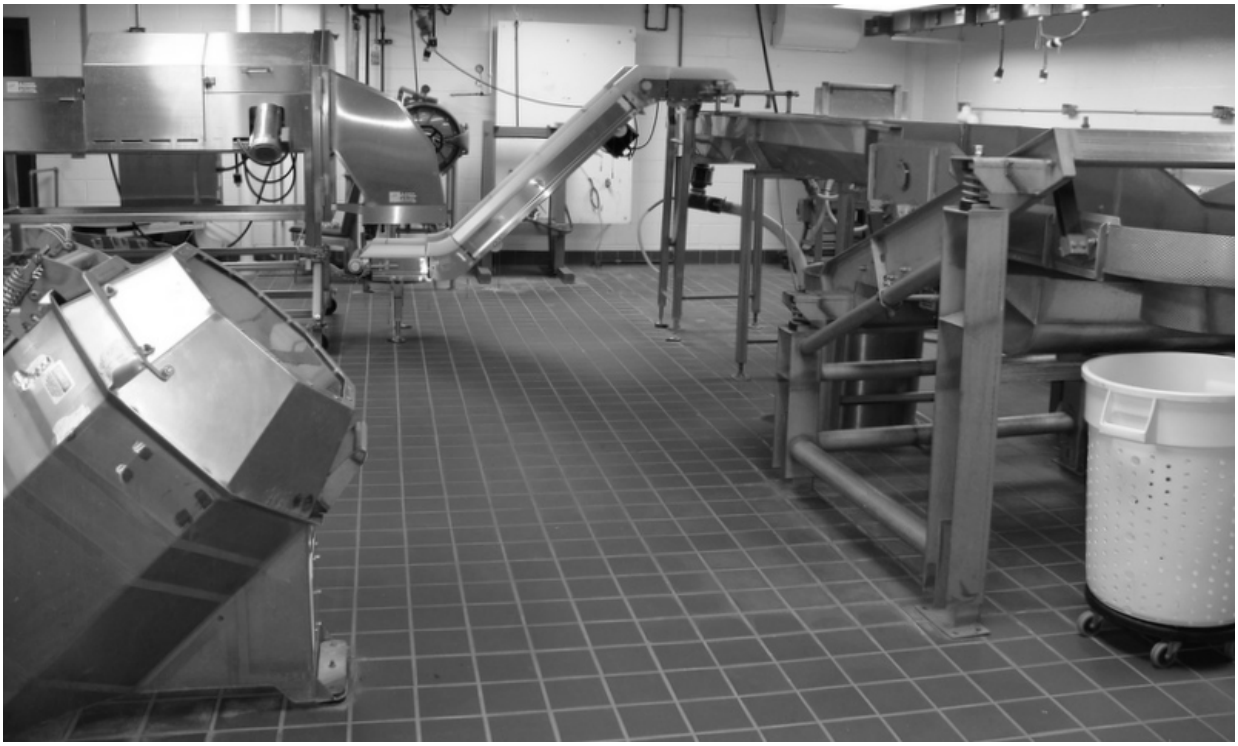


Table 1.2: *Sources of processing contamination*

Stage	**Contamination Sources**	
Post-harvest Processing	Cooling:	Water Ice Vacuum Cooling
	Transport:	Human Handling Shipping Vehicle Contamination Storage Containers
Fresh cut and Value-added Processing	Debris Removal:	Human Handling Processing Equipment: - Sifters, Rollers
	Conveyer:	Conveyer belt
	Washing:	Water
	Value-added Processing:	Processing Equipment: - Shredder, Core Removal, etc.
	Drying:	Processing Equipment: - Shaker table, Centrifugal dryer
	Packaging:	Human handling Packaging materials Packing equipment: - Scales, etc.

Immediately after harvest, many types of produce, including leafy greens must be cooled to remove field heat and preserve product quality (Kays 1997). Rapidly cooling fresh produce to refrigeration temperatures will minimize the growth of both spoilage and pathogenic bacteria. Commonly used commercial precooling methods for leafy greens include vacuum cooling, and hydro cooling or ice cooling (Boyhan 2004). Both spoilage and pathogenic bacteria can be transmitted via air, condensate and water droplets to the produce during the precooling phase. Doering and others found that the amount of time that passes before cooling iceberg lettuce and baby spinach was significant in controlling the *E. coli* population. They showed that the lower field temperature (25 °C) and the shortest time held before cooling (0 h) adversely impacted the *E. coli* counts when compared to the higher field temperature (32 °C) and longer time (10 h) before forced air cooling (2009).

Vacuum cooling, which is based on evaporative cooling of a product under low pressure, can be used to rapidly decrease the field temperature of many fruits and vegetables and is now the method of choice for leafy greens. However, this cooling method may lead to the internalization of both spoilage and pathogenic bacteria. A recent study found that vacuum cooling increased the infiltration of *E. coli* O157:H7 (considering both cell numbers and the depth of penetration) into lettuce tissue by more than 90% when compared to the non-vacuum cooled product with these internalized cells protected during quadruple washing of the product in a produce sanitizer (Li and others 2008). However, Doering and others (2009) failed to see any change in *E. coli* O157:H7 populations before and after vacuum cooling.

Water-based cooling methods carry a much greater risk of contamination, with the microbial populations based on the initial numbers of microorganisms in the water and the numbers of microorganisms transferred between the product and the water. Potable water,

defined as water that has a coliform count of less than 1 CFU/ml, an aerobic plate count of less than 100 CFU/ml and is free of *Cryptosporidium, Giardia lamblia, Legionella* and enteric viruses (EPA 2009), should be used in all cases, including when making ice. In a series of studies looking at the transfer of *E. coli* O157:H7 first from the contaminated lettuce leaves to uncontaminated lettuce leaves via the melting ice and then from melting ice to Romaine lettuce, it was shown that as the ice melted, water transferred *E. coli* O157:H7 from inoculated sites on the leaf to the uninoculated sites on all of the three stacked romaine lettuce heads within one container when the top head contained the inoculated site. Most sites on the Romaine lettuce became contaminated with *E. coli* O157:H7 from the melting ice with populations averaging 3.8 and 5.5 CFU/cm^2 for experiments conducted at 4 and 20 °C, respectively. In the same series of experiments, when the melting ice was contaminated with *E. coli* O157:H7 at 7 log CFU/ml, *E. coli* O157:H7 populations of 3.5 to 3.8 log CFU/cm^2 were seen on the top leaf of the first of the three heads, with lowest counts on the bottom layer of romaine heads with no difference in numbers of *E. coli* O157:H7 recovered from each sampling site at 4 and 20 °C (Kim and Harrison 2008).

Various chemical sanitizers can be added as appropriate to reduce microbial populations in the water (Beuchat and others 2004, Burnett and others 2004, Keskinen and others 2009, Rangaranjan and others 2000, Sapers 2001, Weissinger and others 2000, Zhang and others 2009). However, the sanitizer concentration must always be maintained at an effective level and be carefully monitored to ensure that the organic load does not decrease the efficacy of the sanitizer. Internalization of both spoilage and pathogenic bacteria during water cooling of fresh produce continues to be a major concern, particularly when the temperature of the produce exceeds that of the water by 10 °F or more (Rangaranjan and others 2000).

Human foodborne pathogens, including *E. coli* O157:H7, *Salmonella*, *Listeria* and *Cryptosporidium* that may inadvertently contaminate fresh fruits and vegetables in the field or at harvest, can be readily transferred to much larger quantities during subsequent product handing and further processing, raising even greater food safety concerns. It has been demonstrated that foodborne pathogens can also contaminate the product during processing. In one outbreak of salmonellosis traced to shredded lettuce, Stafford and others (2002) recovered *Salmonella* Bovismorbificans phage type 32 from the cutting wheel of a mechanical shredder during an environmental audit with insufficient cleaning and sanitizing of the shredder cited as a key factor in this outbreak. In a study examining the microbial changes of "Lollo Rosso" lettuce during processing and shelf-life, an increase of 1 log CFU/g was observed after shredding (Allende and others 2004), indicating that the shredder may be an important in-plant vehicle for spreading contamination to leafy greens during processing (Garg and others 1990).

In a survey of Japanese ready-to-eat fresh vegetable processors, populations of mesophilic aerobic bacteria exceeded 5.0 log CFU/cm^2 or ml in many samples collected from product contact surfaces of the washing, slicing, dehydrating, and blending equipment, surfaces of the slicer/shredder blades, and the processing room floor, all of which were obtained at the end of processing (Kaneko and others 1999). In another survey conducted in Spain, populations of mesophilic aerobic bacteria, psychrotrophic bacteria, yeast/ mold, and lactic acid bacteria on unprocessed (whole) vegetables, mainly leafy greens, were 1.1, 1.2, 0.7, and 2.6 logs lower, respectively, than their fresh cut counterparts (Abadias and others 2008).

Leafy green processing leads to plant tissue damage, which Seo and Frank have demonstrated allows *E. coli* to preferentially attach to cut plant edges, including the trichomes, stomata, and cuticle cracks. They showed E. coli 20 to 100 μm below the surface of the leaf,

inside the stomata and cut surfaces (1999). Using confocal scanning laser microscopy, this same group showed that *E. coli* can penetrate the leaf at the cut tissue surfaces that lack a waxy coating (Takeuchi and others 2000). Mechanically damaged Romaine lettuce leaves that were cut or shredded showed a 4.54 and 11.05-fold increase in *E. coli* O157:H7 compared to intact leaves, in which populations only increased by 1.95-fold after a 4 h hold time (Brandl 2008). Romaine lettuce lysates obtained after mechanical damage, promote the growth of *E. coli* O157:H7 with a growth rate equivalent to that of minimal medium with glucose. A microarray-based whole genome transcriptional profiling analysis showed that these *E. coli* expressed genes indicating they were adjusting to the alternate nutrient source and to a variety of stresses, including the presence of reactive oxygen species, antimicrobial compounds, toxic compounds and osmotic stress. Stress adaptation may also result in the enhanced resistance to chemical sanitizers (Kyle and others 2010).

Fruit and vegetable processing typically involves the use of various types of conveyor belt systems manufactured from different belting materials including high density polyethylene, polypropylene and acetyl. Two main conveyor belt designs – interlocking (a series of interlocking pieces that contain many microbial harborage sites) and smooth (a continuous belt with a single seam) can be found in the produce industry with the belting material and design tailored to the specific product and application. Regardless of the belt type or material, all conveyor belts are prone to microbial build-up and the subsequent transfer of microorganisms to incoming product over time. The newer smooth continuous belts, which can be more easily cleaned and sanitized, are now generally preferred over the older interlocking belts that must be dissembled and then manually cleaned and sanitized.

Some produce destined for the ready-to-eat market, including baby spinach and fresh -cut lettuce, can be commercially washed up to three times in various types of flume tanks to remove soil and decrease the levels of microorganisms. In general, washing fresh produce in water alone will only decrease microbial populations by 90 to 99% (Sapers 2001). Therefore, flume and wash water are ideal vehicles for the spread of microorganisms throughout an entire batch of product. This is even truer in Europe where processing leafy greens without the addition of a sanitizer is a common practice *(*Holvoet and others 2012*)*. Unfortunately, when added to flume and wash water, the commonly used chlorine-based sanitizers as well as most others including peroxyacetic acid, chlorine dioxide and ozone have been shown to be only marginally effective (Gil and others 2009; Sapers 2006). Most industrially used sanitizers are best suited for reducing microbial populations in the wash water rather than those on the product being washed. During continued processing, the level of soil and debris, referred to as the organic load, in the recirculating wash water will increase as additional product is processed. Since chlorine will react with any organic material, most chlorinated sanitizers exhibit decreased antimicrobial activity as the organic load in the water increases, with the potential survival of bacterial pathogens and other microorganisms in the wash water becoming a concern. Other alternatives to chlorine include peroxyacetic acid, chlorine dioxide and ozone (Sapers 2001).

When lettuce was sprayed with an aqueous solution containing 200 or 2,000 ppm chlorine, populations of *Salmonella, E. coli* O157:H7, *Listeria monocytogenes,* yeasts and molds, and mesophilic aerobic bacteria on the products decreased only 0.35 and 2.30 log CFU/cm^2 (Beuchat and others 1998). After a 10 min exposure to an aqueous solution of 200 ppm chlorine at 4 and 22°C, *L. monocytogenes* populations decreased only 1.3 and 1.7 log CFU/g on lettuce, respectively (Zhang and Farber 1996). Doering and others (2009) were only able to achieve a <1

to 3 log CFU/g reduction of *E. coli* O157:H7 on iceberg lettuce and baby spinach after washing in a 50 ppm free chlorine solution. These findings indicate that the bacteria which are not being killed on the leafy greens could potentially cross-contaminated uncontaminated product that follows it through the processing line.

After fluming and washing, excess water must be removed before packing through the use of shaker tables, blowers, or centrifugal dryers or by other means to maintain product quality and an acceptable shelf-life. All drying methods are product dependent with the end goal being the preservation of both product quality and shelf-life. Whereas iceberg and romaine lettuce can withstand the forces associated with centrifugal drying, other products such as baby spinach and parsley would be severely damaged. One study conducted at the U.S. Department of Agriculture (USDA) looked at plant lesions and their ability to promote growth of *E. coli* O157:H7 on mechanically bruised/damaged post-harvest Romaine lettuce leaves. Bruised plant tissue, leaves cut into larger pieces and leaves shredded into multiple smaller pieces showed a 3.99, 4.54 and 11.05-fold increase in *E. coli* O157:H7, respectively, compared to the intact leaves which only increased by 1.95-fold after leaves had been held for 4 h at 28°C (Brandl 2008). The increased growth of *E. coli* on the damaged plant tissues may be related to *E. coli*'s enhanced ability to attach to cut surfaces (Takeuchi and others 2000) as well as the availability of nutrients leaching out of the broken plant cells.

The potential for cross-contamination exists during all stages of transport from the field to the consumer's plate. Wooden or plastic totes, crates, and other types of storage containers inevitably come in contact with soil or other debris in the field at the time of harvest. They are an important source of microbial contamination when transported to processing facilities. Many produce growers are now using plastic instead of wooden containers for transport and storage

due to the increased ease of cleaning and sanitizing. Ailes and others (2008) found that when compared to produce samples taken directly from the field, those items collected from the packing bins had over a six-fold increase in likelihood of *E. coli* contamination and only a four-fold increase in likelihood for samples that originated from the box or conveyor belt.

1.6 *E. coli* attachment, persistence and biofilm formation on surfaces

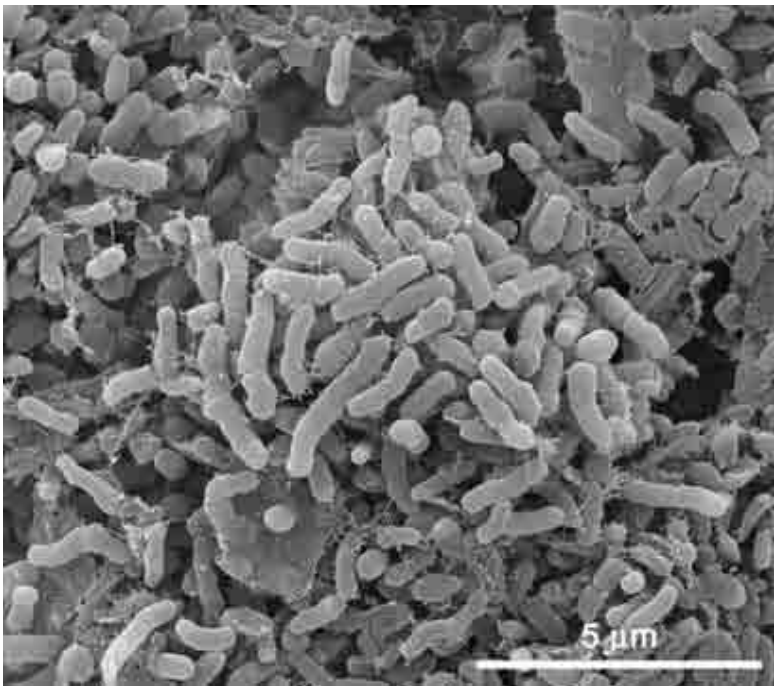
The mechanisms that *E. coli* O157:H7 uses to attach to leafy greens surfaces are an increasing area of interest. One group in the United Kingdom (Shaw and others 2008) has recently demonstrated that EHEC strains of *E. coli* O157:H7 and non-O157:H7 both attach to the leaf surfaces of arugula, spinach and lettuce through the EspA filamentous type III secretion system (fT3SS) that has been shown to also play an important role in the organisms colonization of bovine and human hosts. This finding indicates that EHEC uses plant leaves solely as a transmission vector rather than acting like a plant pathogen. Research conducted at the University of Arizona (Xicohtencatl-Cortes and others 2009) showed that flagella also play a role in attachment because *fliC* gene mutants are significantly impaired in their ability to adhere to the surfaces of spinach and lettuce compared to the parental strains. Crevices and surface wounds on the leafy greens protect the organisms from the environment and any decontamination strategies used during processing. Adhesion was shown to be temperature and time dependent. They also explained that while intimin adhesion is vital for attachment to the host organism, it is not required for colonization of leafy greens. Based on this, colonization of leafy greens by EHEC strains of *E. coli* O157:H7 is likely a means to survive the processing environment and to allow transmission to the human host (Xicohtencatl-Cortes and others 2009).

Biofilms are defined as an aggregate of microbes that are embedded in an extracellular matrix, which encapsulates the cells and provides a physical barrier against environmental stresses such as chemicals (sanitizers), temperature changes, desiccation or ultraviolet rays (Annous and others 2009, Ryu and others 2005, Tarver 2009). A major concern in the food industry is the formation of these biofilms on processing equipment. *E. coli* are capable of producing biofilms on inert surfaces such as: glass, stainless steel, high density polyethylene, polyamide-6, polyvinyl chloride, teflon coupons, glass wool, polystyrene microtiter plates, and glass coverslips (Annous and others 2009). A large portion of a food processing plant uses equipment composed of stainless steel, because of the durability of the material. Specific strains may be more difficult to remove/kill on stainless steel food contact surfaces and more aggressive sanitizing and cleaning procedures are necessary (Ryu and Beuchat 2005, Ryu and others 2004). In a recent study it was shown that when *E. coli* O157:H7 is allowed to become incorporated into a biofilm of other good biofilm-forming companion strains, they show greater resistance to peroxide based sanitizers compared to planktonic cells (Uhlich and others 2010). If biofilms are not removed or prevented portions of these bacterial aggregates persist as transient residents on equipment surfaces throughout the processing line and slough off over time to contaminate large quantities of previously uncontaminated product during processing

Once present on the food contact surfaces of processing equipment, bacteria can transfer to the produce during processing. In a field-coring study conducted at the University of Georgia, when field coring devices were inoculated with soil containing *E. coli* O157:H7 populations of 2.72 and 1.67 log CFU/g the pathogen transferred to 10 and 5 consecutively processed heads of lettuce, respectively (Taormina and others 2009). Moore and others (2003) demonstrated the ability of *Campylobacter jejuni* and *Salmonella enterica* serovar Typhimurium to transfer from

stainless steel surfaces to both wet and dry lettuce via direct contact with 66% percent of the *Salmonella* population transferring from a dry stainless steel surface to a dry product after 60 minutes of direct contact. In contrast, 23 to 31% of the *Salmonella* population transferred from dry stainless steel to wet lettuce after 120 minutes of direct contact. Transfer of *C. jejuni* ranged from 16 to 38% for dry lettuce and 15 to 27% for wet lettuce after 80 minutes of contact. These results show the ability for *C. jejuni* and *Salmonella* Typhimurium to persist on surfaces and maintain the ability to transfer to food surfaces hours after they become contaminated. Bacterial attachment and biofilm formation on the surfaces of leafy greens greatly hinder the removal of microbial contaminants during conventional washing (Carmichael and others 1999, Sapers 2006). When Lang and others (2004) assessed the impact of post-inoculation drying times on *E. coli* O157:H7 survival on iceberg lettuce and parsley, higher (or equal to) populations were recovered from the leafy greens after 2 h of drying at 22°C rather than 2 h drying at 22°C followed by 22 h at 4°C. Using scanning electron microscopy (SEM), another study showed biofilm formation on the stem ends of freshly harvested lettuce leaves after submersion in an *E. coli* cocktail for 1 week at 4°C (Figure 1.4) (Annous and others 2009). SEM is frequently used to observe the survival, colonization and biofilm formation of microbes on the surface of produce (Annous and others 2009, Carmichael and others 1999, Keskinen and others 2009 and Morris and others 1997). These results suggest that transfer of *E. coli* O157:H7 to processing equipment should be examined by SEM and microbiological analysis both after a short and long post-inoculation holding time. This would determine if transfer also follows the same trend as in a study by Lang and others (2004) and decreases after longer holding times.

Figure 1.4: *An SEM (Scanning electron microscopy) showing attachment and biofilm formation of* E. coli *cells on a lettuce leaf (Annous and others 2009)*



1.7 Inoculation of leafy greens and recovery of bacteria

To study the degree to which transfer occurs between leafy greens and the processing environment, product needs to be coated with the inoculum consistently to ensure accurate results that could be replicated and used for predictive modeling. Lang and others (2004) studied the effects of different inoculation methods on survival and recovery of foodborne pathogens on iceberg lettuce and parsley. Their results showed that when using either an *E. coli* O157:H7 or *Salmonella* cocktail, more bacteria were recovered from the dip-inoculated produce than from spot- or spray-inoculated produce, due to a greater numbers of bacterial cells attaching initially.

When inoculating large quantities of product for a pilot scale study, safety is essential to prevent laboratory-acquired infections and avoid escape to the laboratory or research environment. One common work around to avoid using large quantities of a dangerous pathogen

is to use an avirulent strain as a surrogate for the virulent outbreak strain. This technique has become especially common in studies taking place in a processing environment or field. Erickson and others (2010) have demonstrated the application of four avirulent *E. coli* O157:H7 strains (ATCC 43888, CV2B7, 6980-2, and 6982-2) while studying surface and internalization on field grown spinach and lettuce treated with spray-contaminated irrigation water. Shepherd and others (2011) have also applied avirulent strains in a study assessing the use of physical coverings to control *E. coli* at the surface of a compost heap. In another study, a comparison was done between avirulent *E. coli* O157:H7 and generic *E. coli* isolated from irrigation water, soil and lettuce (Tomás-Callejas and others 2011). They showed that populations of both the generic and avirulent strains rapidly declined on the spray inoculated fresh-cut baby leafy greens (Mizuna, Red Chard and Tatsoi), but that minimal processing was not able to disinfect the products (Tomás-Callejas and others 2011). In addition to the avirulent feature, these strains have been transformed with a pGFPuv plasmid containing a green fluorescent protein (GFP) gene and an ampicillin-resistance gene to allow for differentiation from native *E. coli* strains that would be found in the filed environment. To ensure that these labeled strains maintain the plasmid during the study, the stability is tested through a series of propagations without selective pressure and the rate of plasmid-loss is determined. Most GFP labeled *E. coli* O157:H7 have proved to be very stable according to Ma and others (2011). After two consecutive subcultures (~2 generations) in a nonselective media, the rate of plasmid loss for labeled *E. coli* O157:H7, *Salmonella* and *Listeria* strains tested in this study were 0 – 30%, 15.8 – 99.9% and 8.1 – 93.4%, respectively (Ma and others 2011).

Once the inoculated leafy greens are processed, a viable recovery method for pathogens must be implemented. Sampling/swabbing the surfaces of processing equipment pieces creates

unique hurdles. Vorst and others (2004) determined which of four sampling devices, a sterile environmental sponge, a sterile cotton-tipped swab, a sterile calcium alginate fiber-tipped swab, and a one-ply composite tissue, would recover the most *Listeria monocytogenes* from food-grade stainless steel surfaces. They showed that using one-ply composite tissue, rehydrated in 0.1% peptone before swabbing and vortexed in 0.1% peptone for 1 min. after swabbing, showed statistically more recovery that the other methods. As the authors point out, this method is both inexpensive and easy to use and will be very useful for sampling the bacteria that have transferred to the equipment surfaces from the inoculated leafy greens during processing.

1.8 Bacterial transfer in other food environments

Bacterial transfer has also been demonstrated in various other food preparation scenarios including the grinding of meat (Farrell and others 1998; Flores and Stewart 2004, Flores and Tamplin 2002), slicing deli meats (Keskinen and others 2008a; Keskinen and others 2008b; Lin and others 2006; Sheen and Hwang 2008; Vorst and others 2006), between cutting boards (Chai and others 2008; Chen and others 2001; Fravalo and others 2009; Jiménez and others 2009; Ravishankar and others 2010), and through the use of cloths, hands, gloves, utensils and various food products (Chen and others 2001; Gill and Jones 2002; Montville and others 2001; Scott and Bloomfield 1990). Bacteria transfer in a small ground beef table-top grinder demonstrated a linear relationship between the amount of inoculum and the beef trim added to the grinder and the amount of contaminated ground fractions collected. Contaminated ground beef was added to the grinder after an estimated 20% of the beef had entered the grinder, but was collected from the grinder after just 8% of the beef had been collected, so before the point at which the inoculated beef was expected to exit the grinder. This indicates that the bacterial transfer takes

place in part from the conveying, mixing, extrusion and cutting stages of processing and that product does not move though the grinder linearly (Flores and Tamplin 2002). Building on this work, Flores and Stewart conducted an experiment to track *E. coli* O157:H7 during mid-sized commercial grinding of inoculated followed by uninoculated beef. They were able to detect *E. coli* in 12.7 to 86.2 % of the ~6600 g beef trim batch using initial inoculums of 2 to 6 log CFU/g. As the inoculum level increased, the level of contamination in the ground beef increased. Using a Chi-squared algorithm based distribution model, these researchers also showed that the contamination level was a function of the ground beef batch fractions collected (Flores and Stewart 2004).

One notable study measured the distribution of *L. monocytogenes* during slicing of roast turkey breast, Genoa hard salami, and bologna with kitchen knives. These researchers demonstrated that the majority for bacterial transfer takes place within the first 5 to 15 slices depending on the stainless steel type and product being cut (Vorst and others 2006). In a similar fashion, this research group also demonstrated that *L. monocytogenes* counts dropped between 3 and 5 log CFU/g after slicing 16 slices roast turkey breast, even at very low inoculation levels (Keskinen and other 2008b). In agreement with these studies, *L. monocytogenes* was shown to transfer to a larger number of meat samples when the inoculation level was higher (Lin and others 2006). Biofilm-forming ability and inoculation hold time have also been shown to impact transfer of *L. monocytogenes* to deli meats, with significantly more transfer taking place when strong biofilm-formers were used as the inoculum verses the weaker strains and greater transfer taking place 6 h post-inoculation rather than after 12 h (Keskinen and other 2008a). A non-linear model for these *L. monocytogenes* cross contamination scenarios has been developed by Sheen

and Hwang to predict the number of ham slices that may become contaminated by a *L. monocytogenes*-contaminated slicer blade (2008).

Bacterial transfer readily occurs in commercial food processing environments, foodservice establishments and household kitchens. *Campylobacter jejuni* was shown to transfer from vegetables to wash water (30.1 – 38.2%), wash water to cucumbers (26.3 – 47.2%), vegetables to the cutting board (1.6 – 10.3%) and from the cutting board to cucumbers (22.6 – 73.3%), while simulating the preparation of a salad in a household kitchen (Chai and others 2008). The log percent transfer of *Enterobacter aerogenes* from chicken to hands (0.94%), a cutting board to lettuce (0.90%), the faucet to hands (0.36%), hands to lettuce (-0.12%), unwashed hands to washed hands (-0.20%), and from hands to the faucet (-0.80%) has also been recorded (Chen and others 2001). Temperature did not affect the transfer of *Salmonella* to a cutting board, but the amount of time the inoculated product remained in contact with the cutting board did impact transfer (Jimenez and others 2009). One study showed that 60.5% of the *Campylobacter* on naturally contaminated chicken legs transferred to a cutting board with this number increasing to 80.6% if the contaminated chicken legs remained on the cutting board for over 10 min (Fravalo and others 2009). Proper sanitation of kitchen utensils is needed to prevent cross contamination, Ravishankar and others (2010) studied different scenarios where *Salmonella enterica* could move between cutting boards, knives, chicken and lettuce. They showed that when knives and cutting boards are not washed after cutting chicken inoculated with 6 log CFU/g and used for slicing lettuce, ~ 2.97 log CFU/g of the *Salmonella enterica* is able to transfer. If the utensils are quickly rinsed prior to slicing, there is a ~2.36 log CFU/g transfer, while thoroughly washing the utensils with soapy, hot water results in less than 1 log CFU/g detectable on the lettuce. Gloves may be an effective means to reduce the bacterial transfer

between food and the hands of foodservice employees and in transfer from contaminated hands back to food as was demonstrated in a study looking at transfer between chicken, lettuce, bare and gloved hands. Montville and others (2001) showed that 0.01% *Enterobacter aerogenes* (a nonpathogenic surrogate with similar attachment characteristics to *Salmonella*) transfer still takes place from food to hands and from hands to food when the volunteers wore gloves. Significantly more transfer (10%) took place without the glove barrier.

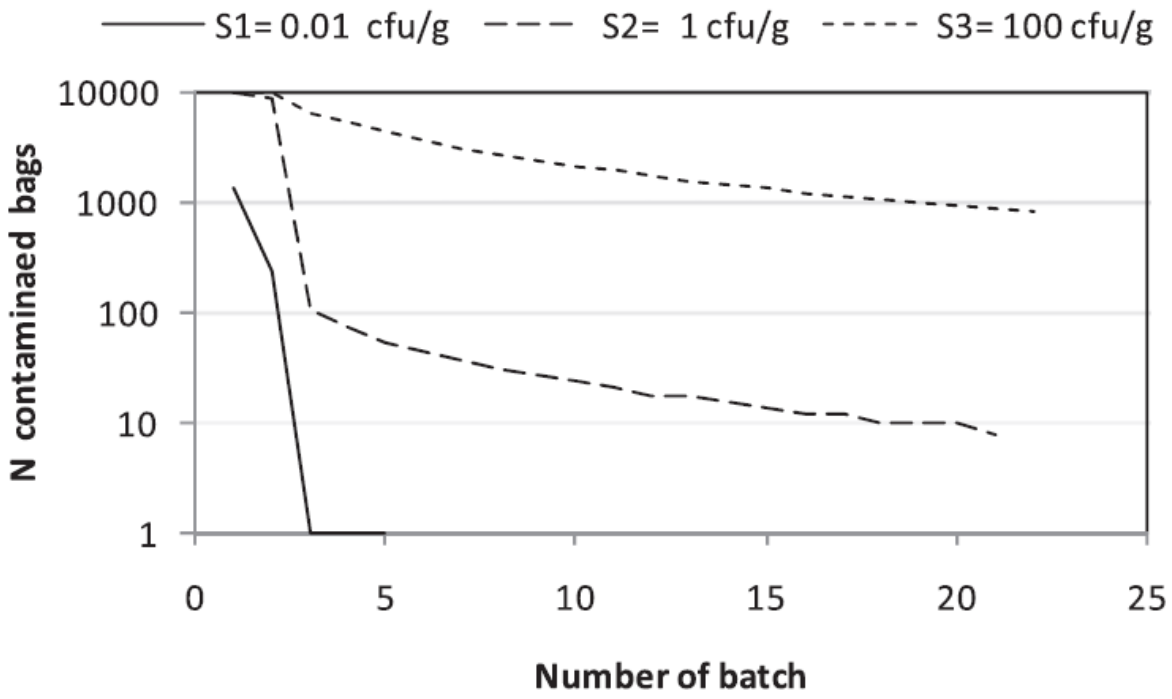
1.9 Predictive modeling of bacterial transfer

A mathematical risk model for use in a leafy green processing line was created by Pérez Rodríguez and others (2011) which looked at *E. coli* O157:H7 cross-contamination in three different contamination scenarios, to estimate the prevalence and percent of *E. coli* O157:H7 transferred during a cross contamination event in fresh-cut bagged leafy greens. In this simulation, the model's process parameters were locked to ensure comparable data among the different simulations. These parameters consisted of 22 batches in a day's product, with a batch size of 1000 kg and a bag size of 100 g. Initial concentrations of the contamination were 0.01, 1 and 100 CFU/g. Based on their simulation, when a low concentration of the contamination enters the processing line, product that is processed in the first hour will become contaminated with subsequent bags of product being sporadically contaminated. The prevalence rate will increase with the inoculum level. This simulation predicts that, when processing a batch of leafy greens inoculated at 1 and 100 CFU/g in sanitizer-free wash water, the prevalence would be ~ 3.05 and 13.39 %, respectively. Based on the simulation, chlorination of the water would reduce this percentage to 0.14 – 0.20 % and 3.28 – 4.00 %, for the 1 and 100 CFU/g inoculation levels, respectively. As shown in Figure 1.5, the predictive model indicates that after a contaminated

batch enters the processing line, the lower the inoculum, the more gradual the decline in the number of cross-contaminated bags. In the simulation using the lowest inoculum, cross-contamination only occurs to the first three batches before dropping off steeply. At the medium inoculation level, cross-contamination to the first two batches was very high (10,000 cross-contaminated bags/batch), with a slight decline to only 100 cross-contaminated bags/batch, followed by a gradual drop-off to only 10 cross-contaminated bags/batch in the batch. The highest inoculation level yielded cross-contamination of between 10,000 and 1000 cross-contaminated bags/batch for the entire production run.

More research will need to be conducted to validate the predictive model by quantifying the extent of cross-contamination to subsequent product by tracking contaminated product though a fresh-cut leafy green processing line. In the event of an outbreak, these finding could be used as a guide to help estimate the amount of product which may have became cross-contaminated during processing and would need to be recalled.

Figure 1.5: *Simulated number of cross-contaminated bags along the production (22 batches) when one contaminated batch enters the processing line (Pérez Rodríguez and others 2011), for these different initial contamination levels (S1, S2, and S3)*



1.10 Overall goals and objectives

The number of documented cases of foodborne illness associated with consumption of produce, such as leafy greens, continues to rise. Consumers expect safe products, and the food industry must make changes to their current processing procedures to be able to provide these products to their customers. It is the researcher's duty to establish where adjustments can be made to help prevent future outbreaks. Without modifications to the present processing methods, leafy green outbreaks will continue to increase in both number and magnitude. The overall objective of this research is to quantify the extent of *E. coli* O157:H7 transfer between leafy greens, wash water and food contact surface areas during simulated commercial processing.

The following series of experiments were conducted to quantify the transfer of *Escherichia coli* O157:H7 between the leafy greens, the wash water and the food contact surfaces in a pilot-scale processing line that included a commercial shredder, conveyor, flume tank, shaker table and centrifugal dryer. Using three different types of leafy greens (iceberg, Romaine lettuce and baby spinach) dip-inoculated with a 4-strain avirulent, GFP-labeled cocktail of *E. coli* O157:H7 at three different levels (10^2, 10^4 and 10^6 CFU/g), transfer coefficients were determined for the numbers of *E. coli* O157:H7 transferred between the product, the water and the equipment surfaces during processing. The effects of post-inoculation holding time and shred size on the transfer coefficients were subsequently evaluated. Inoculated Radicchio - used as a surrogate for contaminated leafy greens, was also tracked through the pilot-scale processing line to visually assess the spread of contaminated product during processing. Finally, the transfer rate between inoculated and uninoculated leafy greens was assessed during shaker table dewatering.

CHAPTER 2:

Quantitative Transfer of *Escherichia coli* O157:H7 to Equipment during Small-Scale Production of Fresh-Cut Leafy Greens

2.1 ABSTRACT

Post-harvest contamination and subsequent spread of *Escherichia coli* O157:H7 can occur during shredding, conveying, fluming and dewatering of fresh-cut leafy greens. This study quantified *E. coli* O157:H7 transfer from leafy greens to equipment surfaces during simulated small-scale commercial processing. Three to five batches (22.7 kg) of baby spinach, iceberg lettuce and romaine lettuce were dip-inoculated with a 4-strain cocktail of avirulent, green fluorescent protein (GFP)-labeled, ampicillin-resistant *E. coli* O157:H7 to contain ~6, 4, and 2 log CFU/g, and then processed after 1 h of draining at ~23°C or 24 h of storage at 4°C. Lettuce was shredded using an Urschel TransSlicer at two different blade and belt speeds to obtain normal (5 x 5 cm) and more finely shredded (0.5 x 5 cm) lettuce. Thereafter, the lettuce was step-conveyed to a flume tank, washed and then dried using a shaker table and centrifugal dryer. Product (25 g) and water (40 ml) samples were collected at various points during processing. After processing, product contact surfaces (100 cm^2) on the shredder (n = 14), conveyer (n = 8), flume tank (n = 11), shaker table (n = 9) and centrifugal dryer (n = 8) were sampled using 1-ply composite tissues. Sample homogenates diluted in phosphate or neutralizing buffer were plated, with or without prior 0.45 μm membrane filtration, on trypticase soy agar containing 0.6% yeast extract supplemented with 100 ppm ampicillin to quantify GFP-labeled *E. coli* O157:H7 under UV light. During leafy green processing, ~90% of the *E. coli* O157:H7 inoculum transferred to the wash water. After processing, *E. coli* O157:H7 populations were highest on the conveyor and shredder ($P < 0.05$), followed by the centrifugal dryer, flume tank and shaker table, with ~29% of the remaining product inoculum lost during centrifugal drying. Overall, a significantly ($P < 0.05$) higher percentage of the inoculum was shed from 1 h (92%) compared to 24 h-held lettuce (58%) after centrifugal drying ,with shred size not impacting the rate of transfer.

2.2 INTRODUCTION

Given the current emphasis on healthier dietary habits in the US, including increased consumption of salads, foodborne outbreaks associated with leafy greens unfortunately are becoming more frequent (HHS/USDA 2005). Consumption of leafy greens increased only 9% from 1996 to 2005 compared to the previous decade; however, the number of leafy green-associated outbreaks increased 38.6% during this same period (Herman and others 2008). Consequently, consumer confidence in the safety of leafy greens has decreased. In 2009, the Center for Science in the Public Interest (CSPI 2009b) ranked leafy greens, including spinach, at the very top of "The 10 Riskiest Foods."

Changes in agricultural practices including large centralized production and harvesting practices are partly responsible for the increasing number of leafy greens outbreaks. Since 1990, 19 outbreaks were specifically associated with the consumption of leafy greens (CSPI 2008a), many of which were traced to produce grown on large-scale farms in the Salinas Valley of California. In September 2006, contaminated pre-bagged baby spinach triggered an *E. coli* O157:H7 outbreak in 26 states and Canada, resulting in five deaths and 205 illnesses (Jay and others 2007). A- few months later, two additional *E. coli* O157:H7 outbreaks were traced to California-grown iceberg lettuce that was shredded and then purchased by two fast-food Mexican restaurant chains in the Midwest and Northeast (FDA 2006, FDA 2007). These two outbreaks sickened a combined total of over 150 individuals (CSPI 2008b) and raised nationwide concerns regarding the safety of leafy greens along with the various routes for contamination.

Microbial pathogens can contaminate fresh produce at any point from farm-to-fork, with sources of contamination in the field including feces from wild and domestic animals, water runoff from livestock operations, green or improperly composted manure, air (dust), insects, and

irrigation water. During and after harvest, pathogens can come in contact with fresh produce through field workers, harvesting equipment, hand coring/trimming, and water used for cooling or washing, as well as improperly cleaned crates, totes and storage bins and the vehicles used for transport (Beuchat 1996, Beuchat and Ryu 1997, Gorny 2006). While most pathogen contamination occurs in the field, conditions during post-harvest processing can intensify and spread what would normally be a small isolated contamination event to far larger quantities of product that can lead to widespread outbreaks (Beuchat and Ryu 1997, Burnett and Beuchat 2001). This was the case in a 2001 Australian outbreak of *Salmonella* Bovismorbificans that was later traced to residual product behind the cutting wheel rim of a lettuce shredder (Stafford and other 2002). Similarly, a Dutch processing plant was implicated in an *E. coli* O157 outbreak associated with shredded and pre-packaged lettuce, which sickened individuals in both the Netherlands and Finland (Friesema and others 2008), and a shigellosis outbreak in Texas caused restaurant patrons to become ill after consuming shredded lettuce, which may have become contaminated during processing and further handling (Davis and others 1988).

Few strategies other than irradiation can ensure the safety of fresh-cut salad greens, with current washing and sanitizing practices reducing *E. coli* O157:H7, *Salmonella* and other pathogens only 1 to 2 log CFU/g, (Sapers 2006). Many factors associated with microbial attachment contribute to the ineffectiveness of these intervention strategies. Fett (2000) suggested that naturally occurring biofilms on sprouts may protect *Salmonella* and *E. coli* O157:H7 from inactivation by chemical sanitizers. During fluming and washing, *E. coli* also can penetrate into the cracks, crevices, cut/injured surfaces and intercellular spaces of lettuce (Seo and Frank 1999, Takeuchi and Frank 2000), making disinfection strategies or physical removal impossible.

Despite the continually increasing market for pre-bagged fresh-cut, ready-to-eat salad greens, the potential for spread of both pathogenic and spoilage microorganisms during commercial shredding, conveying, washing and dewatering of leafy greens is still poorly understood. However, previous studies have quantified the numbers of bacteria transferred during field coring of leafy greens (Taormina and others 2009), grinding of meat (Farrell and others 1998, Flores and Tamplin 2002, Flores and Stewart 2004) and slicing of deli meats (Keskinen and others 2008a, Keskinen and others 2008b, Lin and others 2006, Sheen and Hwang 2008, Vorst and others 2006), as well as between cutting boards (Chai and others 2008, Chen and others 2001, Fravaol and others 2009, Jimenez and others 2009, Ravishankar and others 2010), cloths, hands, gloves, utensils and various food products (Chen and others 2001, Gill and Jones 2002, Montville and others 2001, Scott and Bloomfield 1990).

The objectives of this study were: 1) to quantify the transfer of an avirulent GFP-labeled 4-strain cocktail of *E. coli* O157:H7 from inoculated iceberg lettuce, Romaine lettuce and baby spinach to the water and different pieces of equipment used for commercial processing of fresh-cut leafy greens, and 2) to assess the impact of post-inoculation hold time and lettuce shred size on *E. coli* O157:H7 transfer. However, before beginning this work, the growth rates and attachment forming abilities of the four GFP-labeled, avirulent *E. coli* O157:H7 strains were compared to three virulent *E. coli* O157:H7 leafy green outbreak strains to ensure that the GFP genetically engineered strains behaved identically to the parent strains.

2.3 MATERIALS AND METHODS

2.3.1 Experimental design. Three to five batches (22.7 kg) of baby spinach, iceberg and Romaine lettuce were dip-inoculated with *E. coli* O157:H7 to contain ~10^6, 10^4 and 10^2 CFU/g, and processed by shredding, conveying, fluming, shaker table dewatering and/or centrifugal drying, during and/or after which various product, water and equipment surface samples were collected for *E. coli* O157:H7 quantification. The impact of post-inoculation hold time and shred size on *E. coli* O157:H7 transfer during processing was also determined using triplicate batches of iceberg lettuce inoculated at 10^4 CFU/g.

2.3.2 Leafy greens. Pre-washed, pre-bagged baby spinach (*Spinacia oleracea* L.) from 5 or 10 lb (2.3 or 4.5 kg) bags, as well as individually wrapped heads of iceberg (*Lactuca sativa* L.) (24 heads/case) and Romaine (*Lactuca sativa* L. var. *longifolia*) (12 heads/case) lettuce, were obtained from a local wholesaler (Stan Setas Produce Co., Lansing, MI), stored at 4 °C and used within 5 days of delivery. These three products originated from California or Arizona, depending on the growing season. All heads of lettuce were hand-cored immediately before use.

2.3.3 Bacterial strains. Four avirulent, GFP-labeled, ampicillin-resistant strains of *E. coli* O157:H7 (ATCC 43888, CV2b7, 6980-2, and 6982-2) and three virulent *E. coli* O157:H7 strains from three separate leafy green outbreaks in 2006 (K3995 - spinach, K4492 - lettuce, and K4831 - lettuce) were obtained from Dr. Michael Doyle at the Center for Food Safety, University of Georgia, Griffin, GA. Upon arrival, stock cultures of each strain were prepared in trypticase soy broth (Difco, Becton Dickinson, Sparks, MD) containing 0.6% yeast extract (Difco, Becton Dickinson) (TSBYE) and 10% (v/v) glycerol (Sigma Chemical Co., St. Louis, MO) and stored at -80°C until needed. Working cultures were prepared by streaking each stock culture on trypticase soy agar plates (Difco, Becton Dickinson) containing 0.6% yeast extract

(TSAYE). After 18 - 24 h of incubation at 37°C, a single colony was transferred to 9 ml of TSBYE and similarly incubated. TSAYE and TSBYE used to grow the four avirulent, GFP-labeled ampicillin-resistant *E. coli* O157:H7 strains were supplemented with 100 ppm ampicillin (ampicillin sodium salt, Sigma Life Science, St. Louis, MO) (TSAYE + amp and TSBYE + amp).

2.3.4 *E. coli* O157:H7 growth rates. For the growth rate study, 20 µl aliquots of the 4 avirulent and 3 virulent *E. coli* O157:H7 strains were separately inoculated in triplicate into flasks containing 200 ml of TSBYE + amp and TSBYE, respectively. Immediately after inoculation and again after 2, 4, 6, 15, 18, 21 and 24 h of incubation at 37°C without shaking, 100 µl aliquots were removed, appropriately diluted in sterile 0.1% phosphate buffer solution (PBS) and plated on TSAYE + amp (avirulent strains) or TSAYE (virulent strains) with all plates counted after 18 - 20 h of incubation at 37°C. The equation to calculate Generation time (T_d) was as follows:

$$T_d = (t_2 - t_1) * \log(2)/\log(q_2/q_1),$$

where q_1 is the number of *E. coli* at the t_1 and q_2 is the *E. coli* population at t_2.

2.3.5 Attachment. A modification of the microtiter plate assay described by Jackson and others (Jackson and others 2002) was conducted in triplicate to assess attachment ability. The seven *E. coli* O157:H7 strains were separately grown in 9 ml of colony forming antigen (CFA) medium containing (per liter) 10 g of casamino acids (BD Bacto™, Becton Dickinson), 1.5 g of yeast extract (BD Bacto™, Becton Dickinson), 50 mg of $MgSO_4$ (J.T. Baker, Mallinckrodt Baker Inc.), and 5 mg of $MnCl_2$ (J.T. Baker, Mallinckrodt Baker Inc.) for 18 h at 37°C, with this same medium supplemented with 100 ppm ampicillin for the GFP-labeled strains. Thereafter, each

culture was diluted 1:100 in fresh CFA and pipetted (200 µl) into triplicate wells of a 96-well untreated polystyrene microtiter tissue culture plate (BD Falcon Microtest™ Flat Bottom, Becton Dickinson, Franklin Lakes, NJ) with three wells per plate containing 200 µl of CFA with/without 100 ppm ampicillin serving as negative controls. Following 48 h of incubation at 22°C, the microtiter wells were emptied, stained with 200 µl of 2.0% crystal violet (Remel, Lenexa, KS) for 5 min, rinsed five times with deionized water and air-dried with the remaining dye resolubilized in 160 µl of 33% (v/v) glacial acetic acid (Sigma Chemical Co.). Optical densities were then read at 630 nm using a Synergy HT Microplate Reader (Bio-Tek Instruments, Inc, Winooski, VT).

2.3.6 Inoculation of iceberg lettuce, Romaine lettuce and baby spinach. A 0.2 ml aliquot of each avirulent *E. coli* O157:H7 strain was transferred to 200 ml of TSBYE + amp, incubated for 18 – 20 h at 37°C and then combined to obtain 800 ml of the 4-strain cocktail. Based on similar growth rates, the four strains were combined in equal volumes, after which 800, 8 and 0.08 ml of the cocktail was added to 75 L of tap water (~15^{o}C) in a 121 L plastic container (Rubbermaid, Wooster, OH) to obtain populations of ~10^7, 10^5 and 10^3 CFU/ml, respectively. Cored heads of unwashed iceberg (~24 heads) and Romaine lettuce (~48 heads) or commercially washed and bagged baby spinach leaves, each weighing 22.7 kg, were immersed in the *E. coli* suspension for 15 (iceberg and Romaine) or 1 min (baby spinach) to ensure that each leaf was uniformly inoculated. The heads of iceberg and Romaine lettuce were spun in a dewatering centrifuge (described below) to remove residual inoculum, and then drained/air-dried for 1 h at 22°C before processing. Baby spinach was drained and air-dried for 1 h before processing. Duplicate 25-g samples of each product were then aseptically collected to determine the initial inoculation level at the time of processing.

2.3.7 Impact of post-inoculation hold time on *E. coli* O157:H7 transfer. In order to assess the impact of post-inoculation hold time on *E. coli* O157:H7 transfer, three 22.7 kg batches of iceberg lettuce were inoculated at ~10^4 log CFU/g as just described and then drained/air-dried for 24 h in a 4^{o}C walk-in cooler before processing.

2.3.8 Processing equipment. A small-scale commercial leafy green processing line capable of processing ~3,500 kg/h was assembled that consisted of a lettuce shredder, step conveyer, flume tank, shaker table and dewatering centrifuge (Figures 2.1 – 2.5). The commercial lettuce shredder (model TRS 2500 Urschel TranSlicer, Valparaiso, IN) was operated at a feed belt/slicing wheel speed of 198 m/min and 905 RPM, respectively, to obtain a shred size of approximately 5 x 5 cm. The polyurethane step conveyer belt (ThermoDrive, Mol Industries, Grand Rapids, MI) on the conveyor (Dorner model 736018 mc series, Dorner Manufacturing, Hartland, WI) was operated at 0.11 m/sec. A stainless steel water recirculation tank (~1000 L capacity) was filled with 890 L of sanitizer-free tap water (~15^{o}C), because the objective was to quantify *E. coli* O157:H7 transfer rather than to assess inactivation by sanitizers during processing. This water tank was connected to a 3.6-m long by stainless steel flume tank (Heinzen Manufacturing, Inc., Gilroy, CA) - equipped with two overhead spray jets (1 m from the start) by a 4.14 m long, 10 cm-diameter hard plastic discharge hose and a centrifugal pump (model XB754FHA, Sterling Electric, Inc., Irvine, CA) that circulated the water at ~15 L/sec. The stainless steel shaker table for partial dewatering was operated by a 1 HP Baldor washdown duty motor (Baldor Electric Co., Ft. Smith, AR) at 1760 RPM. Water removed from the leafy greens during mechanical shaking passed through a fine mesh screen and was fed into the water holding tank by a water recirculation spout underneath the shaker table. A 50-lb (22.7-kg)

capacity centrifugal Spin Dryer (model SD50-LT, Heinzen Manufacturing, Inc.) with three internally timed spin cycles totaling 60 s was used for centrifugal drying.

2.3.9 Identification of product contact surfaces. A 0.35% (w/v) suspension of Glo Germ™ (Glo Germ Co., Moab, Utah) was prepared by dissolving 264 g in 1 L of 95% EtOH, which was then added to 75.7 L of distilled water. Uninoculated retail baby spinach leaves and heads of cored iceberg lettuce (22.7 kg each) were divided into five small batches of equal size and immersed in the Glo Germ™ suspension for 1 min. After draining and drying for 18-24 h at 4°C, the lettuce was processed through the entire processing line, beginning with shredding, whereas baby spinach processing began with fluming.

Immediately after processing, the entire line was viewed under ultraviolet light (365 nm, Model SB-100XR , Spectronics Corporation, Westbury, NY) to identify the major product contact points. Based on these observations, 14, 8, 11, 9 and 8 product contact surfaces on the shredder, conveyor, flume tank, shaker table and dewatering centrifuge, respectively, were targeted for subsequent quantitative recovery of *E. coli* O157:H7. Glo Germ™ was removed from the equipment surfaces with 95% EtOH before any *E. coli* O157:H7-inoculated product was processed, so as not to affect bacterial transfer.

2.3.10 Leafy green processing. Inoculated heads of cored iceberg and Romaine lettuce (22.7 kg) were hand-fed into the shredder, with the shredded product then step-conveyed to the flume tank for washing in 890 L of recirculating sanitizer-free water, partially dewatered on the shaker table, collected in a single centrifugation basket and centrifugally dried. Inoculated baby spinach (22.7 kg) was manually dumped into the flume tank, washed in the flume water, partially dried on the shaker table, collected in two centrifuge baskets and then centrifugally dried. Whole heads of iceberg and Romaine lettuce and baby spinach leaves (22.7 kg each) were processed at

a rate of about 0.35 kg per second, with the entire 22.7 kg of product ready for centrifugal drying after 60-70 sec.

2.3.11 Impact of shred size on *E. coli* O157:H7 transfer. Three 22.7 kg batches of cored iceberg lettuce were inoculated with the *E. coli* O157:H7 cocktail at ~10^4 log CFU/g. In order to assess the impact of a smaller shred size of approximately 0.5 x 5 cm on subsequent *E. coli* O157:H7 transfer, the feed belt and slicing wheel speeds were changed from 198 to 76 m/min and from 905 to 2012 RPM, respectively, with the lettuce then processed as previously described.

2.3.12 Sample collection. During processing, five iceberg lettuce, eight Romaine lettuce and ten spinach samples (25 g each) were collected from the conveyer belt (iceberg and Romaine lettuce only) and the end of the flume tank (3 m from the start) at regular intervals, with the number of samples corresponding to the capacity of the centrifugation basket for the three different products. Five 40-ml water samples were also obtained from the flume tank and water recirculation spout. Immediately after shaker table dewatering, five iceberg lettuce, eight Romaine lettuce and ten baby spinach samples (25 g each) were collected through the 2.75 cm-diameter holes that were spaced 10 cm apart along the side of the centrifugation basket. Product in the basket was then dried in the pre-set Spin Dryer. During centrifugal drying, four 40 ml water samples were collected from the centrifuge drain at 15 sec intervals. After centrifugation, four iceberg lettuce, six Romaine lettuce and eight baby spinach samples (25 g each) also were collected from the centrifugation basket as previously described. Twenty-eight baby spinach and 50 iceberg and Romaine lettuce contact areas on the equipment (14 shredder, 8 conveyer, 11 flume tank, 9 shaker table and 8 dewatering centrifuge samples (Figures 2.1 – 2.5)) measuring 100 cm^2 as previously identified using Glo Germ™ were sampled as described by Vorst et al.

(2004) using 1-ply composite tissues moistened with 1 ml of sterile 1% (w/v) phosphate buffer (8.5 g/L NaCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4, J.T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ).

2.3.13 Microbiological analyses. All lettuce and baby spinach samples (25 g) were added to 100 ml of sterile 1% phosphate buffer in a Whirl-Pak™ filter bag (Nasco, Fort Atkinson, WI) and homogenized in a stomacher (Stomacher 400 Circulator, Seward, Worthington, UK) for 1 min at 260 rpm. The only exception was lettuce inoculated at ~10^2 CFU/g, which was processed in a pulsifier (Pulsifier, Filtaflex Ltd., Almonte, Ontario, Canada) rather than the stomacher, to reduce the amount of lettuce exudate/particulates in the samples to be filtered. Preliminary data indicated similar release of bacteria from leafy greens during stomaching and purifying. These sample homogenates were then either appropriately diluted in sterile 1% phosphate buffer and plated on TSAYE + amp or processed using 0.45 μm membrane filters (Millipore, Millipore Corporation, Billerica, MA) (lower detection limit of 0.04 CFU/g), which were placed on 60-mm dia. Petri plates containing TSAYE + amp to quantify *E. coli* O157:H7. The 1-ply composite tissue samples were added to 15 ml of sterile 1% phosphate buffer in a Whirl-Pak™ bag, stomached for 1 min at 260 rpm and then plated identically to the produce samples, giving a lower detection limit of 1 CFU/100 cm^2 when the entire 15 ml sample was passed through the membrane filter. The 40 ml water samples were either appropriately diluted in sterile 1% phosphate buffer and plated on TSAYE + amp or processed by membrane filtration, which gave a minimum detection limit of 0.03 CFU/ml. Following 18 - 20 h of incubation at 37°C, all green fluorescing colonies as seen under ultraviolet light (365 nm, Blak-Ray, Ultra-violet Product Inc. San Gabriel, CA) were counted as *E. coli* O157:H7.

2.3.14 Statistical analysis. *E. coli* O157:H7 counts were converted to log CFU per g, ml or 100 cm^2 and subjected to ANOVA using JMP 8.0 (SAS Institute Inc., Cary, NC). For all tests, $\alpha = 0.05$. *E. coli* generation times were calculated by comparing the time in hours to the CFU/ml during the exponential phase growth. The Tukey-Kramer HSD test was used to compare *E. coli* growth rates and attachment abilities of the strains, identify significant differences in transfer to the individual product contact surfaces and water samples collected at various locations throughout the process. The same tests were applied to identify significant differences in the numbers of *E. coli* O157:H7 shed from iceberg lettuce, Romaine lettuce and baby spinach at the various locations sampled during processing. Impact of shred size and post-inoculation hold time on the numbers of *E. coli* O157:H7 transferred to the water and various equipment surfaces during processing were also assessed using this same procedure.

2.4 RESULTS

2.4.1 *E. coli* O157:H7 growth rate and attachment ability. The three virulent *E. coli* O157:H7 strains (K3995, K4495, and K4831) and three of the four avirulent strains (CV2b7, 6980-2, and 6982-2) exhibited similar growth rates ($P < 0.05$) with generation times at 37°C ranging from 21.5 to 25.1 min. (Figure 2.6). *E. coli* O157:H7 ATCC43888 - one of the four avirulent GFP-labeled strains used in the cocktail for leafy green inoculation, grew significantly more slowly (generation time of 36.8 min) and yielded a higher OD value of 1.321 than the other six strains tested (OD 0.062 to 0.146) ($P < 0.05$) in the microtiter plate attachment assay (Table 2.1).

2.4.2 Lettuce and baby spinach. After inoculating each product at the three different targeted levels, iceberg lettuce, Romaine lettuce and baby spinach contained mean *E. coli* O157:H7 populations of 6.0 – 6.2, 4.0 – 5.0 and 1.8 – 2.5 log CFU/g at the time of processing, with Romaine lettuce containing fewer *E. coli* O157:H7 (1.8 log CFU/g) than baby spinach (2.5 log CFU/g) at the lowest target inoculation level of 2 log CFU/g ($P < 0.05$). At the medium inoculation level, Romaine lettuce (5.0 log CFU/g) retained more of the inoculum than did iceberg lettuce and baby spinach, 4.0 and 4.2 log CFU/g, respectively ($P < 0.05$) (Figure 2.7).

2.4.3 Flume water. Overall, 82.3 to 118.2, 48.5 to 64.6 and 48.9 to 147.2% of the original *E. coli* O157:H7 inoculum was respectively shed from iceberg lettuce, Romaine lettuce and baby spinach into the 890 L of processing water used for fluming (Tables 2.2 – 2.4). These percentages are based on an estimation of the total number of *E. coli* O157:H7 cells having been transferred to the 890 L of flume water from the 50 lbs of inoculated leafy greens. During fluming, *E. coli* O157:H7 populations on inoculated iceberg and Romaine lettuce decreased ($P < 0.05$) by 0.7 - 0.9 and 1.0 - 1.2 log, respectively, with a decrease of 0.9 to 1.3 log seen for baby

spinach directly entering the flume tank. More of the inoculum was removed from baby spinach than from iceberg lettuce ($P < 0.05$). Thereafter, relatively few significant differences ($P < 0.05$) were seen between the different sampling locations, with the numbers of *E. coli* O157:H7 on the three products generally decreasing ~0.1 log CFU/g during shaker table dewatering and centrifugal drying (Figure 2.7), with no significant differences between the three different products. After centrifugal drying, iceberg lettuce, Romaine lettuce, and baby spinach, respectively, retained 7.9 - 12.6, 8.3 - 14.1, and 3.2 - 17.4% of the original *E. coli* O157:H7 inoculum (Tables 2.2 – 2.4).

2.4.4 Centrifugation water. Water exiting the centrifuge drain after spin-drying yielded *E. coli* O157:H7 populations that were 1.1 - 2.1 log CFU/ml higher ($P < 0.05$) compared to the flume water (Figure 2.8). Overall, 30.5 - 38.6, 17.8 - 26.8 and 6.3 - 15.6% of the original inoculum on iceberg lettuce, Romaine lettuce, and baby spinach, respectively, was recovered from the ~3.1 L of water exiting the centrifuge drain (Tables 2.2 – 2.4). At the 6 log CFU/g inoculation level, *E. coli* O157:H7 was more easily shed from iceberg lettuce than from baby spinach ($P < 0.05$) during centrifugation. Similarly, both iceberg and Romaine lettuce shed more *E. coli* O157:H7 during drying than baby spinach, when initially inoculated to contain 2 log CFU/g ($P < 0.05$). At 4 log CFU/g, all three products differed significantly from each other, with iceberg lettuce losing the most and baby spinach the least *E. coli* O157:H7 ($P < 0.05$). As expected, the decrease in numbers of *E. coli* O157:H7 transferred to the water paralleled the three different lettuce inoculation levels (Figures 2.7 and 2.8).

2.4.5 Processing equipment surfaces. After processing iceberg and Romaine lettuce inoculated at the highest and lowest levels, significantly higher percentages of the original *E. coli* O157:H7 inoculum were frequently transferred to surfaces on the shredder (0.20 to 0.53%),

conveyer belt (0.21 to 0.93%) and centrifugal dryer (0.15 to 0.48%), as compared to the flume tank (0.01 to 0.09%) and shaker table (0.01 to 0.15%), with the only exception being iceberg lettuce inoculated at the lowest level, where the surfaces of the shredder yielded higher populations ($P < 0.05$). Baby spinach followed a similar trend, with the most heavily inoculated products retaining the most bacteria after centrifugal drying (0.05%), followed by the flume tank (0.01%) and shaker table (0.00%), while the least inoculated products did not show any significant differences between location ($P < 0.05$) (Tables 2.2 – 2.4). When inoculated at 4 log CFU/g, Romaine and iceberg lettuce transferred higher numbers of *E. coli* O157:H7 to the shredder (0.31 and 0.53%), followed by the conveyer belt (0.35 and 1.39%) and lastly the dewatering centrifuge (0.03 and 0.22%), flume tank (0.02 and 0.04%), and shaker table (0.01 and 0.04%) (Tables 2.2 – 2.4). During spinach processing, highest numbers of *E. coli* O157:H7 transferred to sampling locations on the dewatering centrifuge (0.03%) ($P < 0.05$) followed by the flume tank (0.01%) and shaker table (0.00%) (Tables 2.2 – 2.4). After conveying, transfer was generally greatest when the product initially contacted the slanted front end of the flume tank (Figure 2.1, flume tank sample 1) and when the contaminated water contacted the drain of the centrifugal dryer (Figure 2.5, dewatering centrifuge sample 4) (Tables 2.5 – 2.7). Overall, no significant differences ($P > 0.05$) were observed in the percentage of *E. coli* O157:H7 cells transferred to surfaces on the five different pieces of processing equipment in terms of leafy green variety, except for samples collected from the conveyer belt after processing Romaine and iceberg lettuce at the medium inoculation level and on the centrifugal dryer after processing baby spinach and Romaine lettuce inoculated at 10^2 CFU/g (Tables 2.2 – 2.4).

2.4.6 Impact of shred size and hold time before shredding. Before processing, inoculated iceberg lettuce to be coarse-shredded, fine-shredded, or held for 24 h and then coarse-

shredded contained *E. coli* O157:H7 populations of 4.0, 3.8 and 3.7 log CFU/g, respectively (Figure 2.7B). Overall, no significant differences ($P > 0.05$) in percentages of inocula shed in the flume water were seen between coarse- (86.5%) and fine-shredded lettuce (83.1%) or lettuce held for 24 h at 4°C before processing (49.9%). After centrifugal drying, 42.2% of the original *E. coli* O157:H7 inoculum remained on product that was stored for 24 h before processing, which was greater ($P < 0.05$) than for iceberg lettuce that was processed 1 h after inoculation (8.1%). For coarse and fine-shredded, and 24 h-held lettuce, only 0.0 to 1.4%, 0.0 to 1.5%, and 0.0 to 0.5% of the original inoculum, respectively, transferred to the equipment surfaces, with no significant differences between these values ($P > 0.05$) (Table 2.3).

2.5 DISCUSSION

Four avirulent strains of *E. coli* O157:H7 were used in this study, rather than the virulent outbreak strains, due to the production of potentially infectious aerosols that could have been generated during processing. As has been reported previously (Taormina and others 2009, Wachtel and others 2002), the GFP label that was inserted via a plasmid into these four strains also allowed easy differentiation of our inoculum from the background microflora, which often exceeded 7 log CFU/g. Except for the avirulent GFP-labeled strain ATCC43888, which exhibited slower growth and greater adherence in the microtiter plate assay, the remaining three GFP-labeled strains behaved similarly to the three outbreak strains. While Strain ATCC43888 grew more slowly, it was included in the study to represent the worst case scenario in terms of adherence.

Uniform contamination of the inoculated heads via dipping was essential for obtaining repeatable results. However, the method of inoculation clearly has an impact on bacterial transfer, with Lang et al. (2004) having previously shown that greater numbers of *E. coli* O157:H7 could be recovered from dip- as opposed to spot- or spray-inoculated lettuce when the same numbers of bacterial cells were initially applied.

Three inoculation levels - 6, 4, and 2 log CFU/g were chosen to represent high, medium, and low levels of contamination. While the 6 and 4 log CFU/g levels used in our work may be unrealistically high, previous studies have shown that cattle (the main carriers of *E. coli* O157:H7) normally shed less than 100 CFU/g of feces, with certain high-level shedders, known as "super-shedders," shedding up to 1,000,000 CFU/g in their feces (Chase-Topping and others 2007, Low and others 2005). Hence, the inoculation levels used represent direct contamination of product in the field via fecal matter from domestic and wild animals. In addition, these high

inoculation levels were also needed to obtain quantitative transfer data that could be modeled and incorporated into a subsequent risk assessment as reported elsewhere (Pérez Rodríguez and others 2011).

Direct contact of produce with manure represents only one route of pre-harvest contamination. This same contaminated manure also can enter irrigation water used for crops (Beuchat 2002, Ibekwe and others 2004), resulting in lower numbers of pathogens distributed over a far wider area as opposed to a localized fecal event from an animal. Although closer to realistic estimates for microbial contamination, our results using an inoculation level of 2 log CFU/g yielded considerably greater variability than the medium and high levels.

Commercial washing of leafy greens in sanitizer solutions containing up to 100 ppm chlorine or 80 ppm peroxyacetic acid is now a universally accepted practice (FDA 2009). While most effective in reducing microbial populations in the flume water, these sanitizers typically reduce bacterial populations on fresh produce only 90 to 99%, which represents only a marginal improvement over the use of water alone (Beuchat and others 2004, Burnett and others 2004, Keskinen and others 2009, Sapers 2001, Weissinger and others 2000, Zhang and others 2009). Because the goal of this study was to quantify the numbers of *E. coli* O157:H7 cells transferred during leafy green processing, all lettuce and baby spinach was processed using sanitizer-free water. If chemical sanitizers had been used, the numbers of *E. coli* O157:H7 would have likely decreased to non-detectable levels in the flume water, especially in experiments using low inoculation level, thereby negating our effort to obtain quantitative transfer data for subsequent mathematical modeling.

Our data align well with the typical bacterial reductions of 90 – 99% seen during washing of fresh produce in water alone. During processing of iceberg lettuce inoculated at 2.9 – 3.5 log

CFU/g, approximately 90% of the *E. coli* O157:H7 inoculum was shed during fluming, with populations of 1.1 to 1.6 log CFU/g remaining on the product after centrifugal drying. Hence, given an oral infectious dose for *E. coli* O157:H7 of less than 100 cells (Meng and others 2007), and the fact that our counts, even at the lowest inoculation level, fell within this range, the ineffectiveness of current processing/washing methods is clearly a major concern, as evidenced by the three nationwide spinach and lettuce outbreaks in 2006 (FDA 2006, FDA 2007, Jay and others 2007) and a series of more recent product recalls involving *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* (FDA 2010a, FDA 2010b, FDA 2010c).

During spin drying, about 22% of the remaining inoculum was shed in ~3 L of water exiting the drain of the centrifugal dryer. Even at the lowest inoculation level, *E. coli* O157:H7 was quantifiable at levels of 0.9 to 1.6 log CFU/ml in the centrifugation water. Because microbial contaminants are presumed to be non-uniformly distributed in commercially processed leafy greens, routine monitoring of the spent centrifugation water for *E. coli* or other indicator organisms appears to be a more promising means than product sampling, to better ensure end product safety. Methods are currently being developed to concentrate and rapidly detect pathogens in larger volumes of processing water with ultrafilters yielding *E. coli* recovery rates of 74 to 96% (Hill and others 2005, Morales-Morales and others 2003).

The levels and point at which leafy greens become contaminated are both key factors that partially dictate the extent of *E. coli* O157:H7 transfer during processing. One study conducted at Rutgers University used chicken, lettuce, cutting boards, hands, and a spigot to show that as the inoculum size increased, the number of bacteria transferred remained constant, with the transfer rate decreasing (Montville and Schaffner 2003). In our study, increased bacterial transfer to the water and equipment surfaces paralleled the increase in the produce inoculation level.

When leafy greens were dip-inoculated and processed 1 h later, approximately 83% of the inoculum transferred to the flume water (Tables 2.2 – 2.4). In a bench-top study, Zhang et al. (Zhang and others 2009) showed that a single iceberg lettuce leaf inoculated with *E. coli* O157:H7 at 5.6 log CFU/leaf transferred nearly 99% of the inoculum to 500 ml of sanitizer-free water. These differences in reduction are likely due in part to differences in water turbulence during washing. Such a high reduction is especially surprising, because their leaves were spot inoculated, which has been shown to result in lower *E. coli* O157:H7 recovery as compared to the dip inoculation method that was used in our work (Lang and others 2004). They also held their inoculated lettuce for an additional hour at 4°C before washing, which would suggest more bacterial attachment.

In the present study, a small, but consistent fraction of the original *E. coli* O157:H7 inoculum transferred to the flume tank, shaker table, and centrifugal dryer during processing. Therefore, the populations of *E. coli* O157:H7 recovered from these wet surfaces most likely reflect the number of cells present in the thin film of water that coated the 100 cm^2 surface before sampling. In terms of the wet equipment surfaces, greatest transfer was seen to the interior walls, basket carrier, and drain of the dewatering centrifuge, with the centrifugation water also yielding *E. coli* O157:H7 populations that were 1 to 2 logs higher than the processing water.

In addition to the wet surfaces, direct transfer of *E. coli* O157:H7 was also seen between the product and product contact surfaces of the shredder and conveyor belt. After processing iceberg and Romaine lettuce, the shredder and conveyer belt generally yielded higher *E. coli* O157:H7 counts than the flume tank, shaker table, or centrifugal dryer. These microbial 'hot spots' are not surprising, since exudate from shredded lettuce was visible on the cutting wheel and discharge chute of the shredder, as well as on all product contact areas of the conveyor. This

liquid likely enhanced *E. coli* O157:H7 transfer from lettuce to the stainless steel and polyurethane surfaces of the shredder and conveyor belt, as was also shown by Moore et al. (Moore and others 2003). In a survey of fresh vegetable processing facilities in Japan, populations of mesophilic aerobic bacteria exceeded 5.0 log CFU/cm^2 or ml in many samples collected from washing, slicing, dehydrating, and blending equipment, surfaces of slicer/shredder blades, and the processing room floor after processing (Kaneko and others 1999). Although the percentage of the *E. coli* O157:H7 inoculum transferred to equipment surfaces was minimal in our study, only a few cells are needed to cross-contaminate subsequent product during continuous processing. In support of these observations, contaminated lettuce particulates lingering behind the cutting wheel rim of a commercial lettuce shredder were the probable cause of an Australian *Salmonella* Bovismorbificans outbreak that resulted in 41 cases of illness (Stafford and other 2002).

Lettuce shred size did not significantly impact *E. coli* O157:H7 transfer during subsequent processing. These results were somewhat surprising, given that an increase in the cut surface area would be expected to release more lettuce exudates, which would in turn increase *E. coli* O157:H7 transfer during shredding and conveying. One reason for this unexpected outcome may be the modest difference in shred size – approximately 5 x 5 cm for large-shredded compared to 0.5 x 5 cm for small-shredded lettuce.

Bacterial attachment and biofilm formation on the surfaces of leafy greens are two key factors that negatively impact the removal of microorganisms during conventional washing (Carmichael amd others 1999, Sapers 2006). When Lang et al. (2004) assessed the impact of different post-inoculation drying times on recovery of *E. coli* O157:H7 from iceberg lettuce and parsley, higher (or similar) populations were obtained from leafy greens that were dried for 2 h at

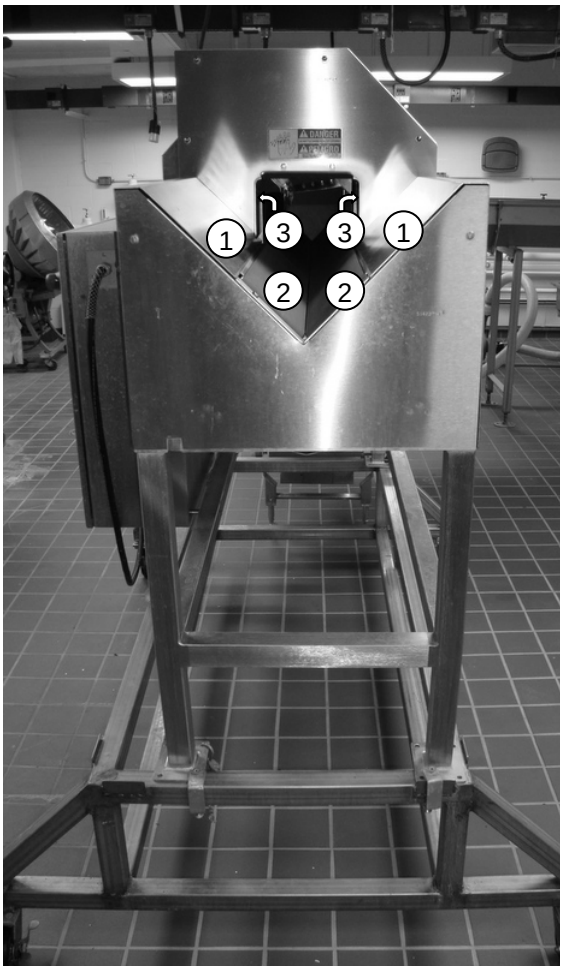
22°C as opposed to 2 h at 22°C followed by 22 h at 4°C. Consistent with those findings, our results also showed that increasing the time from inoculation to processing from 1 h at 23°C to 1 h at 23°C followed by 24 h at 4°C decreased the number of *E. coli* O157:H7 cells transferred from iceberg lettuce to the water and equipment surfaces during processing. While this implies that refrigerating the product for 1 day post-harvest could decrease the potential spread of *E. coli* O157:H7 during processing and lead to potentially smaller-scale outbreaks, current commercial produce washing/sanitizing practices are still not sufficiently efficacious against foodborne pathogens. Based on several surveys indicating *Salmonella* and *Listeria monocytogenes* incidence rates of 0 to 35.7% and 4.8%, respectively, on farm and retail produce (Doyle and Erickson 2008), ample opportunity exists for foodborne pathogens to survive, grow, and enter produce packing houses on product coming from the field.

This is the first report to quantify the number of *E. coli* O157:H7 cells transferred from product to equipment surfaces during pilot-scale production of fresh-cut leafy greens. Based on our findings, approximately 90% of the *E. coli* O157:H7 inoculum was shed in the sanitizer-free water, with this pathogen also contaminating the product contact surfaces of the shredder, conveyor, flume tank, shaker table, and dewatering centrifuge to various degrees during processing. Routine microbial monitoring of the centrifugation water may prove to be a useful strategy for minimizing and preventing the continued spread of bacterial pathogens to large quantities of product, with these findings providing important insight into potential lapses in current cleaning/sanitizing practices and improved equipment designs.

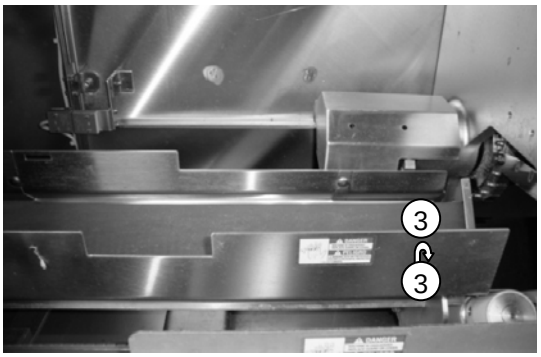
2.6 FIGURES

Figure 2.1: *Lettuce shredder sampling locations: (A) loading end, (B) interior feed belt and guides, and (C) blade and discharge chute*

A



B



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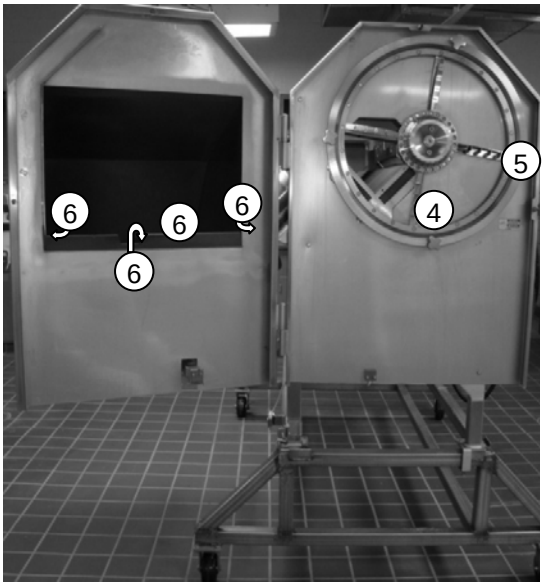


Figure 2.2: *Conveyer belt sampling locations*

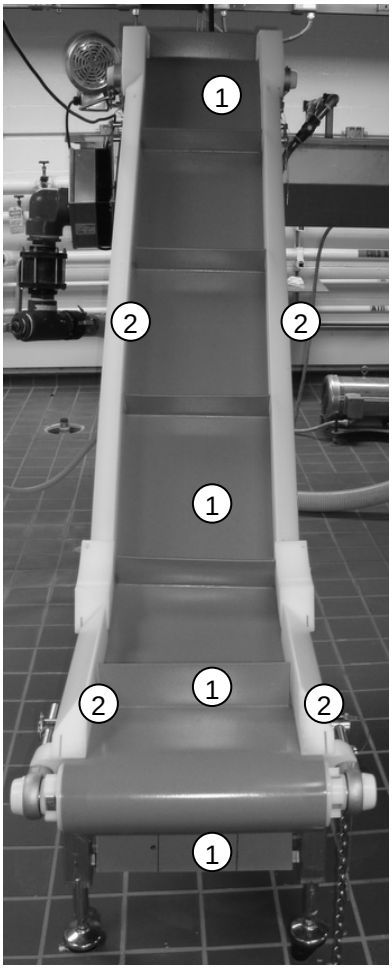


Figure 2.3: *Flume tank sampling locations*

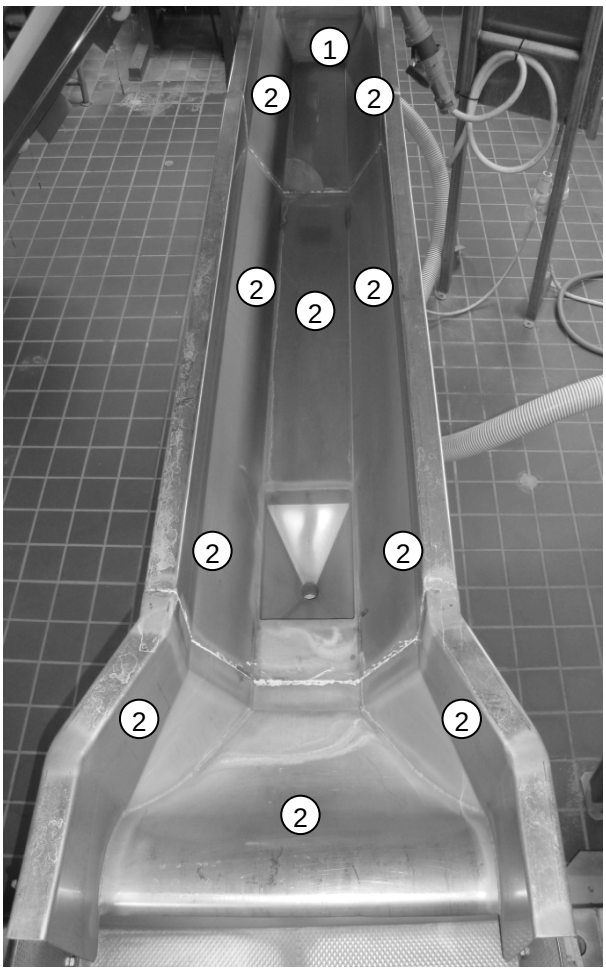


Figure 2.4: *Shaker table sampling locations*

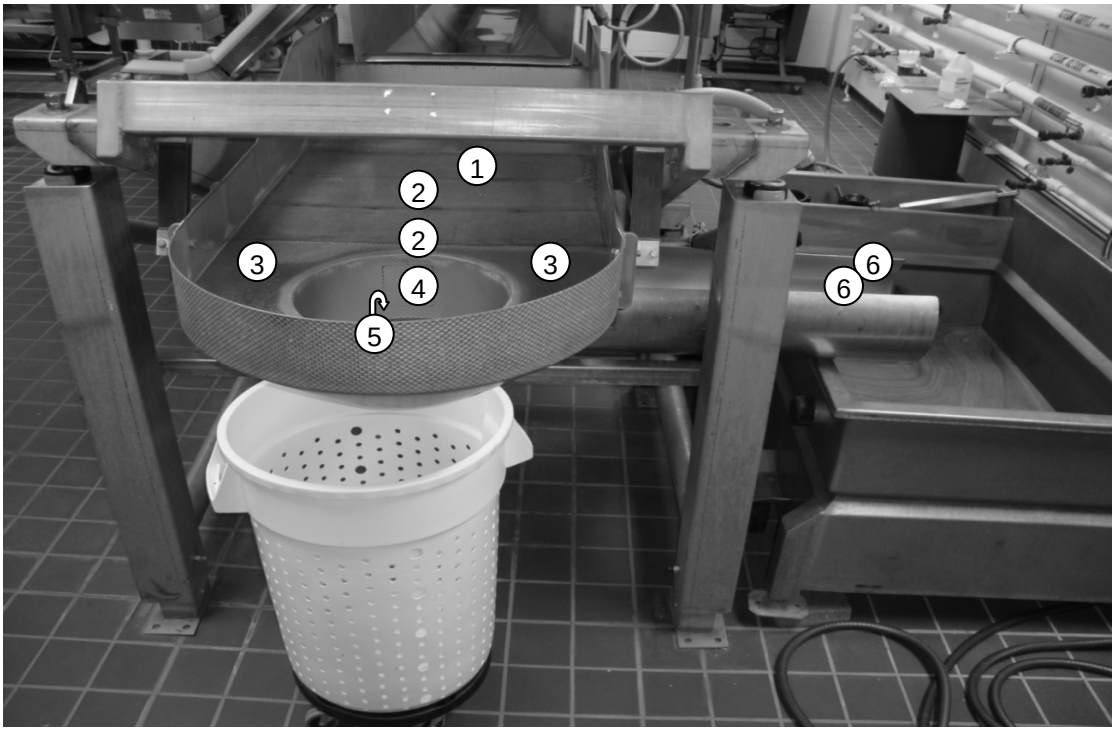


Figure 2.5: *Dewatering centrifuge sampling locations*

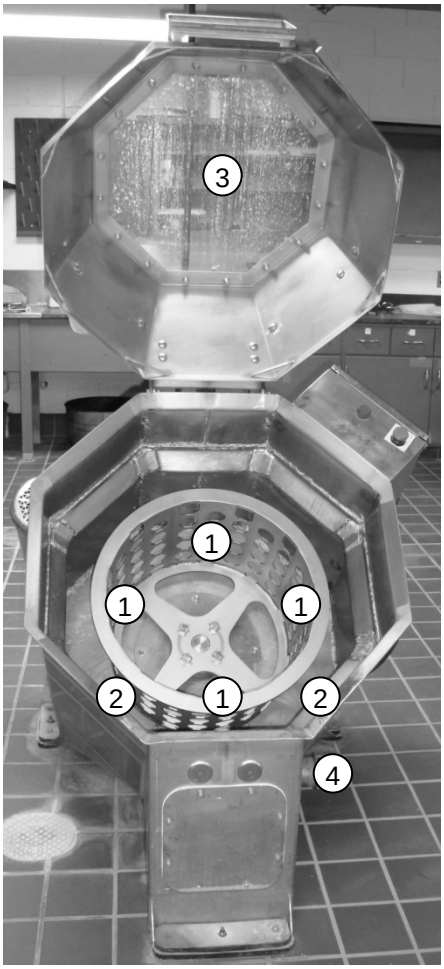


Figure 2.6: *Growth curves at 37°C for four avirulent, GFP-labeled, ampicillin-resistant strains of* E. coli *O157:H7 (ATCC 43888, CV2b7, 6980-2, and 6982-2) in TSBYE + amp and three virulent strains of* E. coli *O157:H7 (K3995, K4495, and K4831) TSBYE (n=3)*

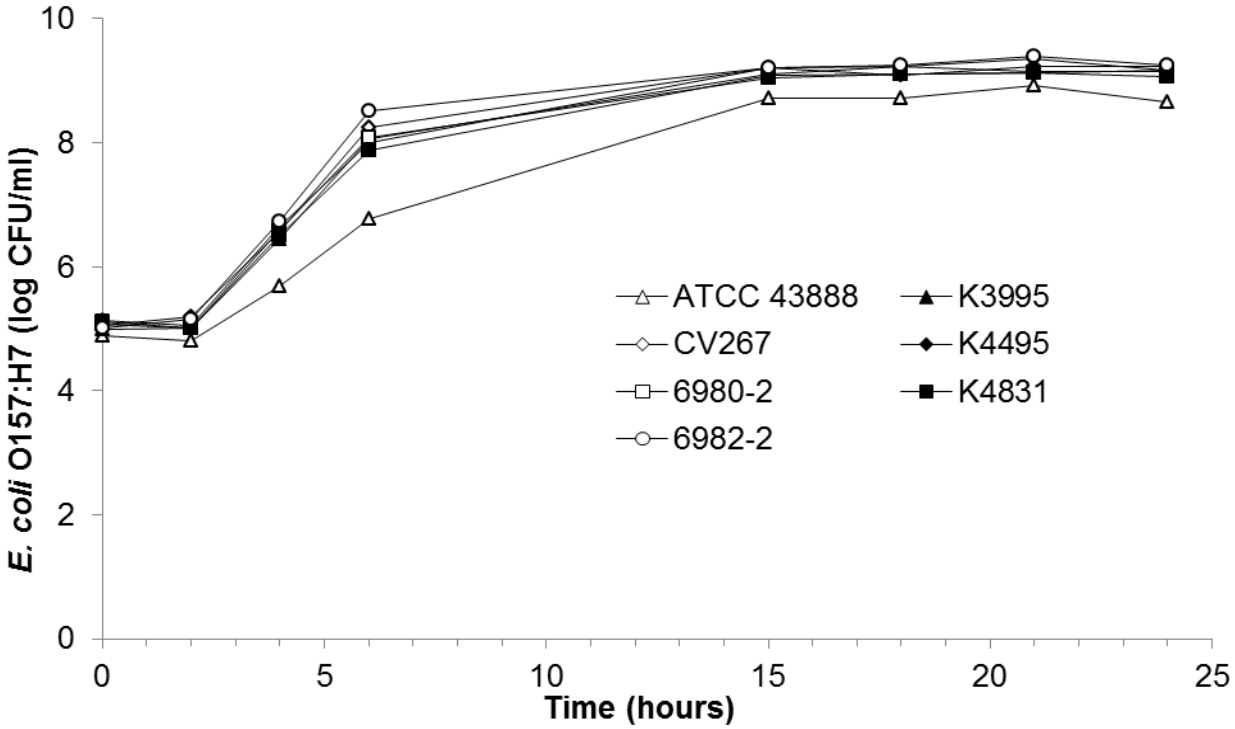


Figure 2.7: E. coli *O157:H7 populations (mean ± SD)*[a] *on the product during processing* of *leafy greens inoculated at: (A) ~6 log CFU/g (n = 3), (B) ~4 log CFU/g (n = 3), and (C) ~2 log CFU/g (n = 5).*

A

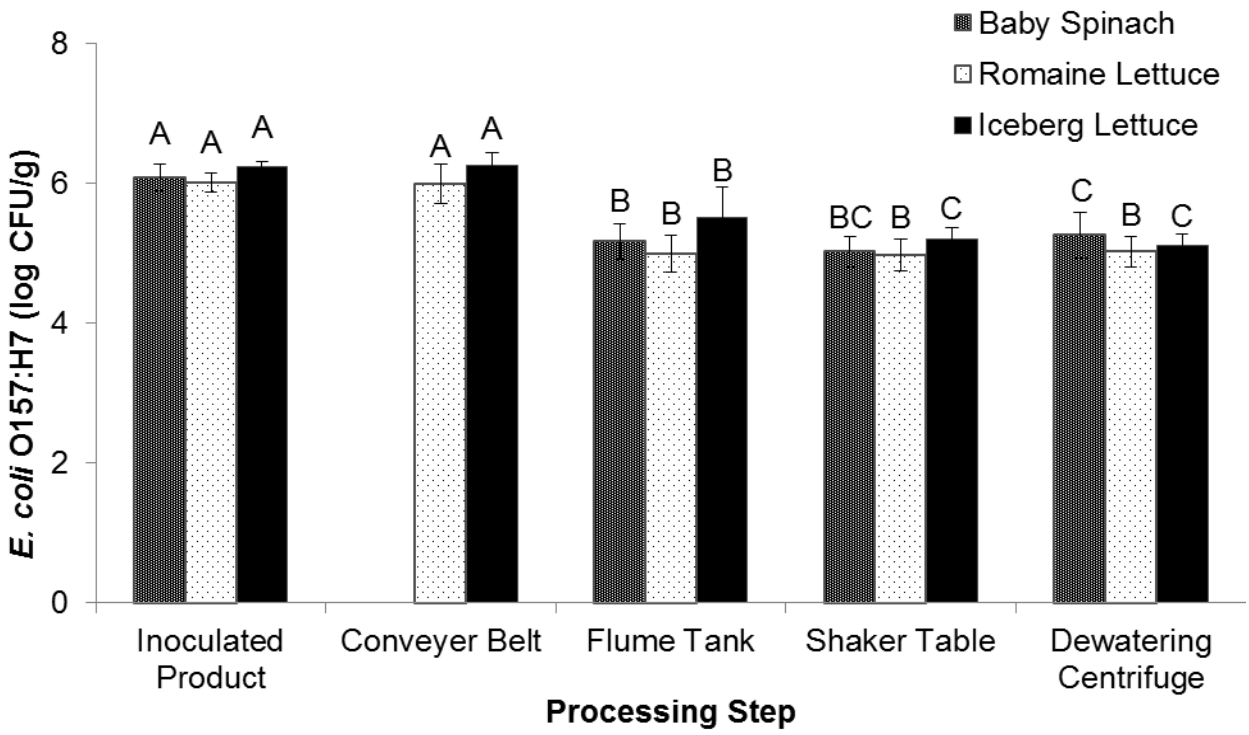


Figure 2.7 (cont'd)

B

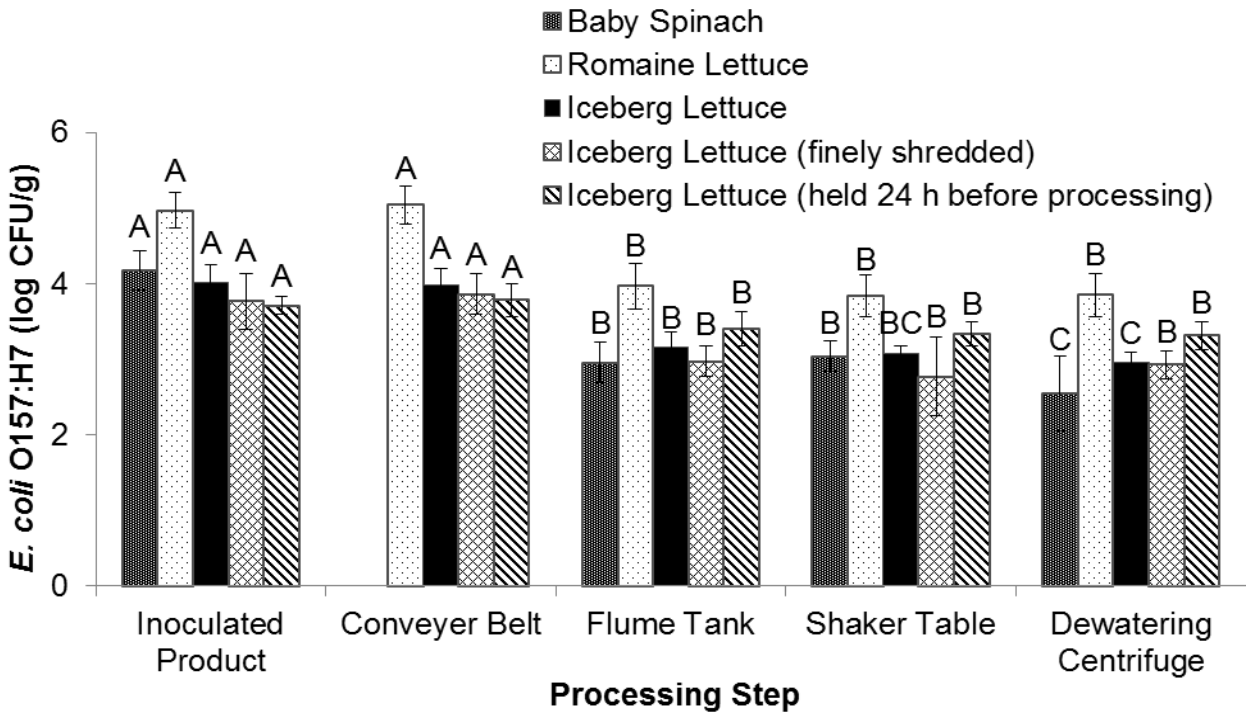
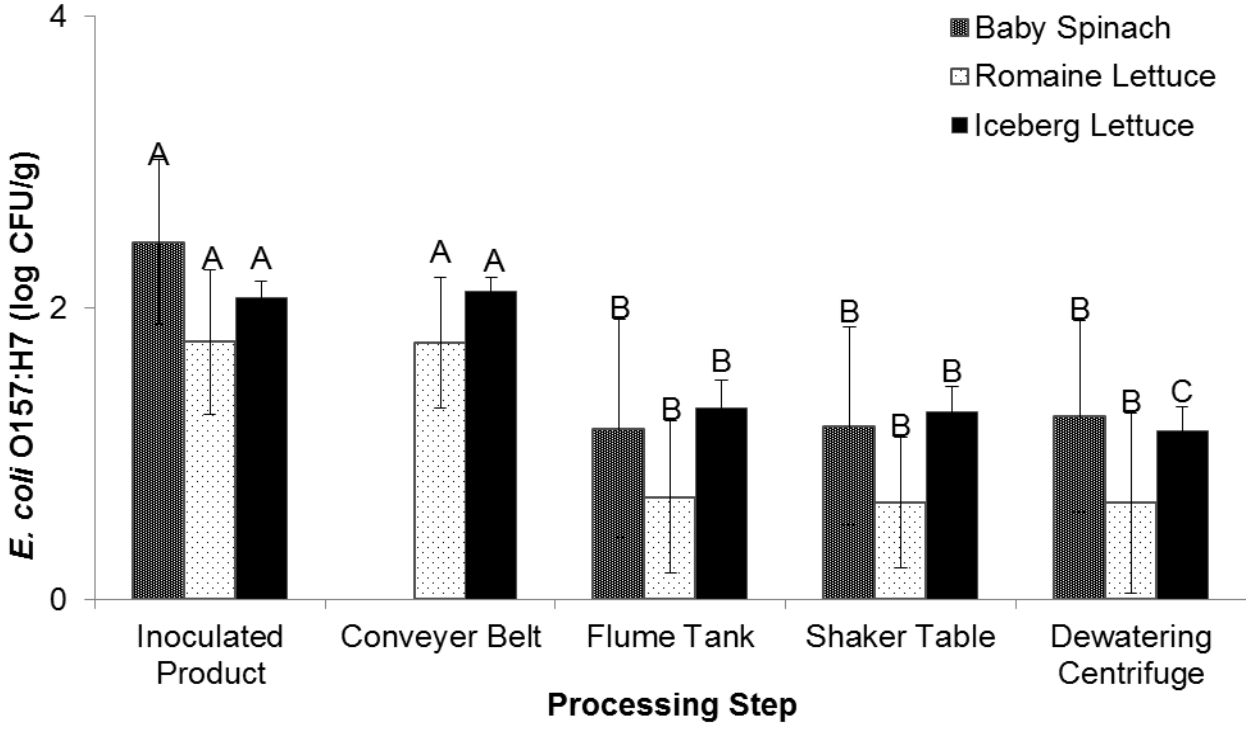


Figure 2.7 (cont'd)

C



[a] One over the detection limit was used to calculate the mean log value for samples not yielding colonies by direct plate count. Means of the same product type with different letters are significantly different ($P \leq 0.05$)

Figure 2.8: E. coli *O157:H7 populations (mean ± SD)*[a] *in water during processing of leafy green inoculated at: (A) ~6 log CFU/g (n = 3), (B) ~4 log CFU/g (n = 3), and (C) ~2 log CFU/g (n = 5).*

A

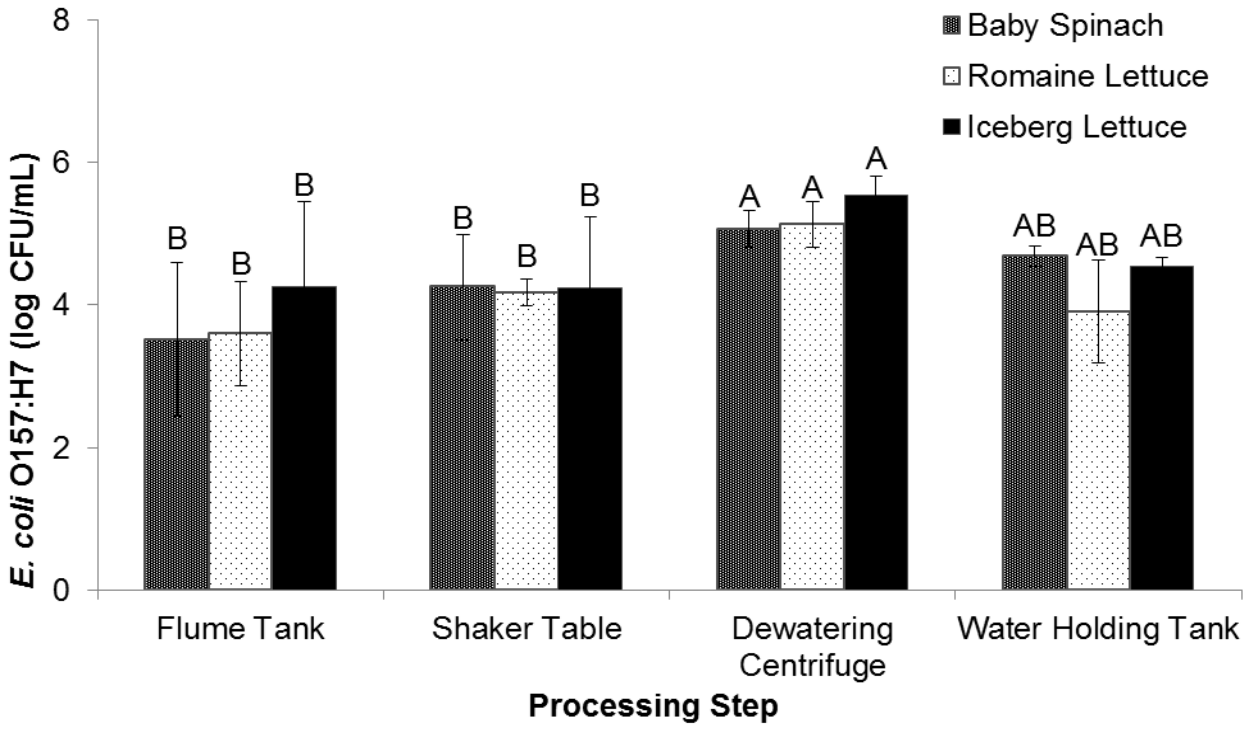


Figure 2.8 (cont'd)

B

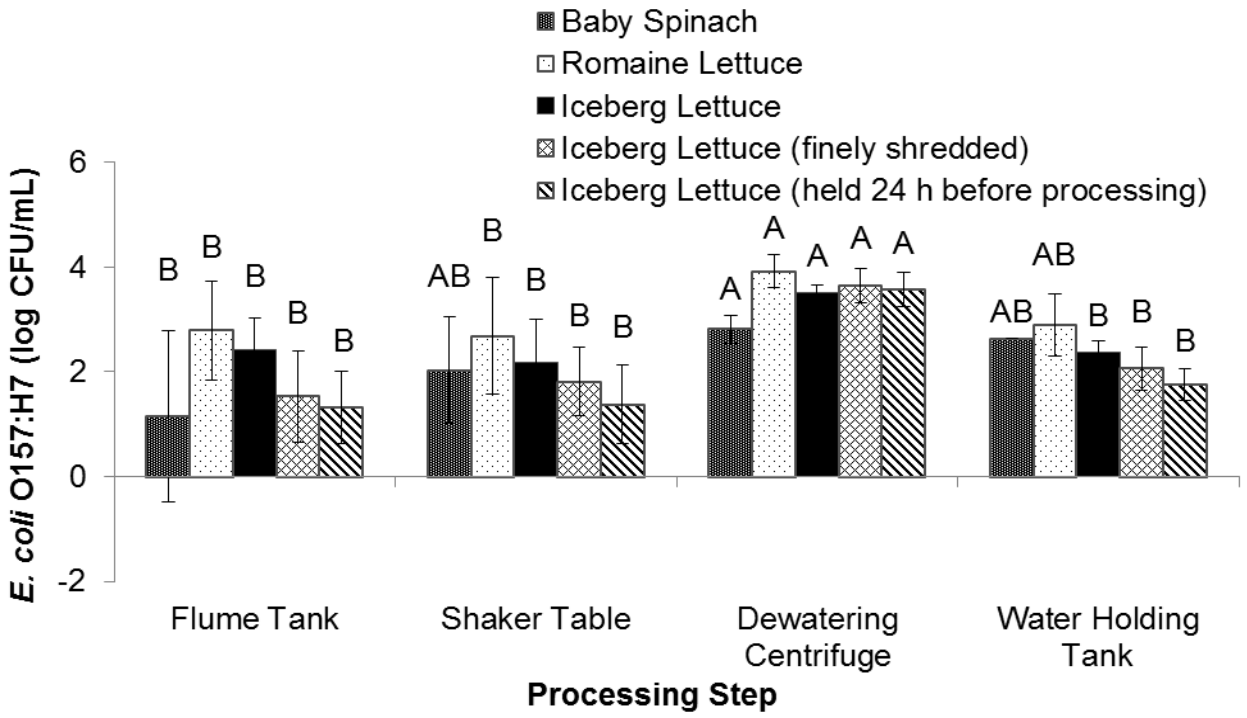
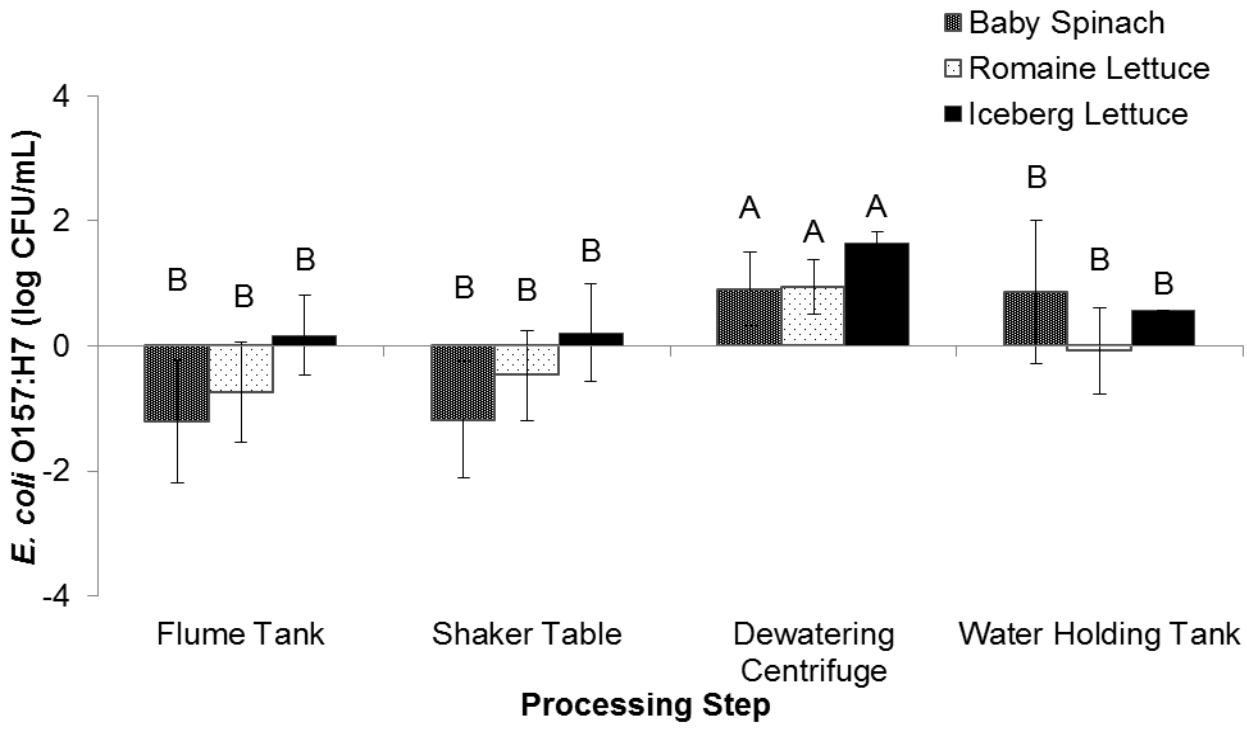


Figure 2.8 (cont'd)

C



[a] One over the detection limit was used to calculate the mean log value for samples not yielding colonies by direct plate count. Means of the same product type with different letters are significantly different ($P \leq 0.05$).

2.7 TABLES

Table 2.1. Bacterial attachment, as measured by the optical density at 630 nm for four avirulent, GFP-labeled, ampicillin-resistant strains of *E. coli* O157:H7 (ATCC 43888, CV2b7, 6980-2, and 6982-2) and three virulent strains of *E. coli* O157:H7 (K3995, K4495, and K4831) (n=3) [a]

Strains	Mean Optical Density ± SD	
Virulent		
K3995	0.062 ± 0.007	B
K4831	0.081 ± 0.012	B
K4492	0.061 ± 0.007	B
Control	0.065 ± 0.005	B
Avirulent, GFP-labeled, ampicillin-resistant		
CV2b7	0.059 ± 0.003	B
ATCC43888	1.321 ± 0.232	A
6982-2	0.095 ± 0.009	B
6980-2	0.146 ± 0.012	B
Control with 100 ppm ampicillin	0.065 ± 0.014	B

[a] Means of the same product type with different letters are significantly different ($P \leq 0.05$).

Table 2.2: E. coli *transfer (%) during processing of leafy green inoculated at 6 log CFU/g*

	Mean ± SD *E. coli* O157:H7 recovered (%)[b,c]		
Processing Step	Baby Spinach	Romaine Lettuce	Iceberg Lettuce
Flume Water[a]	147.2 ± 50.4	52.6 ± 43.0	82.3 ± 24.4
Shredder[a]	NA	0.2 ± 0.4 Aa	0.2 ± 0.3 Aa
Conveyor Belt[a]	NA	0.2 ± 0.2 Aa	0.7 ± 0.8 ABa
Flume Tank[a]	0.0 ± 0.0 Ba	0.0 ± 0.1 Ba	0.0 ± 0.0 Ba
Shaker Table[a]	0.0 ± 0.0 Ba	0.0 ± 0.0 Ba	0.0 ± 0.0 Ba
Dewatering Centrifuge[a]	0.1 ± 0.1 Aa	0.2 ± 0.2 ABa	0.4 ± 0.8 ABa
% Original Inoculum on Product after Processing	17.4 ± 10.8	11.1 ± 5.5	7.9 ± 2.6

[a] The total equipment surface areas for the shredder, conveyer belt, flume tank, shaker table and dewatering centrifuge were 1.74 m^2, 2.88 m^2, 2.60 m^2, 2.47 m^2, and 4.55 m^2, respectively.

[b] Means with different capital letter represent *E. coli* O157:H7 counts that differ significantly in terms of processing equipment location while means with different lowercase letter differ significantly in terms of leafy green variety ($P \leq 0.05$).

Table 2.2 (cont'd)

[c] These percentages are based on an estimation of the total number of *E. coli* O157:H7 cells having been transferred to the 890 L of flume water or equipment surfaces from the 50 lbs of inoculated leafy greens.

Table 2.3: E. coli *transfer (%) during processing of leafy green inoculated at 4 log CFU/g*

Processing Step	Mean ± SD *E. coli* O157:H7 recovered (%)[b,c]				
	Baby Spinach	Romaine Lettuce	Iceberg Lettuce	Iceberg Lettuce (finely shredded)	Iceberg Lettuce (held 24 h before processing)
Flume Water[a]	96.9 ± 2.4	48.5 ± 47.6	86.5 ± 38.4	83.1 ± 78.6	49.9 ± 29.8
Shredder[a]	NA	0.1 ± 0.1 Aa	0.4 ± 0.5 Aa	0.0 ± 0.1 B	0.1 ± 0.4 B
Conveyor Belt[a]	NA	0.4 ± 0.3 Bb	1.4 ± 1.0 Ba	0.2 ± 0.2 B	0.4 ± 0.8 AB
Flume Tank[a]	0.0 ± 0.0 Ba	0.0 ± 0.1 Ca	0.0 ± 0.1 Ca	0.1 ± 0.1 B	0.2 ± 0.5 AB
Shaker Table[a]	0.0 ± 0.0 Ca	0.0 ± 0.0 Ca	0.0 ± 0.0 Ca	0.4 ± 0.6 B	0.0 ± 0.1 B
Dewatering Centrifuge[a]	0.0 ± 0.1 Aa	0.0 ± 0.0 Ca	0.2 ± 0.4 BCa	1.6 ± 2.0 A	0.5 ± 0.5 A
% Original Inoculum on Product after Processing	3.2 ± 3.1	8.3 ± 6.1	8.1 ± 2.8	12.5 ± 5.7	42.2 ± 19.8

Table 2.3 (cont'd)

[a] The total equipment surface areas for the shredder, conveyer belt, flume tank, shaker table and dewatering centrifuge are 1.74 m^2, 2.88 m^2, 2.60 m^2, 2.47 m^2, and 4.55 m^2, respectively.

[b] Means with different capital letter represent *E. coli* O157:H7 counts that differ significantly in terms of processing equipment location while means with different lowercase letter differ significantly in terms of leafy green variety ($P \leq 0.05$).

[c] Percentages are based on an estimation of the total number of *E. coli* O157:H7 cells having been transferred to the 890 L of flume water or equipment surfaces from the 50 lbs of inoculated leafy greens.

Table 2.4: E. coli *transfer (%) during processing of leafy green inoculated at 2 log CFU/g*

	Mean ± SD *E. coli* O157:H7 recovered (%)[b,c]		
Processing Step	Baby Spinach	Romaine Lettuce	Iceberg Lettuce
Flume Water[a]	48.9 ± 21.8	64.6 ± 64.2	118.2 ± 4.1
Shredder[a]	NA	0.5 ± 1.4 Aa	0.3 ± 0.3 Aa
Conveyor Belt[a]	NA	0.8 ± 1.5 ABa	0.9 ± 0.8 Ba
Flume Tank[a]	0.1 ± 0.2 Aa	0.0 ± 0.1 Ba	0.1 ± 0.2 Ba
Shaker Table[a]	0.0 ± 0.0 Aa	0.0 ± 0.0 Ba	0.2 ± 0.5 Ba
Dewatering Centrifuge[a]	0.0 ± 0.0 Ab	0.5 ± 0.9 ABa	0.4 ± 0.7 Bab
% Original Inoculum on Product after Processing	7.3 ± 9.6	14.1 ± 24.3	12.6 ± 4.5

[a] The total equipment surface areas for the shredder, conveyer belt, flume tank, shaker table and dewatering centrifuge are 1.74 m^2, 2.88 m^2, 2.60 m^2, 2.47 m^2, and 4.55 m^2, respectively.

[b] Means with different capital letter represent *E. coli* O157:H7 counts that differ significantly in terms of processing equipment location while means with different lowercase letter differ significantly in terms of leafy green variety ($P \leq 0.05$).

Table 2.4 (cont'd)

[c] Percentages are based on an estimation of the total number of *E. coli* O157:H7 cells having been transferred to the 890 L of flume water or equipment surfaces from the 50 lbs of inoculated leafy greens.

Table 2.5: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces during processing of leafy greens inoculated at 6 log CFU/g (n=3)*

	Mean ± SD *E. coli* O157:H7 recovered (log CFU/100 cm^2)[a]		
Location on Processing Equipment	Baby Spinach	Romaine Lettuce	Iceberg Lettuce
Shredder			
1. front, stainless steel (n=2)	ND	5.0 ± 0.5 $_{AB}$	5.7 ± 0.3 $_{AB}$
2. feed belt, plastic (n=2)	ND	4.6 ± 0.9 $_{ABC}$	5.3 ± 0.3 $_{ABC}$
3. feed belt guide, stainless steel (n=4)	ND	3.5 ± 2.1 $_{BC}$	4.8 ± 0.8 $_{BCDE}$
4. below the cutting wheel, stainless steel (n=1)	ND	4.2 ± 0.9 $_{ABC}$	5.0 ± 0.7 $_{ABCDE}$
5. cutting wheel, stainless steel (n=1)	ND	4.6 ± 0.3 $_{ABC}$	4.7 ± 0.3 $_{ABCDE}$
6. discharge chute, stainless steel (n=4)	ND	5.6 ± 0.5 $_{A}$	5.8 ± 0.5 $_{A}$
Conveyer Belt			
1. conveyer belt, polyurethane (n=4)	ND	5.1 ± 0.6 $_{A}$	5.7 ± 0.4 $_{A}$

Table 2.5 (cont'd)

2. belt guide, plastic (n=4)	ND	4.8 ± 0.6 AB	5.7 ± 0.5 A
Flume Tank			
1. slanted front end, stainless steel (n=1)	5.0 ± 0.5 A	5.3 ± 0.4 AB	5.1 ± 0.2 ABCDE
2. interior wall, stainless steel (n=10)	2.6 ± 0.8 D	1.9 ± 0.9 DE	2.5 ± 0.8 F
Shaker table			
1. front, stainless steel (n=1)	3.1 ± 0.6 BCD	3.9 ± 0.3 ABC	3.7 ± 0.3 CDEF
2. perforated dewatering screen, stainless steel (n=2)	3.6 ± 0.7 ABCD	4.4 ± 0.2 ABC	4.7 ± 0.0 ABCDE
3. center, stainless steel (n=2)	3.8 ± 0.3 ABC	4.5 ± 0.2 ABC	4.6 ± 0.1 BCDE
4. end (vertical portion), stainless steel (n=1)	3.0 ± 0.5 BCDE	3.6 ± 0.2 ABCD	3.8 ± 0.5 DEF
5. end (slanted portion), stainless steel (n=1)	2.5 ± 1.3 CDE	2.4 ± 0.2 CDE	3.5 ± 0.4 EF
6. recirculation spout, stainless steel (n=2)	1.5 ± 0.5 E	1.3 ± 0.5 E	2.6 ± 0.3 F
Dewatering Centrifuge			

Table 2.5 (cont'd)

1. interior basket carrier, stainless steel (n=4)	4.1 ± 0.3 $_{AB}$	4.8 ± 0.3 $_{AB}$	4.9 ± 0.6 $_{ABCD}$
2. interior wall, stainless steel (n=2)	4.0 ± 0.2 $_{ABC}$	4.1 ± 0.2 $_{ABC}$	4.2 ± 0.5 $_{CDE}$
3. viewing window in the lid, plastic (n=1)	2.6 ± 1.3 $_{CDE}$	1.0 ± 0.0 $_{E}$	2.1 ± 0.3 $_{F}$
4. drain, stainless steel (n=1)	5.2 ± 0.2 $_{A}$	5.5 ± 0.1 $_{AB}$	6.0 ± 0.4 $_{AB}$

[a] One over the detection limit was used to calculate the mean log value when a sample did not yield any colonies by direct plate count.

Means of the same product type with different letters are significantly different ($P \leq 0.05$). The numbers preceding the equipment location descriptions correspond with locations identified in Figures 2.1 through 2.5.

Table 2.6: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces during processing of leafy greens inoculated at 4 log CFU/g (n=3)*

Location on Processing Equipment	Mean ± SD *E. coli* O157:H7 recovered (log CFU/100 cm^2)[a]				
	Baby Spinach	Romaine Lettuce	Iceberg Lettuce	Iceberg Lettuce (finely shredded)	Iceberg Lettuce (held 24 h before processing)
Shredder					
1. front, stainless steel (n=2)	ND	$4.2 \pm 0.2_{AB}$	$3.8 \pm 0.2_{AB}$	$1.8 \pm 1.1_{ABC}$	$2.0 \pm 0.8_{ABC}$
2. feed belt, plastic (n=2)	ND	$4.2 \pm 0.1_{AB}$	$3.7 \pm 0.3_{ABC}$	$1.1 \pm 0.7_{ABC}$	$1.7 \pm 0.7_{ABC}$
3. feed belt guide, stainless steel (n=4)	ND	$3.8 \pm 0.7_{AB}$	$3.4 \pm 0.9_{ABCDE}$	$1.2 \pm 0.5_{BC}$	$1.8 \pm 0.9_{ABC}$
4. below the cutting wheel, stainless steel (n=1)	ND	$3.0 \pm 0.9_{ABCD}$	$3.3 \pm 0.4_{ABCDEF}$	$0.9 \pm 0.1_{ABC}$	$2.2 \pm 1.7_{ABC}$

Table 2.6 (cont'd)

5. cutting wheel, stainless steel (n=1)	ND	$3.9 \pm 0.1_{ABC}$	$2.3 \pm 0.2_{DEFG}$	$1.3 \pm 1.1_{ABC}$	$2.3 \pm 0.3_{ABC}$	
6. discharge chute, stainless steel (n=4)	ND	$4.5 \pm 0.3_{A}$	$3.7 \pm 0.7_{AB}$	$2.4 \pm 1.0_{ABC}$	$2.1 \pm 1.1_{AC}$	
Conveyer Belt						
1. conveyer belt, polyurethane (n=4)	ND	$4.4 \pm 0.4_{A}$	$4.0 \pm 0.5_{A}$	$2.8 \pm 0.6_{AC}$	$2.6 \pm 0.9_{A}$	
2. belt guide, plastic (n=4)	ND	$4.3 \pm 0.3_{AB}$	$3.9 \pm 0.4_{A}$	$1.9 \pm 1.2_{ABC}$	$2.5 \pm 0.6_{A}$	
Flume Tank						
1. slanted front end, stainless steel (n=1)	$2.9 \pm 0.9_{AB}$	$4.5 \pm 0.3_{AB}$	$3.3 \pm 0.1_{ABCDEF}$	$2.5 \pm 0.1_{ABC}$	$2.6 \pm 0.7_{ABC}$	
2. interior wall, stainless steel (n=10)	$0.7 \pm 0.9_{D}$	$1.7 \pm 1.1_{DE}$	$1.1 \pm 0.6_{GH}$	$1.3 \pm 1.7_{B}$	$0.6 \pm 1.6_{B}$	
Shaker table						
1. front, stainless steel (n=1)	$1.2 \pm 1.0_{ABCD}$	$1.2 \pm 1.0_{DE}$	$2.2 \pm 0.3_{EFG}$	$2.2 \pm 0.5_{ABC}$	$0.7 \pm 1.5_{ABC}$	

Table 2.6 (cont'd)

2.	perforated dewatering screen, stainless steel (n=2)	1.8	± 0.8	ABCD	2.9 ± 0.1	ABCDE	2.8 ± 0.1	ABCDEF	0.8 ± 1.6	ABC	1.0	± 1.7	ABC
3.	center, stainless steel (n=2)	1.7	± 0.4	ABC	3.1 ± 3	BC	2.9 ± 0.2	BCDEF	2.7 ± 0.8	ABC	1.5	± 1.9	ABC
4.	end (vertical portion), stainless steel (n=1)	1.4	± 0.3	ABCD	3.1 ± 0.1	ABCD	2.3 ± 0.2	CDEFG	1.7 ± 0.1	ABC	1.4	± 0.8	ABC
5.	end (slanted portion), stainless steel (n=1)	0.9	± 0.9	BCD	3.2 ± 0.2	ABCD	2.0 ± 0.5	FG	3.0 ± 1.6	ABC	1.3	± 0.4	ABC
6.	recirculation spout, stainless steel (n=2)	1.0	± 1.3	CD	0.8 ± 1.2	E	0.4 ± 0.9	H	2.3 ± 2.5	ABC	0.3	± 1.6	BC
Dewatering Centrifuge														
1.	interior basket carrier, stainless steel (n=4)	2.3	± 0.3	ABC	2.4 ± 1.5	CD	2.7 ± 0.2	CDEF	3.2 ± 1.0	A	3.0	± 0.6	A
2.	interior wall, stainless steel (n=2)	1.7	± 0.2	ABCD	3.1 ± 0.2	ABCD	2.1 ± 0.3	F	3.1 ± 0.8	ABC	2.4	± 0.5	ABC
3.	viewing window in the lid, plastic (n=1)	0.9	± 0.6	BCD	3.1 ± 0.3	ABCD	3	± 1.6	H	3.1 ± 1.9	ABC	1.8	± 0.8	ABC

Table 2.6 (cont'd)

4. drain, stainless steel (n=1)	3.1	0.3 $_{A}$	0.9 ± 0.8 $_{DE}$	3.9 ± 0.1 $_{ABCD}$	3.5 ± 0.7 $_{ABC}$	3.5 ± 0.3 $_{A}$

[a] One over the detection limit was used to calculate the mean log value when a sample did not yield any colonies by direct plate count.

Means of the same product with different letters are significantly different ($P \leq 0.05$). The numbers preceding the equipment location descriptions correspond with locations identified in Figures 2.1 through 2.5.

Table 2.7: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces during processing of leafy greens inoculated at 2 log CFU/g (n=5)*

Location on Processing Equipment	Mean ± SD E. coli O157:H7 recovered (log CFU/100 cm^2)[a]		
	Baby Spinach	Romaine Lettuce	Iceberg Lettuce
Shredder			
1. front, stainless steel (n=2)	ND	1.3 ± 0.6 $_{ABC}$	1.6 ± 0.6 $_{ABCD}$
2. feed belt, plastic (n=2)	ND	0.9 ± 1.1 $_{ABCD}$	1.3 ± 0.6 $_{ABCDEF}$
3. feed belt guide, stainless steel (n=4)	ND	0.0 ± 1.2 $_{DEF}$	1.0 ± 1.2 $_{ABCDEF}$
4. below the cutting wheel, stainless steel (n=1)	ND	0.2 ± 0.8 $_{BCDEFG}$	1.3 ± 0.6 $_{ABCDEFG}$
5. cutting wheel, stainless steel (n=1)	ND	0.9 ± 0.4 $_{ABCDE}$	1.0 ± 0.6 $_{ABCDEFGH}$
6. discharge chute, stainless steel (n=4)	ND	1.8 ± 0.6 $_{A}$	1.5 ± 0.6 $_{ABC}$
Conveyer Belt			
1. conveyer belt, polyurethane (n=4)	ND	1.1 ± 1.1 $_{ABC}$	1.8 ± 0.5 $_{A}$

Table 2.7 (cont'd)

2. belt guide, plastic (n=4)	ND	1.0 ± 0.9 ABC	1.7 ± 0.5 AC
Flume Tank			
1. slanted front end, stainless steel (n=1)	1.9 ± 1.0 A	0.3 ± 1.2 BCDEFG	1.5 ± 0.2 ABCDEF
2. interior wall, stainless steel (n=10)	-0.7 ± 0.8 C	-1.1 ± 0.3 G	-0.4 ± 1.0 H
Shaker table			
1. front, stainless steel (n=1)	-0.3 ± 1.3 BC	-0.8 ± 0.8 EFG	-0.1 ± 0.7 DEFGH
2. perforated dewatering screen, stainless steel (n=2)	-0.4 ± 1.3 BC	0.0 ± 0.8 BCDEFG	0.5 ± 1.0 ABCDEFGH
3. center, stainless steel (n=2)	0.1 ± 1.2 BC	0.1 ± 0.9 CDEF	0.6 ± 1.0 BDEFG
4. end (vertical portion), stainless steel (n=1)	-0.5 ± 1.0 BC	-0.8 ± 0.7 EFG	-0.3 ± 0.8 FGH
5. end (slanted portion), stainless steel (n=1)	0.1 ± 1.3 ABC	-1.1 ± 0.1 FG	0.2 ± 1.6 BCDEFGH
6. recirculation spout, stainless steel (n=2)	-1.0 ± 0.7 C	-1.0 ± 0.5 FG	-0.2 ± 1.3 GH
Dewatering Centrifuge			

Table 2.7 (cont'd)

1. interior basket carrier, stainless steel (n=4)	0.5 ± 0.7 $_{AB}$	0.6 ± 1.1 $_{BCDE}$	1.0 ± 0.7 $_{ABCDEFG}$
2. interior wall, stainless steel (n=2)	0.0 ± 0.9 $_{BC}$	-0.1 ± 0.8 $_{DEFG}$	0.0 ± 0.9 $_{EFGH}$
3. viewing window in the lid, plastic (n=1)	-0.9 ± 0.5 $_{BC}$	-1.0 ± 0.0 $_{FG}$	-0.1 ± 1.1 $_{EFGH}$
4. drain, stainless steel (n=1)	0.8 ± 1.2 $_{AB}$	1.8 ± 0.6 $_{AB}$	1.7 ± 0.5 $_{ABCDE}$

[a] One over the detection limit was used to calculate the mean log value when a sample did not yield any colonies by direct plate count.

Means of the same product with different letters are significantly different ($P \leq 0.05$). The numbers preceding the equipment location descriptions correspond with locations identified in Figures 2.1 through 2.5.

CHAPTER 3:

Transfer of *Escherichia coli* O157:H7 from Equipment Surfaces to Iceberg and Romaine Lettuce during Pilot-Plant Production of Fresh-Cut Leafy Greens

3.1 ABSTRACT

Transfer of *Escherichia coli* O157:H7 during commercial production of ready-to-eat salad greens has become a public health concern due to several large foodborne outbreaks. The goal of this study was to quantify *E. coli* O157:H7 transfer from product-inoculated equipment surfaces to uninoculated lettuce during post-harvest processing. Uninoculated cored heads of iceberg and Romaine lettuce (22.7 kg) were processed using a commercial shredder, step conveyor, 3.3-m flume tank with sanitizer-free tap water, shaker table and centrifugal dryer, followed by 22.7 kg of product that was dip-inoculated to contain ~6, 4, or 2 log CFU/g of a 4-strain avirulent, GFP-labeled, ampicillin-resistant *E. coli* O157:H7 cocktail. After draining the flume tank and refilling the holding tank with tap water, 90.8 kg of uninoculated product was similarly processed and collected in ~5 kg aliquots. After processing, 42 equipment surface samples along with 46 iceberg or 36 romaine lettuce samples (25 g each) from the collection baskets were quantitatively examined for GFP-labeled *E. coli* O157:H7 by direct plating or membrane filtration using trypticase soy agar containing 0.6% yeast extract and 100 ppm ampicillin. Initially, greatest *E. coli* O157:H7 transfer was seen from inoculated lettuce to the shredder and conveyer belt, with all equipment surface populations decreasing 90 - 99% after processing 90.8 kg of uncontaminated product. After processing lettuce containing 10^6 or 10^4 *E. coli* O157:H7 CFU/g followed by uninoculated lettuce, *E. coli* O157:H7 was quantifiable throughout the entire 90.8 kg of product. At an inoculation level of 2 log CFU/g, *E. coli* O157:H7 was consistently detected in the first 21.2 and 68.0 kg of previously uninoculated lettuce at 2 - 3 log CFU/100 g and transferred out to 75.6 and 86.9 kg product, respectively. These findings demonstrate the persistence of *E. coli* O157:H7 on equipment and subsequent contamination of potentially large quantities of uncontaminated product during processing.

3.2 INTRODUCTION

Changes in food production and consumption patterns are repeatedly blamed for the increasing frequency of produce-associated outbreaks. In the United States between 1998 and 2007, fresh produce was implicated in 684 outbreaks that included 26,735 cases of illness (CSPI 2009). Norovirus was linked to 51% of these outbreaks, whereas *Salmonella* and *E. coli* O157:H7 were respectively responsible for 17 and 7% of the outbreaks (CSPI 2009). The food industry has evolved from many local producers to a far smaller number of large-scale operations with national distribution patterns. This change means that one contamination event at a large centralized grower or processor can have far-reaching consequences. This was the case in September of 2006, when contaminated pre-bagged baby spinach was linked to an *E. coli* O157:H7 outbreak in 26 states and Canada that resulted in 205 illnesses and five deaths (Jay and others 2007). Only three months later, two additional nationwide *E. coli* O157:H7 outbreaks were traced to California-grown iceberg lettuce that was shredded and then purchased by two fast-food Mexican restaurant chains in the Midwest and Northeast (FDA 2006, FDA 2007), with these two outbreaks involving over 150 individuals (CSPI 2009).

Leafy greens are prone to contamination throughout the farm-to-fork continuum (Beuchat 2002), including during growth in the field (Erickson and others 2010, Solomon and others 2002), harvesting (Taormina and others 2009), post-harvest handling and processing (Kaneko and others 1999, Stafford and others 2002), distribution (Beuchat and Ryu 1997), and preparation in the home or food service settings (Chai and others 2008, Chen and others 2001, Montville and others 2001, Ravishankar and others 2010). Even though *E. coli* O157:H7 contamination from fecal material is presumed to be highly localized in the field, this organism can be spread easily to previously pathogen-free produce during and after harvest, thus

intensifying what was once a small contamination event (Beuchat and Ryu 1997, Burnett and Beuchat 2001).

The introduction and spread of bacterial pathogens during commercial leafy green processing remains a major industry concern. In May and June of 2001, 10 cases of *Salmonella* Bovismorbificans infection phage type 32 were traced to commercially shredded lettuce in Australia (Stafford and others 2002). Investigators eventually recovered the outbreak strain of *S.* Bovismorbificans from the back rim of the lettuce shredder cutting wheel, with improper maintenance of the shredder being a key factor in this outbreak. More recently, shredded and pre-packaged lettuce from a Dutch processing plant was traced to an *E. coli* O157 outbreak that sickened a total of 50 individuals in the Netherlands and Iceland (Friesema and others 2008). This outbreak, which again was likely due to the spread of the contaminant during processing, exemplifies the problems that can occur when a contaminated product is simultaneously distributed across borders.

Despite these recent large-scale outbreaks, the extent of bacterial transfer and cross-contamination that can occur during commercial processing of leafy greens remains poorly understood, with most bacterial transfer work previously focused on slicing of deli meats (Keskinen and others 2008a, Keskinen and others 2008b, Lin and others 2006, Sheen and Hwang 2008, Vorst and others 2006), grinding of beef (Farrell and others 1998, Floras and Tamplin 2002, Floras and Stewart 2004), and various food service and consumer food preparation/handling practices (Chai and others 2008, Chen and others 2001, Fravalo and others 2009, Gill and Jones 2002, Montville and others 2001, Ravishankar and others 2010, Scott and Bloomfield 1990). In limited work with *E. coli* O157:H7-inoculated leafy greens, our laboratory demonstrated that approximately 90% of the inoculum was shed in sanitizer-free

water, with this pathogen also contaminating the product contact surfaces of a shredder, conveyor, flume tank, shaker table and dewatering centrifuge to various degrees during processing *(*Buchholz and others 2012b*).* When researchers in Japan examined factories that processed ready-to-eat fresh vegetables, samples from equipment surfaces used for trimming, washing, slicing, soaking, dehydrating, blending, packaging, the surfaces of workers' plastic gloves and from the floor and air of processing rooms proved to be most heavily contaminated *(*Kaneko and others 1999*).* Elsewhere in the food chain, *E. coli* O157:H7 was shown to transfer from inoculated coring blades to multiple heads of iceberg lettuce during field coring *(*Taormina and others 2009*)*, with this pathogen also shown to remain viable on dry stainless steel surfaces for up to 4 days *(*Kusumaningnum and others 2003*).*

The overall goal of this study was to determine the numbers of *E. coli* O157:H7 transferred from inoculated to uninoculated iceberg and Romaine lettuce during small-scale processing, which included shredding, conveying, fluming/washing, shaker table dewatering and centrifugal drying, with these findings needed in the development of more accurate risk assessments for leafy greens.

3.3 MATERIALS AND METHODS

3.3.1 Experimental design. Transfer of *E coli* O157:H7 from various surfaces of a small-scale commercial leafy green processing line to iceberg and Romaine lettuce was assessed by first processing 22.7 kg of uninoculated lettuce to prime the processing line, followed by 22.7 kg of lettuce inoculated with *E. coli* O157:H7 at 10^6 and 10^4 (3 replicates each) or 10^2 CFU/g (5 replicates). After draining and refilling the water recirculation tank with tap water, 90.8 kg of uninoculated lettuce was processed, collected in a series of baskets and quantitatively examined for *E. coli* O157:H7 as described below.

3.3.2 Leafy greens. Individually wrapped heads of iceberg (*Lactuca sativa* L.) (24 heads per case) and Romaine (*Lactuca sativa* L. var. *longifolia*) (12 heads per case) lettuce were obtained from a local wholesaler (Stan Setas Produce Co., Lansing, MI), stored in a 4°C walk-in-cooler and used within 5 days of delivery. Products originated from California or Arizona, depending on the growing season. All heads were hand-cored immediately before use.

3.3.3 Bacterial strains. Four avirulent, GFP-labeled, ampicillin-resistant strains of *E. coli* O157:H7 (ATCC 43888, CV2b7, 6980-2, and 6982-2) were obtained from Dr. Michael Doyle at the Center for Food Safety, University of Georgia, Griffin, GA. Upon arrival, stock cultures of each strain were grown in trypticase soy broth (Difco, Becton Dickinson, Sparks, MD) containing 0.6% yeast extract (Difco, Becton Dickinson) supplemented with 100 ppm ampicillin (ampicillin sodium salt, Sigma Life Science, St. Louis, MO) (TSBYE + amp). After 24 h of incubation at 37^{o}C, 10% (v/v) glycerol (Sigma Chemical Company, St. Louis, MO) was added to these cultures which were then stored at -80°C until needed. Working cultures were prepared by streaking each stock culture on trypticase soy agar (Difco, Becton Dickinson) containing 0.6% yeast extract and 100 ppm ampicillin (TSAYE + amp). After 18 - 24 h of

incubation at 37°C, cells from a single colony were transferred to 9 ml of TSBYE + amp and similarly incubated. Thereafter, a 0.2 ml aliquot of each culture was transferred to 200 ml of TSBYE + amp, incubated for 18 - 20 h at 37°C and then combined in equal volumes to obtain the 4-strain cocktail.

3.3.4 Inoculation of iceberg and Romaine lettuce. *E. coli* O157:H7 suspensions containing 10^7, 10^5 and 10^3 CFU/ml were respectively obtained by adding 800, 8 and 0.08 ml of the 4-strain cocktail to 75 L of tap water (~15^{o}C) in a 121 L plastic container (Rubbermaid, Wooster, OH). Thereafter, 22.7 kg of iceberg (~24 heads) or Romaine lettuce (~48 heads) was immersed in the *E. coli* suspension for 15 min, removed, spun in a 50-lb capacity centrifugal Spin Dryer (model SD50-LT, Heinzen Manufacturing, Inc., Gilroy, CA), with one internally timed spin cycle totaling ~20 sec to remove the residual inoculum, and then drained/air-dried for 1 h at 22°C before processing. Using sterilized tongs, two representative 25-g samples were taken from two inoculated lettuce heads to determine the average inoculation level at the time of processing.

3.3.5 Processing equipment. The pilot-scale size leafy green processing line housed in the Department of Food Science and Human Nutrition consisted of a lettuce shredder, conveyer, flume tank, shaker table, and water recirculation tank. The commercial lettuce shredder (model TRS 2500 Urschel TranSlicer, Valparaiso, IN) operated at a feed belt speed of 198 m/min and slicing wheel speed of 905 RPM, which yielded shreds of lettuce measuring approximately 5 x 5 cm. The motorized conveyor (Dorner model 736018 mc series, Hartland, WI), which was equipped with a smooth polyurethane step conveyer belt (ThermoDrive, Mol Industries), operated at 0.11 m/sec. The stainless steel water recirculation tank (~1000 L capacity) was filled with 890 L of sanitizer-free tap water (~15^{o}C). This water was centrifugally pumped (model

XB754FHA, Sterling Electric, Inc., Irvine, CA) at a rate of ~15 L/sec through a 4.14 m long, 10-cm diameter hard plastic connector hose to a 3.6-m long stainless steel flume tank (Heinzen Manufacturing, Inc.) that was equipped with two overhead spray jets located 1 m from the start of the flume tank. The stainless steel shaker table for partial dewatering was equipped with a wash down duty motor (1 HP, Baldor Electric Co., Ft. Smith, AR) that operated at 1760 rpm. Water exiting the flume tank passed through a screen on the shaker table into a water recirculation spout, which returned the water to the holding tank for recirculation.

3.3.6 Iceberg and Romaine lettuce processing and sample collection. Cored heads of uninoculated iceberg and Romaine lettuce (22.7 kg) were fed into the shredder for continuous processing followed by 22.7 kg of inoculated lettuce. The lettuce was then step-conveyed to the flume tank and washed, with ~5 kg aliquots of the partially dried product exiting the shaker table then collected in each of ~12 plastic mesh laundry baskets (United Plastics, Leomister, MA). After draining the flume and recirculation tanks, 11 previously identified *(*Buchholz and others 2012b*)* product contact surface samples (100 cm^2) on the equipment (3 shredder, 2 conveyer belt, 3 flume tank and 3 shaker table samples) were collected using one-ply composite tissues (Kimwipes®, Kimberly-Clark Corp., Irving, TX) moistened in 1 ml of sterile 1% (w/v) phosphate buffer (8.5 g/L NaCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4, J.T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ) as described by Vorst et al. (2006). The water recirculation tank was then refilled with ~890 L of sanitizer-free tap water (~15^oC), after which 90.8 kg of uninoculated lettuce was similarly processed. During processing, 200-ml aliquots of water (~13 from iceberg and ~18 Romaine from lettuce processing) were collected from the water return spout on the shaker table. After processing, the 90.8 kg of previously uninoculated lettuce was collected in ~24 baskets for later enumeration of *E. coli* O157:H7. Finally, all 50 product contact surface

areas (14 shredder, 8 conveyer belt, 11 flume tank and 9 shaker table samples) previously identified by Buchholz et al. (2012b) using Glo Germ™ (Glo Germ Company, Moab, Utah) were sampled using one-ply composite tissues moistened with 1 ml of sterile phosphate buffer.

3.3.7 Microbiological analyses. Romaine and iceberg lettuce samples (25 g) composited from 5 separate locations within each of the ~24 baskets were added to 100 ml of sterile phosphate buffer in a Whirl-Pak™ filter bag (Nasco, Fort Atkinson, WI) and pulsified (Pulsifier, Filtaflex Ltd., Almonte, Ontario, Canada) for 1 min. Lettuce samples were either serially diluted in sterile phosphate buffer and plated on TSAYE + amp or processed by filtration using 0.45 μm membrane filters (Millipore, Millipore Corporation, Billerica, MA) (lower detectable limit of 0.04 CFU/g), which were placed on 60-mm dia. Petri plates containing TSAYE + amp to quantify *E. coli* O157:H7. The one-ply composite tissue samples were added to 15 ml of phosphate buffer in a Whirl-Pak™ bag, homogenized by stomaching (Stomacher 400 Circulator, Seward, Worthington, UK) for 1 min at 260 rpm and then plated on TSAYE + amp as well, giving a lower detectable limit of 1 CFU/100 cm^2. The 200 ml water samples were either appropriately diluted and plated on TSAYE + amp or processed by membrane filtration, which gave a minimum detectable limit of 0.01 CFU/ml. After 18 – 20 h of incubation at 37°C, all green fluorescing colonies as seen under ultraviolet light (Blak-Ray, Ultra-violet Product Inc., San Gabriel, CA) were counted as *E. coli* O157:H7.

3.3.8 Statistical analyses. Bacterial counts were converted to log CFU per gram, ml or 100 cm^2 to determine the numbers of *E. coli* O157:H7 transferred to uncontaminated product, equipment surfaces, and water, respectively, during processing of iceberg and Romaine lettuce. An ANOVA with the Tukey-Kramer HSD test were then performed using JMP 8.0 (SAS

Institute Inc., Cary, NC) to determine statistically significant differences ($P < 0.05$) in the numbers of *E. coli* O157:H7 transferred between the equipment surfaces, lettuce and water.

3.4 RESULTS

3.4.1 Initial inoculation levels. The three four-strain *E. coli* O157:H7 cocktails used to inoculate iceberg lettuce contained 6.98 ± 0.07, 4.98 ± 0.11 and 2.96 ± 0.24 log CFU/ml, and the cocktails for Romaine lettuce contained 7.07 ± 0.47, 5.96 ± 0.15 and 3.37 ± 1.16 log CFU/ml. After immersing for 15 min followed by 1 h of air drying and 20 sec of centrifugal drying to remove excess inoculum, iceberg lettuce contained *E. coli* O157:H7 populations of 6.08 ± 0.63, 4.14 ± 0.45 and 2.21 ± 0.71 log CFU/g, and Romaine lettuce contained 6.44 ± 0.59, 4.47 ± 0.65 and 1.98 ± 0.18 log CFU/g (Figure 3.1, 3.2 and 3.3). No significant differences in inoculation levels ($P > 0.05$) were seen between product types.

3.4.2 Flume water. When 22.7 kg of lettuce containing three different levels of *E. coli* O157:H7 was processed, approximately 90% of the inoculum transferred to the sanitizer-free water. This water, which was subsequently drained from the flume and water recirculation tanks to avoid re-contaminating the uninoculated product to follow, contained *E. coli* O157:H7 populations of 2.23, 2.21 and -0.27 log, and 2.95, 1.94 and -0.25 log CFU/ml after processing iceberg and Romaine lettuce inoculated at the three levels, respectively. After refilling the recirculation tank with ~890 L of tap water (~15^{o}C) this same water yielded *E. coli* O157:H7 populations of -1.40, 0.01 and -2.18 log CFU/ml, and 1.98, 0.12 and -1.61 log CFU/ml after processing the remaining 90.8 kg of uninoculated iceberg and Romaine lettuce, respectively.

3.4.3 Processing equipment surfaces. After processing, populations of *E. coli* O157:H7 were higher ($P < 0.05$)on the conveyer belt for both products at all three inoculation levels as well as on the shredder after processing Romaine and iceberg lettuce at the medium and low inoculation levels, respectively. Statistically higher ($P < 0.05$) *E. coli* populations were seen on the polyurethane conveyer belt compared to the other equipment surfaces (Tables 3.1, 3.2, and

3.3). Overall, *E. coli* O157:H7 populations decreased 0.24 to 2.51 and 0.11 to 1.98 log CFU/100 cm^2 on equipment surfaces after processing the uninoculated iceberg and Romaine lettuce, respectively, with greatest losses seen for the shredder and shaker table (Table 3.4). Based on these observations, processing additional uninoculated product would likely have resulted in further cross-contamination from the equipment.

3.4.4 Lettuce. GFP-labeled *E. coli* O157:H7 was sporadically recovered from uninoculated lettuce used to prime the processing line immediately, before introducing inoculated product. This phenomenon can be explained by pieces of inoculated lettuce sporadically exiting the processing line with uninoculated lettuce since not all shreds of lettuce moved through the line at the same rate. In addition, some GFP-labeled cells of *E. coli* O157:H7 likely transferred from the inoculated lettuce to the recirculating wash water and then to the last portion of uninoculated lettuce exiting the processing line. When 22.7 kg of lettuce inoculated at 2 log CFU/g was processed followed by 90.8 kg of uninoculated lettuce, *E. coli* O157:H7 transferred to the 78^{th} - 82^{nd} kg of uninoculated iceberg lettuce (the 127^{th} - 131^{th} kg of total processed iceberg lettuce) at -1.29 log CFU/g, whereas at inoculation levels of 4 and 6 CFU/g, the last kg of previously uninoculated product processed (the 91^{st} kg) yielded *E. coli* O157:H7 populations of 0.34 and 1.51 log CFU/g, respectively. Regardless of inoculation level, contamination was seen throughout the uninoculated Romaine lettuce after processing. *E. coli* O157:H7 counts after Romaine lettuce processing were 2.90, 0.72 and -1.10 log CFU/g for the high, medium, and low inoculation levels, respectively. Differences in *E. coli* O157:H7 contamination levels were observed between replicates for each inoculation level; however, the distribution followed the same pattern (Figure 3.4, 3.5, and 3.6).

A 2 log reduction was observed after 6.98 and 16.21 kg of uninoculated Romaine lettuce was processed in the high and medium inoculation level experiments, respectively. Similarly, in the high and medium inoculation level experiments, 5.92 and 8.88 kg of uninoculated iceberg lettuce needed to be processed to achieve a 2 log reduction, respectively. However, *E. coli* O157:H7 reductions of only a 1.74 and 1.98 log were observed in the low inoculation level experiments using Romaine and iceberg lettuce, respectively. After processing uninoculated followed by inoculated product, mean recovery was significantly greater ($P > 0.05$) for Romaine as compared to iceberg lettuce regardless of the inoculation level.

3.5 DISCUSSION

Quantitative bacterial transfer has been previously assessed during field coring of iceberg lettuce (Taormina and others 2009), production of ground beef (Farrell and others 1998, Floras and Tamplin 2002, Floras and Stewart 2004), and slicing of delicatessen meats (Keskinen and others 2008a, Keskinen and others 2008b, Lin and others 2006, Sheen and Hwang 2008, Vorst and others 2006). In one study modeling the distribution of *E. coli* O157:H7 during commercial grinding of inoculated followed by uninoculated beef, results were obtained using initial inoculums of 2, 4 and 6 log CFU/g with *E. coli* detected in 12.7, 32.1, and 86.2% of ~6600 g of beef trim, respectively (Floras and Stewart 2004). In terms of leafy greens, Taormina et al. (2009) at the University of Georgia demonstrated that *E. coli* O157:H7 can transfer to successive uncontaminated heads of iceberg lettuce during harvesting. When field coring implements were inoculated through contact with contaminated soil containing *E. coli* O157:H7 at 2.72 and 1.67 log CFU/g and then used to cut and core 10 lettuce heads, the pathogen transferred to 10 and 5 consecutively processed heads, respectively, again demonstrating a direct correlation between increasing bacterial load and subsequent transfer.

This, however, is the first study to systematically quantify the extent of product cross-contamination for key unit operations, including shredding, conveying, fluming, shaker table dewatering, and centrifugal drying, that are found in most commercial processing lines for production of fresh-cut salad greens. In this work, three inoculation levels were evaluated - 6, 4 and 2 log CFU/g, which represent high, medium, and low bacterial loads on leafy greens, respectively. One study conducted at Rutgers University showed that as the inoculum size increases, the number of bacteria transferred remains constant and the apparent percent transfer rate will decrease (Montville and Schaffner 2003). In their study focusing on cross-

contamination between chicken, lettuce, cutting boards, hands, and a spigot, a significant correlation was seen between inoculum size and percent transfer during a cross-contamination event. While our starting inocula of 6 and 4 log CFU/g might be considered unusually high, these levels were needed to generate the necessary quantitative data for subsequent predictive models and eventual incorporation into risk assessments for leafy greens. The two higher inoculation levels may represent direct contamination in the field from fecal matter, because cattle are a major source of this pathogen in the environment (Williams and others 2008). Although direct contact with manure is one source of pre-harvest contamination, the same contaminated manure can also be introduced into the farm's irrigation water, which may be lead to far lower numbers of bacteria being distributed to a much wider area of the field (Beuchat 2002, Ibekwe and others 2004). As expected, the *E. coli* O157:H7 transfer rates were more variable using lettuce inoculated at 2 log CFU/g, as compared to the two higher levels.

A uniform inoculum, as could be achieved only by dipping, was essential for generating repeatable transfer data. While other inoculation methods also exist, Lang et al. (2004) reported that higher *E. coli* O157:H7 populations were recovered from dip- as opposed to spot- or spray-inoculated lettuce when the same populations were initially applied, because a larger surface area of the leaf could be covered. In our study, we chose to evaluate products held for one hour after dip inoculation to simulate contamination at the processing facility. However, extending the post-inoculation hold time before processing enhances the attachment of *E. coli* O157:H7 to leafy greens and lowers the transfer rate during processing, as previously reported by Buchholz et al. (2012b). Lang et al. (2004) reported similar findings when comparing *E. coli* O157:H7 recovery from leafy greens that were dried for either 2 h at 22°C or 2 h at 22°C followed by 22 h

at 4°C. Additional work is needed to determine the extent of cross-contamination during processing of field-contaminated leafy greens.

In the United States, commercially prepared leafy greens intended for the fresh-cut ready-to-eat market undergo multiple washings in sanitizer-containing flume systems to remove soil and decrease the microbial load. However, microbial contaminants can be easily spread to previously uncontaminated product via water and equipment surfaces during processing. Because the goal of this study was to quantify *E. coli* O157:H7 transfer during leafy green processing, both iceberg and Romaine lettuce were processed without the use of a chemical sanitizer, which is also common practice in Europe (Holvoet and others 2012). If sanitizers had been used, *E. coli* O157:H7 populations in the flume water would have most likely been reduced to near non-detectable levels, decreasing our ability to quantify the transfer of viable cells to previously uncontaminated product. When iceberg and Romaine lettuce were inoculated to contain ~2 log CFU/g, *E. coli* was consistently detected in the flume water at levels between -1.5 and -2.0 log CFU/g. However, given an estimated oral infectious dose of less than 100 cells for *E. coli* O157:H7 (Meng and others 2007), presence of any viable *E. coli* O157:H7 cells in flume water, as seen in our study, is reason for concern if appropriate intervention strategies are not taken to prevent cross-contamination. The potential for cross-contamination during flume washing was also previously demonstrated by immersing apples in a dump tank (Buchanan and others 1999); however, a few years later a group of USDA researchers showed negligible cross-contamination from inoculated to uninoculated apples via the wash water (Annous and others 2001).

Not only was *E. coli* O157:H7 quantifiable during and after processing of the 22.7 kg of inoculated lettuce, but small numbers of the pathogen were also present in water collected from

the shaker table before the inoculated product reached that same point in the process. This phenomenon may be attributed to the tumbling movement of the product through the flume tank, and the velocity differential between the water and the product, with the overhead jets also forcing the leafy greens below the waterline. Since the uninoculated and inoculated products were of the same type and therefore visually indistinguishable, the exact point at which the inoculated leaves exited the shaker table could only be estimated based on knowing that the initial uninoculated and inoculated batches were of equal size at the start.

As the inoculation level increased, both the amount of contaminated product and the *E. coli* O157:H7 populations on the product increased. The design of this study provides an overall estimate of the numbers of *E. coli* O157:H7 accumulating over time after a contaminated batch of product is processed, rather than the numbers of *E. coli* O157:H7 exclusively transferred to previously uncontaminated lettuce. Cross contamination of uninoculated leafy greens during processing may have occured through contact with 1) the water (~2 L) remaining in the flume tank, shaker table, centrifugal pump, hose and water recirculation tank after draining, 2) inoculated lettuce clinging to the shredder and conveyer belt surfaces, and 3) any contaminated leafy greens lingering in the processing line.

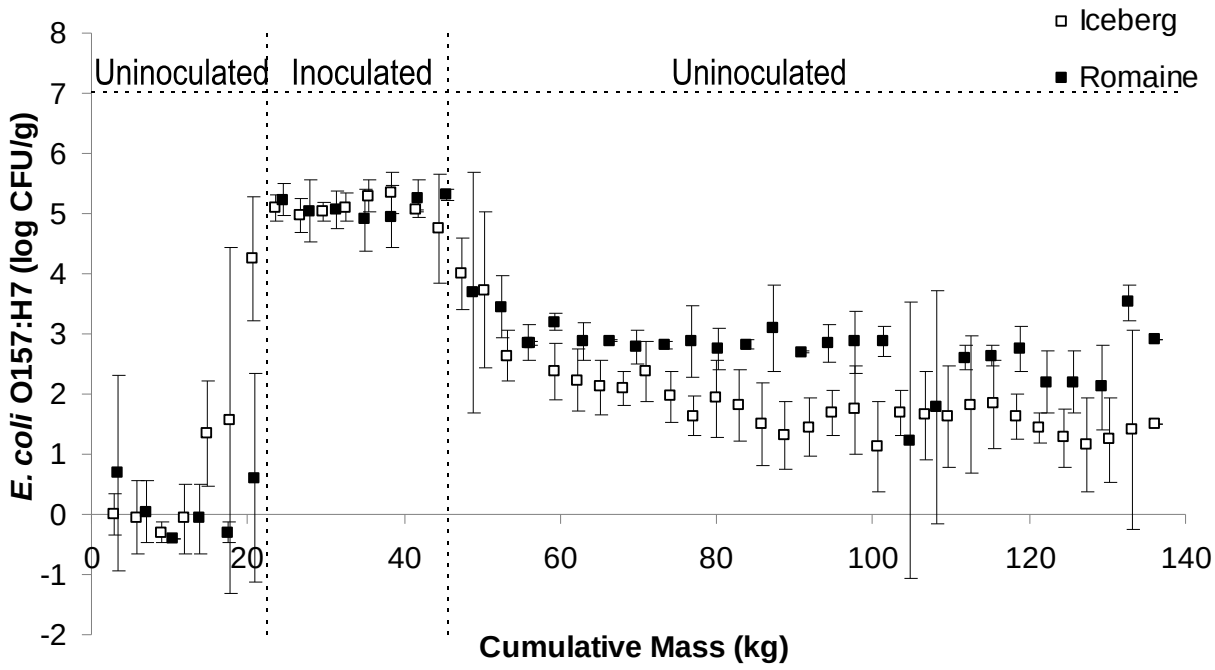
In terms of equipment surfaces, the conveyer belt, shredder discharge chute and front panel of the flume tank were frequent collection points for *E. coli* O157:H7 after uninoculated product was processed. These locations, which were not exposed to water, were frequently coated with lettuce juice and many small shreds of residual product after processing, providing ideal bacterial harborage sites. This finding was not surprising, since two studies showed that relatively high numbers of *Salmonella* and *Campylobacter* were transferred to Romaine lettuce even 1 to 2 h after the food contact surface became contaminated *(*Moore and others 2003*)*, with

Salmonella remaining viable on surfaces for up to four days *(*Kusumaningnum and others 2003*)*. In our study, some of the small shreds of lettuce lingering on the processing equipment could still be the initial inoculated product, which could break free from the equipment surface as additional product is processed. In a study examining the microbial changes of "Lollo Rosso" lettuce during processing and subsequent storage, an increase of 1 log CFU/g was observed after shredding *(*Allende and others 2004*)*, reaffirming the role of the shredder in spreading contamination to additional product during processing *(*Garg and others 1990*,* Stafford and others 2002*)*. Locations that were constantly exposed to the wash water, such as the interior walls of the flume tank and the water recirculation spout on the shaker table, yielded the lowest *E. coli* O157:H7 populations after processing. Comparing these findings with data collected after processing 22.7 kg of contaminated iceberg or Romaine lettuce though the same processing line *(*Buchholz and others 2012b*)* illustrates how easily this pathogen can persist on food contact surfaces and cross-contaminate product still to be processed, especially if sanitizers are not used or monitored properly.

In summary, this study shows that one contaminated batch of leafy greens can easily contaminate subsequent batches of previously uncontaminated product in a processing facility if an effective microbial intervention strategy is not implemented. Such cross-contamination can occur even when *E. coli* O157:H7 is present in very low levels, with 0.06 and 0.34% of the original 2 log CFU/g inoculum spread to previously uninoculated product after processing. This study supports the importance of sanitizers in wash waters, or other interventions, to adequately reduce the microbial load on the product and in flume water during commercial processing to prevent future product recalls and outbreaks.

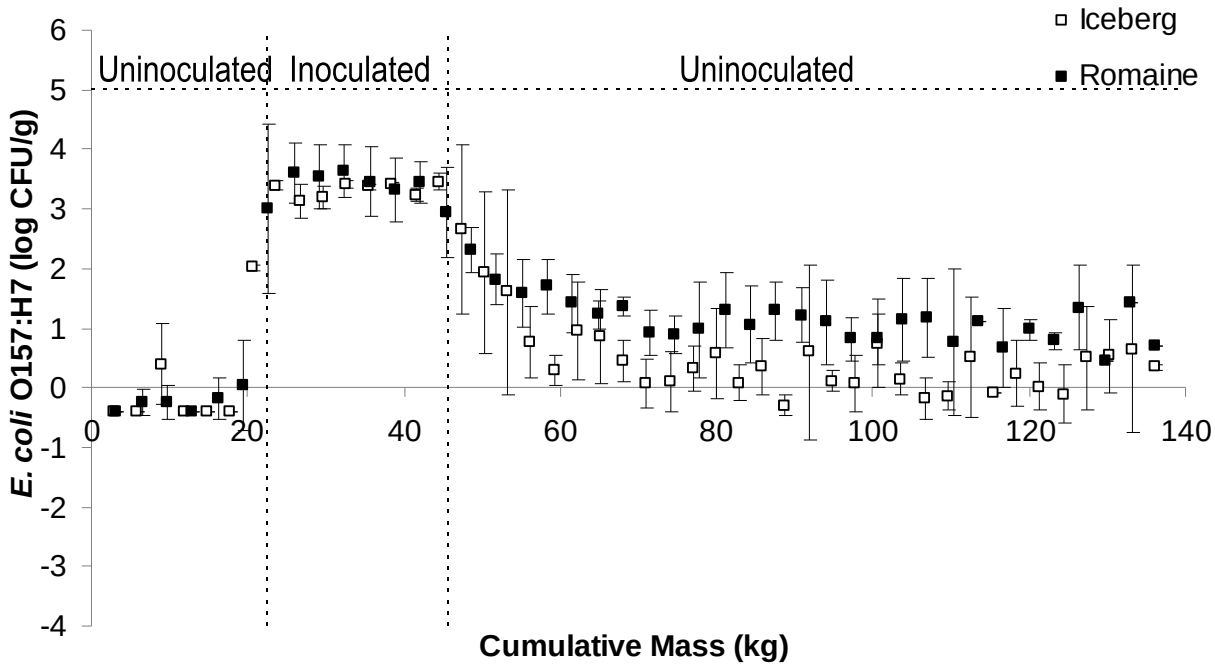
3.6 FIGURES

Figure 3.1: E. coli *O157:H7 populations (mean ± SD) on the product during processing of iceberg and Romaine lettuce inoculated at ~6 log CFU/g (n = 3).*



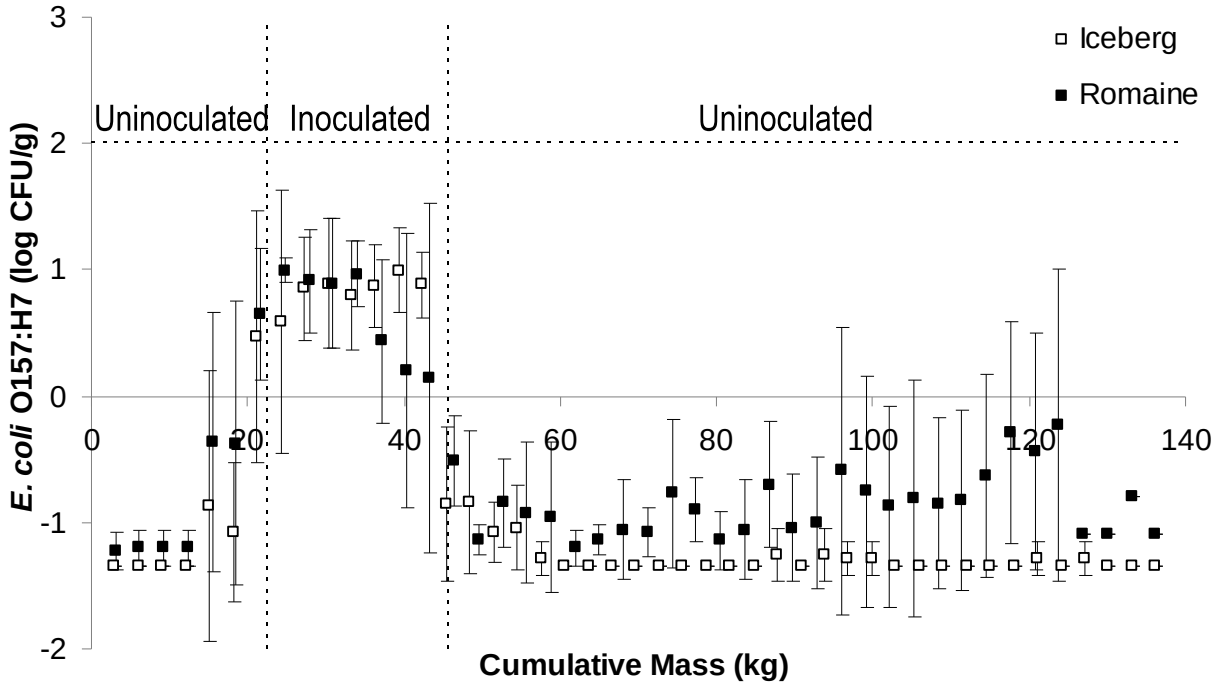
[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit.

Figure 3.2: E. coli *O157:H7 populations (mean ± SD)*[a] *on the product during processing of iceberg and Romaine lettuce inoculated at ~ 4 logs CFU/g (n = 3).*



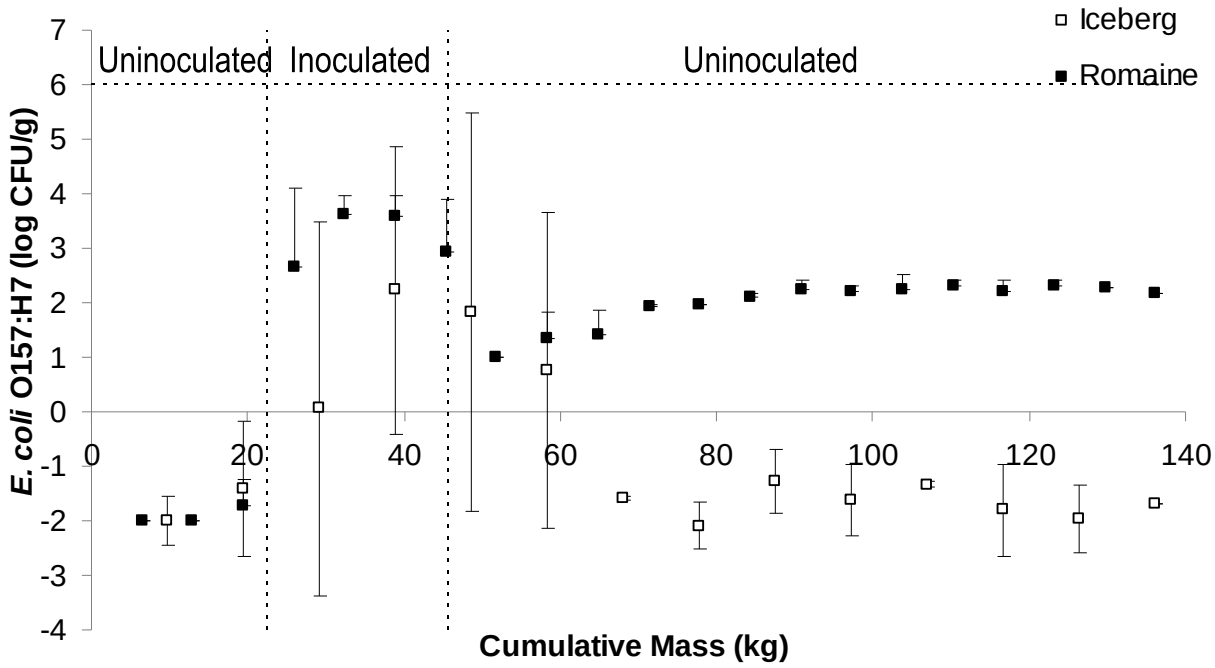
[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit.

Figure 3.3: E. coli *O157:H7 populations (mean ± SD)*[a] *on the product during processing of iceberg and Romaine lettuce inoculated at ~ 2 logs CFU/g (n = 5).*



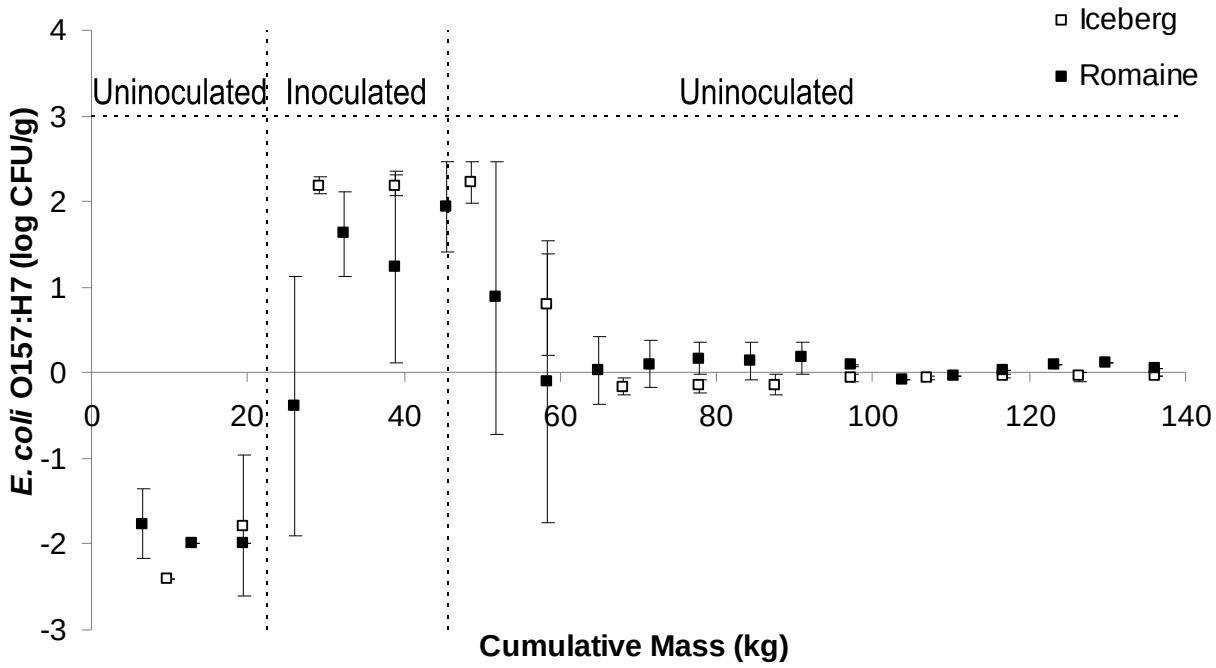
[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit.

Figure 3.4: E. coli *O157:H7 populations (mean ± SD)*[a] *in water during processing of iceberg and Romaine lettuce inoculated at ~6 logs CFU/g (n = 3).*



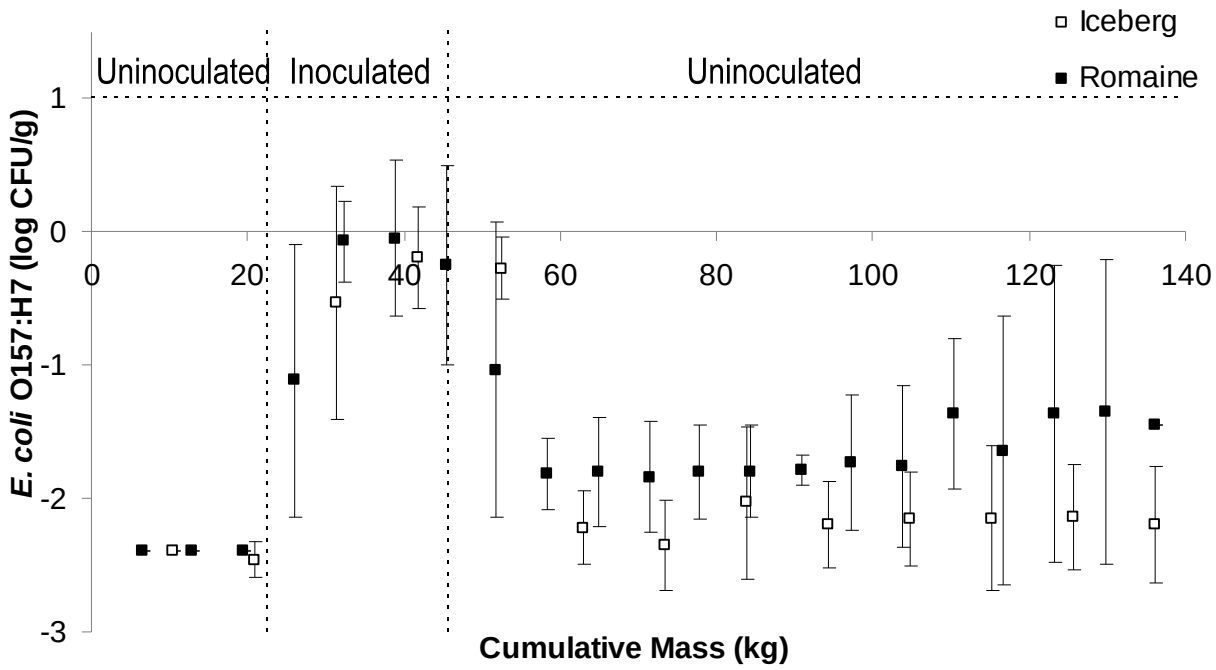
[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit.

Figure 3.5*:* E. coli *O157:H7 populations (mean ± SD)*[a] *in water during processing of iceberg and Romaine lettuce inoculated at ~4 logs CFU/g (n = 3).*



[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit.

Figure 3.6*:* E. coli *O157:H7 populations (mean ± SD)*[a] *in water during processing of iceberg and Romaine lettuce inoculated at ~2 logs CFU/g (n = 5).*



[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit.

3.7 TABLES

Table 3.1: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces during processing of leafy greens inoculated at 6 log CFU/g (average of 3 replicates)*

Location on Processing Equipment	Mean (± SD) *E. coli* O157:H7 (log CFU/g)			
	Romaine Lettuce		Iceberg Lettuce	
Shredder				
1. front, stainless steel (n=2)	3.7 ± 1.8	ABC	2.1 ± 0.5	BCD
2. feed belt, plastic (n=2)	1.2 ± 0.4	DE	2.9 ± 0.4	ABCD
3. feed belt guide, stainless steel (n=4)	1.6 ± 0.7	DE	2.0 ± 1.0	D
4. below the cutting wheel, stainless steel (n=1)	3.5 ± 1.0	ABC	2.8 ± 0.5	ABCD
5. cutting wheel, stainless steel (n=1)	3.9 ± 0.6	ABC	1.6 ± 0.7	BCDE
6. discharge chute, stainless steel (n=4)	4.0 ± 1.3	AB	3.2 ± 1.0	ABC
Conveyer Belt				
1. conveyer belt, polyurethane (n=4)	4.4 ± 0.8	A	3.8 ± 0.8	A
2. belt guide, plastic (n=4)	3.7 ± 0.8	AB	3.1 ± 0.7	AB
Flume Tank				
3. slanted front end, stainless steel (n=1)	4.2 ± 0.8	ABC	3.6 ± 0.0	ABCD
4. interior wall, stainless steel (n=10)	0.8 ± 0.9	E	0.6 ± 1.0	EF

Table 3.1 (cont'd)

Shaker table				
1. front, stainless steel (n=1)	2.2 ± 0.2	BCDE	1.4 ± 0.3	CDEF
2. perforated dewatering screen, stainless steel (n=2)	2.7 ± 0.2	BCD	2.4 ± 0.2	BCD
3. center, stainless steel (n=2)	2.7 ± 0.4	BCD	2.2 ± 0.6	BCD
4. end (vertical portion), stainless steel (n=1)	2.6 ± 0.56	BCD	2.0 ± 0.3	BCDE
5. end (slanted portion), stainless steel (n=1)	1.9 ± 0.5	CDE	1.9 ± 0.1	BCDE
6. recirculation spout, stainless steel (n=2)	0.5 ± 0.8	E	-0.5 ± 0.8	F

[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit. Means of the same product not followed by the same letter are significantly different ($P \leq 0.05$).

[b] The numbers preceding the equipment location descriptions correspond with locations previously identified by Buchholz et al. (2012b).

Table 3.2: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces during processing of leafy greens inoculated at 4 log CFU/g (average of 3 replicates)*

Location on Processing Equipment	Mean (± SD) *E. coli* O157:H7 (log CFU/g)	
	Romaine Lettuce	Iceberg Lettuce
Shredder		
1. front, stainless steel (n=2)	1.9 ± 0.5 $_{ABCD}$	0.4 ± 1.0 $_{BCDE}$
2. feed belt, plastic (n=2)	1.1 ± 1.1 $_{BCD}$	1.0 ± 1.1 $_{ABCD}$
3. feed belt guide, stainless steel (n=4)	1.2 ± 0.6 $_{CD}$	-0.4 ± 1.0 $_{CE}$
4. below the cutting wheel, stainless steel (n=1)	1.9 ± 1.1 $_{ABCD}$	1.0 ± 0.6 $_{ABCD}$
5. cutting wheel, stainless steel (n=1)	1.3 ± 0.5 $_{ABCDE}$	1.0 ± 0.5 $_{ABCD}$
6. discharge chute, stainless steel (n=4)	2.5 ± 0.7 $_{AB}$	0.7 ± 1.3 $_{ABCD}$
Conveyer Belt		
1. conveyer belt, polyurethane (n=4)	2.6 ± 0.7 $_{A}$	1.7 ± 0.7 $_{A}$
2. belt guide, plastic (n=4)	1.6 ± 1.2 $_{ABCD}$	0.9 ± 1.1 $_{ABD}$
Flume Tank		
1. slanted front end, stainless steel (n=1)	3.0 ± 0.5 $_{ABC}$	2.2 ± 0.3 $_{AB}$
2. interior wall, stainless steel (n=10)	-0.3 ± 1.0 $_{EF}$	-0.8 ± 0.6 $_{E}$
Shaker table		

Table 3.2 (cont'd)

1. front, stainless steel (n=1)	1.0 ± 0.2	BCDE	0.1 ± 0.2	ABCDE
2. perforated dewatering screen, stainless steel (n=2)	1.3 ± 0.2	BCD	0.3 ± 0.7	BCDE
3. center, stainless steel (n=2)	1.3 ± 0.2	BCD	0.3 ± 0.87	BCDE
4. end (vertical portion), stainless steel (n=1)	1.6 ± 0.2	ABCD	0.3 ± 0.2	ABCDE
5. end (slanted portion), stainless steel (n=1)	0.5 ± 0.6	DEF	-0.7 ± 0.9	CDE
6. recirculation spout, stainless steel (n=2)	-1.2 ± 0.0	F	-1.2 ± 0.0	E

[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit. Means of the same product not followed by the same letter are significantly different ($P \leq 0.05$).

[b] The numbers preceding the equipment location descriptions correspond with locations previously identified by Buchholz et al. (2012b).

Table 3.3: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces during processing of leafy greens inoculated at 2 log CFU/g (average of 5 replicates)*

Location on Processing Equipment	Mean (± SD) *E. coli* O157:H7 recovered (log CFU/g)					
	Romaine Lettuce			Iceberg Lettuce		
Shredder						
1. front, stainless steel (n=2)	-0.4	± 0.9	ABC	-1.2	± 0.0	AB
2. feed belt, plastic (n=2)	-0.7	± 0.8	ABC	-0.9	± 0.5	AB
3. feed belt guide, stainless steel (n=4)	-0.6	± 0.9	BC	-1.2	± 0.0	B
4. below the cutting wheel, stainless steel (n=1)	-0.3	± 1.3	ABC	-1.2	± 0.0	AB
5. cutting wheel, stainless steel (n=1)	-0.9	± 0.7	ABC	-1.2	± 0.0	AB
6. discharge chute, stainless steel (n=4)	0.5	± 1.3	A	-0.9	± 0.7	AB
Conveyer Belt						
1. conveyer belt, polyurethane (n=4)	0.4	± 1.1	A	-0.6	± 0.8	A
2. belt guide, plastic (n=4)	0.2	± 1.1	AB	-1.1	± 0.5	B
Flume Tank						
1. slanted front end, stainless steel (n=1)	0.4	± 1.0	ABC	-0.7	± 0.6	AB
2. interior wall, stainless steel (n=10)	-1.1	± 0.4	C	-1.2	± 0.0	B

Table 3.3 (cont'd)

Shaker table

1.	front, stainless steel (n=1)	-0.5	± 0.9	ABC	-1.2	± 0.0	AB
2.	perforated dewatering screen, stainless steel (n=2)	-0.5	± 0.9	ABC	-1.2	± 0.0	B
3.	center, stainless steel (n=2)	-0.7	± 1.1	ABC	-1.1	± 0.4	AB
4.	end (vertical portion), stainless steel (n=1)	-0.4	± 1.1	ABC	-0.9	± 0.5	AB
5.	end (slanted portion), stainless steel (n=1)	-1.2	± 0.0	BC	-1.2	± 0.0	AB
6.	recirculation spout, stainless steel (n=2)	-1.2	± 0.0	C	-1.2	± 0.0	B

[a] Mean log values for samples without counts by direct plating were calculated as 1/detection limit. Means of the same product not followed by the same letter are significantly different ($P \leq 0.05$).

[b] The numbers preceding the equipment location descriptions correspond with locations previously identified by Buchholz et al. (2012b).

Table 3.4: E. coli *O157:H7 populations (mean ± SD) recovered from equipment surfaces* [a] *before and after processing 90.7 kg of uninoculated leafy greens*

Product	Inoculation Level	Processing Step	Mean (± SD) *E. coli* O157:H7 (log CFU)		
			Before	After	Reduction
Romaine Lettuce	6 log CFU/g[b]	Shredder	8.3 ± 7.8	6.6 ± 7.0	1.7 ± 0.8
		Conveyor Belt	8.6 ± 8.4	7.3 ± 7.3	1.3 ± 1.1
		Flume Tank	6.6 ± 7.0	6.0 ± 6.8	0.6 ± 0.2
		Shaker Table	6.9 ± 6.8	4.9 ± 5.0	2.0 ± 1.8
	4 log CFU/g[b]	Shredder	6.7 ± 6.8	4.7 ± 5.0	2.0 ± 1.8
		Conveyor Belt	6.9 ± 6.9	5.4 ± 5.6	1.5 ± 1.3
		Flume Tank	4.7 ± 4.8	4.4 ± 5.1	0.3 ± -0.3
		Shaker Table	5.3 ± 5.1	3.6 ± 3.6	1.7 ± 1.5
	2 log CFU/g[c]	Shredder	4.2 ± 4.1	3.4 ± 3.8	0.9 ± 0.3
		Conveyor Belt	4.4 ± 4.2	3.5 ± 3.7	1.0 ± 0.5
		Flume Tank	2.5 ± 2.8	2.4 ± 3.0	0.1 ± -0.2

Table 3.4 (cont'd)

		Shaker Table	3.1	±	2.9	2.8	±	3.5	0.2	± -0.6
Iceberg Lettuce	6 log CFU/g	Shredder	7.5	±	7.8	5.7	±	6.2	1.8	± 1.6
		Conveyor Belt	7.8	±	8.0	6.8	±	7.0	1.0	± 1.0
		Flume Tank	5.8	±	5.8	4.6	±	5.3	1.3	± 0.6
		Shaker Table	7.0	±	7.1	4.6	±	4.7	2.4	± 2.4
	4 log CFU/g	Shredder	7.4	±	7.7	3.7	±	4.1	3.7	± 3.6
		Conveyor Belt	5.6	±	5.5	4.6	±	5.0	1.0	± 0.5
		Flume Tank	3.9	±	3.9	3.7	±	4.2	0.2	± -0.3
		Shaker Table	5.2	±	5.1	2.7	±	2.7	2.5	± 2.4
	2 log CFU/g	Shredder	3.2	±	3.3	1.8	±	2.4	1.4	± 0.9
		Conveyor Belt	3.7	±	3.9	2.4	±	2.7	1.3	± 1.2
		Flume Tank	3.0	±	3.3	1.4	±	1.7	1.6	± 1.6
		Shaker Table	3.3	±	3.6	1.4	±	1.7	1.9	± 1.9

[a] Total product contact surface areas for the shredder, conveyer belt, flume tank and shaker table are 1.74, 2.88, 2.60, and 2.47 m^2, respectively.

Table 3.4 (cont'd)

[b] $n = 3$ for 6 and 4 log CFU/g

[c] $n = 5$ for 2 log CFU/g

CHAPTER 4:

Tracking an *Escherichia coli* O157:H7 Contaminated Batch of Leafy Greens through a Commercial Processing Line

4.1 ABSTRACT

Cross-contamination of fresh-cut leafy greens with small quantities of *Escherichia coli* O157:H7-contaminated product during commercial processing is likely to be at least partially responsible for several recent multistate outbreaks. In this study, Radicchio (9.1 kg) was dip-inoculated to contain 10^6 CFU/g of a 4-strain avirulent, GFP-labeled, ampicillin-resistant *E. coli* O157:H7 cocktail and then used as a visual marker to track and quantify the spread of *E. coli* O157:H7 to iceberg lettuce in a pilot-scale processing line that included a commercial shredder, step conveyor, flume tank, shaker table and centrifugal dryer. After priming this line with 45 kg of uninoculated iceberg lettuce, the inoculated Radicchio was processed followed by 907 kg of iceberg lettuce. Forty water samples (500 ml), 50 equipment swabs (100 cm^2) and ~38 bags of lettuce/Radicchio (~22.7 kg/bag) were collected. All visible shreds of Radicchio were retrieved from the bags of shredded product, the equipment, and the floor and were weighed and counted. Twenty-five grams of iceberg lettuce was collected from each bag of product and from 10 of the bags after centrifugal drying. All samples were examined for *E. coli* O157:H7 by direct plating or membrane filtration with trypticase soy agar containing 0.6% yeast extract and 100 ppm ampicillin. After processing, the weight of inoculated Radicchio in shredded lettuce averaged 614.9 g (93.6 %), 6.9 g (1.3 %), 5.0 g (0.8 %) and 2.8 g (0.5 %) for bags 1 - 10, 11 - 20, 21 - 30, and 31 – 40, respectively. These same bag groupings contained average *E. coli* O157:H7 populations of 1.69, 1.22, 1.10 and 1.11 log CFU/g. After processing, shreds of Radicchio were most prevalent on the conveyor (9.8 g) followed by the shredder (8.3 g), flume tank (3.5 g) and shaker table (0.1 g), showing that contaminated product can continually spread during leafy green processing long after the contamination event.

4.2 INTRODUCTION

Leafy greens from the Salinas Valley of California have been implicated in nine *Escherichia coli* O157:H7 outbreaks from 1995 to 2006, with the last three receiving considerable national attention. In September 2006, contaminated pre-bagged baby spinach triggered an *Escherichia coli* O157:H7 outbreak in 26 states and Canada, resulting in five deaths and 205 illnesses (Jay and others 2007). Only a few months later, two additional *E. coli* O157:H7 outbreaks were traced to California-grown iceberg lettuce that was shredded and then purchased by two fast-food Mexican restaurant chains in the Midwest and Northeast (FDA 2006; FDA 2007). These two outbreaks sickened a combined total of over 150 individuals (CSPI 2009a). Changes in food production and consumption patterns are repeatedly blamed for the increasing frequency of produce-associated outbreaks. Evolution of this industry from many small local to far fewer large-scale operations now means that one contamination event at a large centralized grower or processor can have a lasting negative impact on the industry as seen by the spinach outbreak that decimated baby spinach sales for months (Calvin 2007).

Leafy greens are prone to contamination throughout the farm-to-fork continuum. Pre-harvest sources include feces from wild and domestic animals, water runoff from livestock operations, green or improperly composted manure, air, dust, insects, and irrigation water. Pathogens can also come in contact with fresh produce through field workers, harvesting equipment, hand coring/trimming, water used for cooling/washing, improperly cleaned crates, totes and storage bins and the vehicles used for transport (Beuchat 1996; Beuchat and Ryu 1997; Gorny 2006). A significant portion of these leafy-greens become fresh-cut ready-to-eat products that place consumers at increased risk due to the potential spread of microbial pathogens during commercial processing (Beuchat and Ryu 1997; Burnett and Beuchat 2001). This situation is

exacerbated by the fact that most commonly used commercial sanitizers are only capable of decreasing the microbial load 90 – 99% on the product during flume washing (Beuchat and others 2004; Burnett and others 2004; Keskinen and others 2009; Sapers 2001; Weissinger and others 2000; Zhang and others 2009).

Shredded lettuce can easily remain in a leafy green processing line and contaminate subsequent product as is supported by a 2001 outbreak of *Salmonella* Bovismorbificans in Australia that sickened 10 people. This outbreak was later attributed to residual shredded lettuce that was found behind the cutting wheel of the shredder during an environmental audit (Stafford and others 2002). Previous work in our laboratory has confirmed that *E. coli* O157:H7 can readily transfer to the product contact surfaces of a leafy green processing line (Buchholz and others 2012b) and persist on these equipment surfaces, subsequently contaminating large quantities of previously uncontaminated product during processing (Buchholz and others 2012d).

The goals of this study were to track an *E. coli* O157:H7-inoculated batch of Radicchio through a pilot-scale processing line to determine: 1) the numbers of *E. coli* O157:H7 transferred to a subsequently processed much larger batch of iceberg lettuce as well as to the equipment and water during processing, and 2) the amount of inoculated Radicchio transferred to the shredded lettuce and the various pieces of equipment after processing.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design. Initially 45.4 kg of uninoculated iceberg lettuce was processed in a small-scale commercial processing line, which included shredding, conveying, fluming/washing, shaker table dewatering and centrifugal drying. Thereafter, Radicchio heads inoculated at 10^6 CFU/g *E. coli* O157:H7 and held for 1 h at 22°C were similarly processed followed by 907.2 kg of uninoculated lettuce, with the red color of Radicchio allowing easy differentiation between inoculated and uninoculated product. All experiments were conducted in triplicate.

4.3.2 Produce. Pallets of pre-cored iceberg lettuce (*Lactuca sativa* L.) heads (~250 kg/pallet) were received from Aunt Mid's Produce Company (Detroit, MI) and stored at 4°C at Michigan State University Food Stores and used with 2 days of delivery. Radicchio heads (*Cichorium intybus*) (12 heads per case) were obtained from a local wholesaler (Stan Setas Produce Co., Lansing, MI), stored at 4°C in a walk-in-cooler and used within 5 days of delivery. Both products originated from California or Arizona, depending on the growing season.

4.3.3 Bacterial strains. Four avirulent, GFP-labeled, ampicillin-resistant strains of *E. coli* O157:H7 (ATCC 43888, CV2b7, 6980-2, and 6982-2) were obtained from Dr. Michael Doyle at the Center for Food Safety, University of Georgia, Griffin, GA. Upon arrival, stock cultures of each strain were prepared in trypticase soy broth (Difco, Becton Dickinson, Sparks, MD) containing 0.6% (w/v) yeast extract (Difco, Becton Dickinson) supplemented with 100 ppm ampicillin (ampicillin sodium salt, Sigma Life Science, St. Louis, MO) (TSBYE + amp) and 10% (v/v) glycerol (Sigma Chemical Company, St. Louis, MO) and stored at -80°C until needed. Working cultures were prepared by streaking each stock culture on plates of trypticase soy agar (Difco, Becton Dickinson) containing 0.6% yeast extract and 100 ppm ampicillin (TSAYE +

amp). After 18 - 24 h of incubation at 37°C, a single colony was transferred to 9 ml of TSBYE + amp and similarly incubated. Thereafter, a 0.2 ml aliquot of each culture was transferred to 200 ml of TSBYE + amp, incubated for 18 – 20 h at 37°C and then combined in equal volumes to obtain the 4-strain cocktail.

4.3.4 Inoculation of Radicchio. An *E. coli* O157:H7 suspension containing 10^7 CFU/mL was obtained by adding 800 mL (quantities were determined based on the growth rates from Buchholz and others 2012b) of the 4-strain cocktail to 75 L of tap water (~15°C) in a 121 L plastic container (Rubbermaid, Wooster, Ohio). Hand-cored heads of Radicchio (9.1 kg) were then immersed in the *E. coli* suspension for 15 min, spun in the dewatering centrifuge to remove residual inoculum and drained/air-dried for 1 h at 22°C before processing. Using sterilized tongs, two 25-g samples were taken from two inoculated Radicchio heads to determine the average inoculation level at the time of processing.

4.3.5 Processing equipment. The pilot-scale size leafy green processing line consisted of a lettuce shredder, conveyer belt, flume tank, shaker table and centrifugal dryer. The commercial lettuce shredder (model TRS 2500 Urschel TranSlicer, Valparaiso, IN) was set to operate at a feed belt speed of 198 m/min and a slicing wheel speed of 905 RPM. The polyurethane step conveyer belt (ThermoDrive, Mol Industries, Grand Rapids, MI) on the conveyor (Dorner model 736018 mc series, Hartland, WI) operated at 0.11 m/sec. A stainless steel, ~1000 L capacity, water holding tank was filled with 890 L of tap water (~15°C) for fluming. This water holding tank was connected to a 3.6 m stainless steel flume tank (Heinzen Manufacturing, Inc., Gilroy, CA) equipped with two overhead spray jets (1 m from the start) by a 4.14 m long, 10 cm diameter hard plastic circulation hose with a centrifugal pump (model XB754FHA, Sterling Electric, Inc., Irvine, CA) that operated at a rate of ~15 L/sec. The stainless

steel shaker table, which partially dewatered the leafy greens, was equipped with a 1 HP Baldor washdown duty motor (Baldor Electric Co., Ft. Smith, AR) that operated at 1760 RPM. Water drained from the leafy greens through a screen on the shaker table and was transferred to the water holding tank via a water recirculation spout on the shaker table. A 50-lb capacity centrifugal Spin Dryer (model SD50-LT, Heinzen Manufacturing, Inc.) with three internally timed spin cycles totaling 60 sec. was used for centrifugal drying.

4.3.6 Radicchio and iceberg lettuce processing and sample collection. Intact heads of uninoculated cored iceberg lettuce (45.4 kg dry weight) were processed using the previously described pilot scale processing line, beginning with the shredder, followed by 9.1 kg (dry weight) of inoculated Radicchio and collected in three 121 L containers (~23 kg wet weight/ container). After draining and refilling the water recirculation tank with ~900 L of tap water (~15^{o}C), 907.2 kg of uninoculated iceberg lettuce were similarly processed in about 22 min with an additional 121-L of tap water (~15^{o}C) added to the water recirculation tank midway through processing to maintain proper water flow. During processing ~38 containers (~23 kg wet weight/container) of lettuce/Radicchio were sequentially collected for analysis along with ~38 500-mL water samples from the water return spout on the shaker table. One 25-g sample of exclusively iceberg lettuce was removed from each container for microbiological analysis after which all visible shreds of Radicchio in the ~38 containers and on the shredder, conveyor, flume tank, shaker table and the floor were retrieved and weighed. Iceberg lettuce container numbers 1, 3, 5, 10, 15, 20, 25, 30, 35 and 40 were centrifugally dried, after which one 25-g sample/container was collected for analysis. Finally, 42 equipment surface samples (100 cm^2) previously identified using Glo Germ™ (14 shredder, 8 conveyer belt, 11 flume tank and 9 shaker table samples) were collected using one-ply composite tissues moistened in 1 mL of

sterile phosphate buffer and composited based on the piece of equipment to obtain a total of four samples.

4.3.7 Microbiological analyses. Iceberg lettuce samples (25 g) were added to 100 ml of phosphate buffer in a Whirl-Pak™ filter bag and homogenized in a pulsifier (Pulsifier, Filtaflex Ltd., Almonte, Ontario, Canada) for 1 min. Radicchio pieces from containers 1 - 2, 3 - 4, 5 - 9, 10 - 19, 20 - 24, 25 - 29, 30 - 34, 35 - ~38 were composited to obtain a maximum sample size of 25 g, diluted 1:5 in phosphate buffer in Whirl-Pak™ bag (Nasco, Fort Atkinson, WI) and then either homogenized in a stomacher (Stomacher 400 Circulator, Seward, Worthington, UK) for 1 min at 260 rpm or, for very small samples, hand-massaged in the bag for 1 min. Following the same procedure described in Buchholz and others (2012b), the lettuce, Radicchio and water samples were either appropriately diluted or 10 to 100 ml were filtered through a 0.45 μm membrane filter (Millipore, Millipore Corporation, Billerica, MA) and then plated on TSAYE + amp to quantify *E. coli* O157:H7. The one-ply composite tissue samples were added to 100 ml of phosphate buffer in a Whirl-Pak™ bag, homogenized in a Stomacher for 1 min at 260 rpm and then plated on TSAYE + amp. The 100 ml water samples were processed by membrane filtration and placed on TSAYE + amp. The plates were incubated for 18 – 20 h at 37°C, after which all green fluorescing colonies seen under ultraviolet light were counted as *E. coli* O157:H7.

4.3.8 Statistical analysis. *E. coli* O157:H7 populations transferring from the inoculated Radicchio to the equipment surfaces, uninoculated iceberg lettuce and water during processing were converted to log CFU/g, ml or 100 cm^2. The wet-weight percentage of inoculated Radicchio in each container of shredded iceberg lettuce was also determined. An ANOVA followed by the Tukey-Kramer HSD test ($\alpha = 0.05$) was conducted using JMP 8.0 software (SAS

Institute Inc., Cary, NC) to determine significant differences in *E. coli* O157:H7 numbers transferred between the equipment surfaces, water and iceberg lettuce.

4.3.9 Modeling of non-linear curves. JMP 8.0 software was also used to conduct a non-linear regression analysis and calculate 95% confidence intervals for the curves fitted to the *E. coli* O157:H7 counts on the Radicchio, lettuce and water, as well as the weights of Radicchio transferred over time. The cumulative Weibull model was used as follows:

$$\log N/N_0 = -bt^n,$$

where b and n are the scale and shape parameters, respectively (Chen 2007, Gonzalez and others 2009, Marfart and others 2002, Peleg and Cole 1998).

4.4 RESULTS

4.4.1 Radicchio. A four-strain *E. coli* O157:H7 suspension containing 7.2 ± 0.5 log CFU/mL was used to inoculate the heads of Radicchio. After immersion, the Radicchio contained 5.9 ± 0.5 log CFU/g *E. coli* O157:H7. Numbers of *E. coli* O157:H7 decreased to 5.0 ± 0.4 log CFU/g on the Radicchio after shredding, conveying, washing and shaker table partial dewatering with similar populations seen on Radicchio shreds recovered from the ~38 containers of iceberg lettuce after processing (Figure 4.1).

After single 45.4 kg batches of uninoculated iceberg lettuce and Radicchio were processed following by 907.2 kg of uninoculated lettuce, shreds of Radicchio were found in all but one bag of the ~38 bags for each of the three independent experiments. The weight of Radicchio ranged from 858.3 to 0.001 g (Figure 4.2) with most of the inoculated Radicchio appearing in the first of the ~38 containers of the uncontaminated iceberg collected for each of three replicates. When the shredded lettuce bags were divided into four groups including bags 1 - 10, 11 - 20, 21 - 30, and 31 – 40, then after processing, the weight of inoculated Radicchio averaged 614.9 g (93.6 %), 6.9 g (1.3 %), 5.0 g (0.8 %) and 2.8 g (0.5 %) in the shredded lettuce bag groupings, respectively.

4.4.2 Iceberg lettuce. *E. coli* O157:H7 populations on the 907.2 kg of previously uninoculated lettuce ranged from -0.1 to 3.0 log CFU/g, with an overall average of 1.2 log CFU/g (Figure 4.3). Slight differences in contamination levels were observed between replicates; however, the distribution followed the same pattern. Using the same bag grouping system as described above, bags 1 - 10, 11 - 20, 21 - 30, and 31 – 40, contained average *E. coli* O157:H7 populations of 1.69, 1.22, 1.10 and 1.11 log CFU/g, respectively.

The weight of the of iceberg lettuce increased from 907.2 kg to ~1194 kg due to the water that remained on the product after partial dewatering on the shaker table. No significant decreases ($\alpha = 0.05$) in *E. coli* O157:H7 populations were observed on the iceberg lettuce following centrifugal drying for each sample basket pair (Figure 4.4). Unlike the findings in Buchholz and others (2012b), which showed a 30% *E. coli* population decrease on the iceberg lettuce after centrifugal drying, this study indicates between a 3.8 and 82.0 % decrease. This difference in findings could be due to the large standard deviation and the very low levels of *E. coli* on this Iceberg lettuce in this particular study.

4.4.3 Processing equipment surfaces. While a few pieces of shredded Radicchio remained on each piece of processing equipment (Figure 4.5), a larger percentage in weight of these pieces ($P < 0.05$) remained on the conveyer belt as compared to the shredder, flume tank and shaker table (Table 4.1). Radicchio build-up was most evident on the discharge chute and area surrounding the cutting wheel of the shredder, the front of the flume tank and the polyurethane conveyer belt, especially the junction between the guard rails and the belt.

No significant differences in average numbers of *E. coli* O157:H7 (1.88 – 3.15 log CFU/100 cm^2) remaining at the end of processing ($P > 0.05$) were seen between the four equipment surface areas (Table 4.2). These findings clearly indicate that *E. coli* O157:H7 persisted in this processing line after 907.2 kg of clean uncontaminated iceberg lettuce was processed.

4.4.4 Flume water. After processing 907.2 kg of uninoculated iceberg lettuce, *E. coli* O157:H7 populations in the sanitizer-free flume water ranged from the limit of detection (LOD) to 3.6 log CFU/100 ml. Differences between the three replicates highlight the variability in

transfer during processing (Figure 4.6). This finding is most likely related to the amount of residual water remaining in the system prior to refilling the water holding tank.

4.5 DISCUSSION

Previous studies have demonstrated the potential for bacterial cross-contamination during field coring (Taormina and others 2009) and post-harvest processing of leafy greens (Buchholz and others 2012b and 2012d) as well as washing of produce (Buchanan and others 1999; Lopez-Galvez and others 2009; Lopez-Galvez and others 2010; Nou and Lou 2010). Bacterial transfer has also been demonstrated in various other food preparation scenarios, including grinding of meat (Farrell and others 1998; Flores and Stewart 2004, Flores and Tamplin 2002) and slicing deli meats (Keskinen and others 2008a; Keskinen and others 2008b; Lin and others 2006; Sheen and Hwang 2008; Vorst and others 2006), as well as between lettuce, chicken, and cutting boards (Chai and others 2008; Chen and others 2001; Fravalo and others 2009; Jiménez and others 2009; Ravishankar and others 2010), and cloths, hands, gloves, utensils and various food products (Chen and others 2001; Gill and Jones 2002; Montville and others 2001; Scott and Bloomfield 1990).

One notable study measured the distribution of *Listeria monocytogenes* during slicing of roast turkey breast, Genoa hard salami, and bologna with kitchen knives. These researchers demonstrated that most bacteria transfer to the first 5 to 15 slices depended on the stainless steel type and product being cut (Vorst and others 2006). Work conducted at University of Georgia also demonstrated that *E. coli* O157:H7 transferred to successive uncontaminated heads of iceberg lettuce during harvesting. When devices used in field coring were inoculated with soil containing 2.72 and 1.67 log *E. coli* O157:H7 CFU/g, the pathogen transferred to 10 and 5 consecutively processed heads of lettuce, respectively (Taormina et al. 2009). Flores and Stewart (2004) tracked the spread of *E. coli* O157:H7 during commercial grinding of inoculated

followed by uninoculated beef with *E. coli* present in 12.7 to 86.2 % of the ~6600 g beef trim batch using initial inoculums of 2 to 6 log CFU/g.

Work in our own laboratory has also shown that after processing lettuce containing 10^6 or 10^4 *E. coli* O157:H7 CFU/g followed by uninoculated lettuce, *E. coli* O157:H7 was quantifiable throughout the entire 90.8 kg of product. At a lower inoculation level of 10^2 CFU/g, *E. coli* O157:H7 was consistently detected in the first 21.2 and 68.0 kg of previously uninoculated iceberg and romaine lettuce at 3 and 2 CFU/100 g and transferred out to 75.6 and 86.9 kg product, respectively (Buchholz and others 2012d). Using this data, a simulation model was created to estimate the prevalence and percent of *E. coli* O157:H7 transferred during a cross contamination event in fresh-cut bagged leafy greens (Pérez Rodríguez and others 2011). This simulation predicts that when processing without sanitized wash water a batch of leafy greens inoculated at 1 and 100 CFU/g the prevalence would be ~ 3.05 and 13.39 %, respectively. Based on the simulation, chlorination of the water would reduce this percentage to 0.14 – 0.20 % and 3.28 – 4.00 %, for the 1 and 100 CFU/g inoculation levels, respectively (Pérez Rodríguez and others 2011). Keeping in mind that grinding of meat and post-harvest processing of leafy greens are fundamentally different processes, it is interesting to note that washing produce with non-chlorinated wash water results in similar *E. coli* cross-contamination as when a contaminated batch of ground beef enters a commercial grinding operation.

As seen in the previously mentioned studies involving ground beef and post-harvest leafy green processing, inoculated and uninoculated food products will inevitably become commingled during processing, which complicates any assessment of transfer from equipment and contaminated product to previously uncontaminated product. In this study *E. coli* O157:H7-

inoculated Radicchio was used as an iceberg lettuce surrogate to track the spread of this pathogen and contaminated product during small-scale commercial processing of leafy greens. Various methods for differentiating product are available based on colored dyes, physical markings (Zhang and others 2009), and similar products of different colors with Annous and others (2001) having used both inoculated Golden Delicious and uninoculated Fuji apples to assess the efficacy of a commercial flatbed brush washer (Annous and others 2001). Similarly, Nou and Luo (2010) used inoculated "Lollo Rossa" and uninoculated green-leaf lettuce leaves to evaluate the efficacy of a chlorine wash in the production of mixed salad greens. In our study, Radicchio - a red colored leaf chicory, was used as a surrogate for iceberg lettuce in order to visually track the inoculated product through the processing line. Although not a member of the same genus as lettuce, Radicchio is in the same family and both are considered part of the "leafy vegetable" category. Radicchio is frequently included in fresh-cut leafy green mixtures such as spring mix, Italian salad and fancy romaine salad packages. The vivid red color of the Radicchio allowed us to visually track the product through the processing line and quickly retrieve the small pieces from the processed lettuce, while the head shape mimicked that of a small head of iceberg, which was used as the uninoculated product. Preliminary unpublished experiments showed that Radicchio acted as a better iceberg lettuce surrogate than red leaf lettuce, which clung to the equipment surfaces (unlike iceberg). Dyeing the iceberg heads was also investigated, but it was quickly determined that the color uptake was not sufficient to allow for quick visual differentiation between inoculated and uninoculated pieces.

Radicchio was inoculated at the ~6 log CFU/g, to represent a high pathogen load and a worst-case scenario for incoming product from an animal contamination event in the field. This targeted inoculation level at the time of processing was based on previous work in Buchholz and

others (2012b and 2012d), which showed the needed low variability for future mathematical modeling and leafy green risk assessments. While less common in a real world scenario than lower pathogen loads, our high inoculation levels represent direct contamination from fecal matter in a field, because cattle (the main carriers of *E. coli* O157:H7) can present a major contamination source of the environment (Williams and others 2008). While direct contact with produce is one source of pre-harvest contamination, contaminated manure can also enter irrigation water to become a source for the spread of pathogens (Beuchat 2002; Ibekwe and others 2004). Compared to the work in Buchholz and others (2012d), this study used a much smaller inoculated to uninoculated batch size ratio. This was done to simulate only a few heads of lettuce (or a small area of baby spinach) out of an entire field experiencing a contamination event, as would be seen with direct fecal contamination. While direct contact with the produce is one source of pre-harvest contamination, manure can also contaminate a produce field's water supply, causing the irrigation or rain water to become a source for the spread of pathogens (Beuchat 2002; Ibekwe and others 2004). Dip inoculation provided the needed uniformity for obtaining repeatable cross-contamination results. Ensuring that the entire Radicchio head was coated in the inoculum was essential because once these heads passed through the shredder each shred acted as its own contaminated piece.

This study determined the *E. coli* O157:H7 levels transferred to one batch of previously uncontaminated leafy greens over time after processing a contaminated batch of product. These findings are similar to those discussed in Buchholz and others (2012d) where *E. coli* O157:H7 was quantifiable throughout 90.8 kg of iceberg and Romaine lettuce with average counts of 1.51 and 2.90 log CFU/g, respectively. The main differences between these studies were batch size and the ability to remove the inoculated pieces before microbial analysis. Spread of *E. coli*

O157:H7 from inoculated to uninoculated product during processing occurred through contact with residual water in the leafy green processing line, inoculated Radicchio retained on the shredder and conveyer belt, and contaminated product lingering throughout the processing line.

Since the number of collected lettuce containers varied in the three replicates, 95% confidence intervals were calculated for the *E. coli* O157:H7 counts based on non-linear regression analysis. When fitted to the data, the nonlinear Weibull model produced an initial decline in counts followed by diminishing numbers of *E. coli* O157:H7 as processing continued. As expected, the curve for the percentage of Radicchio recovered from each container of iceberg lettuce followed a similar pattern, indicating that transfer decreases as smaller amounts of inoculated product remain in the system.

Like other types of fresh-cut produce, leafy greens must be washed during post-harvest processing to remove any dirt and debris as well as to reduce the microbial load. The Weibull model fit to the *E. coli* counts in the process water shows a very different trend compared to those on the Iceberg lettuce. In this case, the line curves upward showing an increased transfer of the pathogen from contaminated equipment surfaces, from residual pieces of Radicchio, to the wash water. If low bacterial populations remain in flume water as a result of decreased sanitizer efficacy, any product will immediately be contaminated by direct contact with this water. Greater variability was seen in the *E. coli* O157H:7 counts from water as opposed to lettuce. Cross-contamination of apples during flume washing was also demonstrated by immersing apples in a dump tank (Buchanan and others 1999).

Many research studies have shown that tradition of leafy green washing practices are only capable of reducing microbial populations by at best 90 to 99% (Burnett and others 2006; Keskinen and others 2009; Sapers 2001; Weissinger and others 2000; Zhang and others 2009).

Most industrially used sanitizers are best suited for reducing microbial populations in the wash water rather than on the product being washed. The organic load in recirculating flume water will increase as additional product is processed. Unless properly monitored, the antimicrobial activity of chlorinated sanitizers will similarly decrease as the organic load increases, with the increasing bacterial load in the wash water negatively impacting end product shelf-life and safety. However, if a sanitizer had been used, fewer *E. coli* O157:H7 cells would have been available for cross-contamination during processing, and thereby reducing our ability to obtain quantitative data for subsequent mathematical modeling.

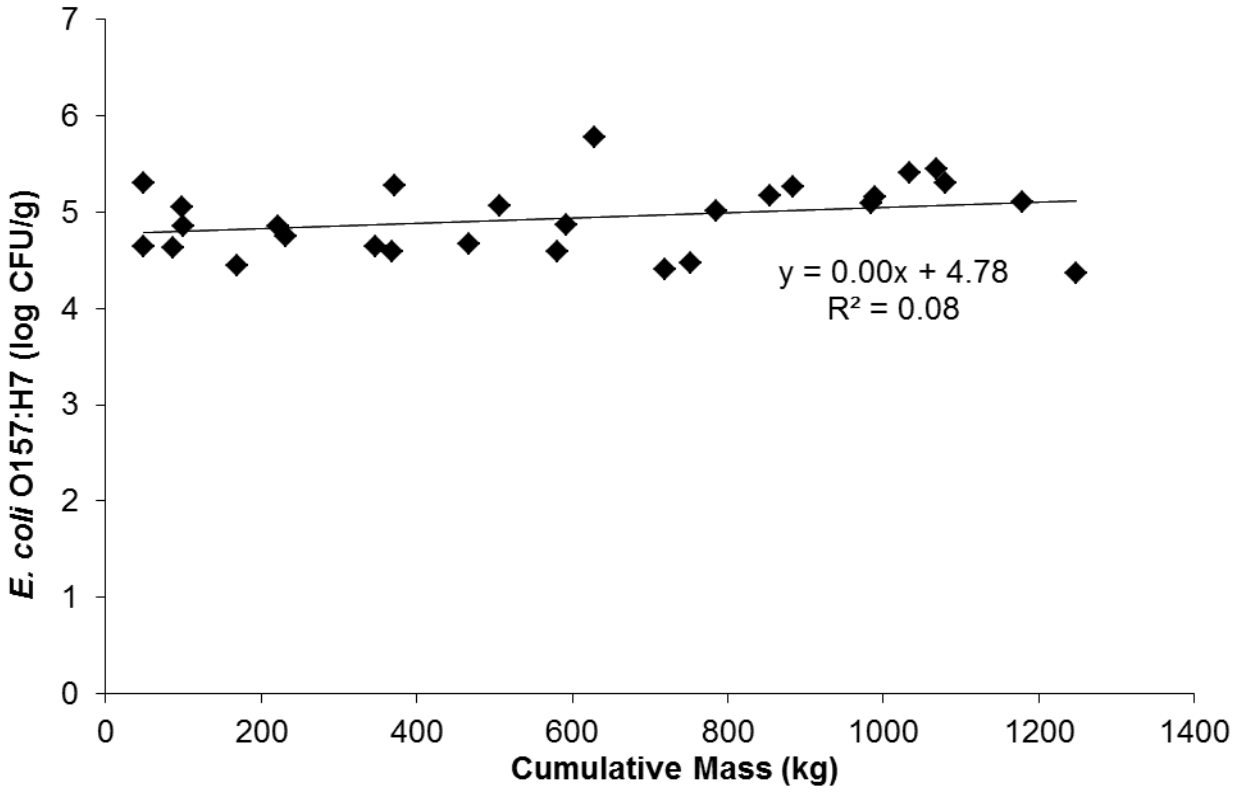
Hundreds of contaminated Radicchio shreds were recovered from the leafy green processing line after 907.2 kg of uninoculated iceberg lettuce had been processed. Over time, these lingering pieces of inoculated Radicchio will eventually break free from the equipment surface and contaminate additional product as evidenced by the presence of Radicchio in the last container of uninoculated lettuce. Although the *E. coli* O157:H7 counts were relatively high on these Radicchio pieces, only low numbers of *E. coli* O157:H7 transferred to the product contact surfaces. Unlike in Buchholz and others (2012b and 2012d) where the shredder and conveyer belt yielded higher populations of *E. coli* O157:H7, in this study no significant differences ($P > 0.05$) were seen between the shredder, conveyor, flume tank or shaker table. This difference may be linked to the fact that in this experiment processed a much larger quantity of uninoculated product after the inoculated batch, which could have 'cleaned' the equipment surfaces.

In summary, this study quantified the extent of cross-contamination to subsequent product by tracking contaminated product though a fresh-cut leafy green processing line. Visually demonstrating the spread of contaminated product during processing illustrates the ease

by which *E. coli* O157:H7 can contaminate multiple batches of product. In the event of an outbreak these finding could be used as a guide to help estimate the amount of product which may have become cross-contaminated during processing and would need to be recalled.

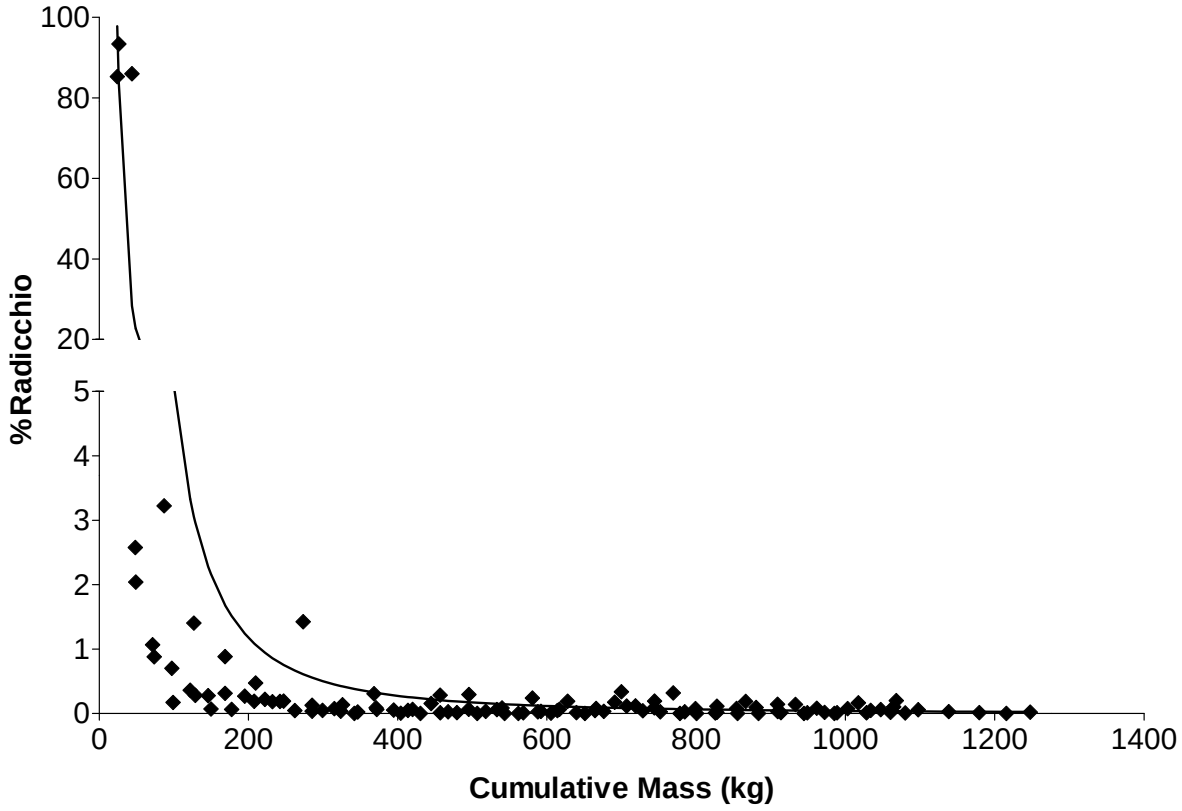
4.6 FIGURES

Figure 4.1: *Linear trend line fitted to mean (± SD)* E. coli *O157:H7 populations on Radicchio after leafy green processing* [a].



[a] The wet weight of the iceberg lettuce after processing is the cumulative mass.

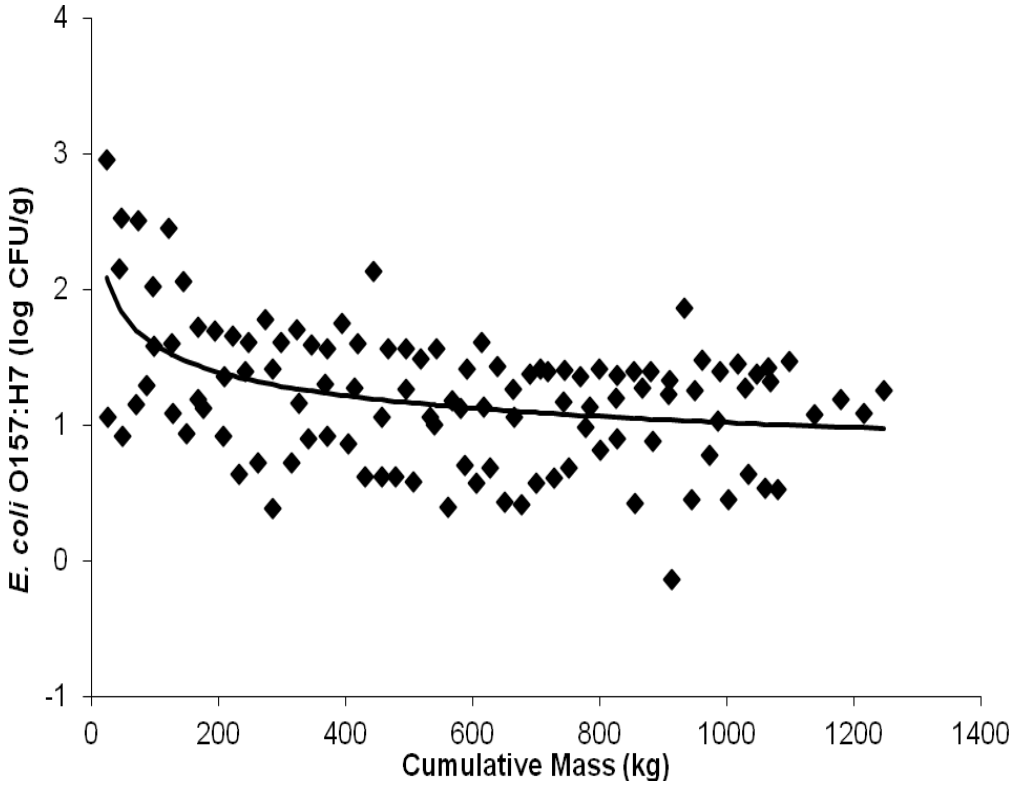
Figure 4.2: *Weibull model fitted to percentage (± SD)* [a] *of Radicchio recovered from iceberg lettuce after leafy green processing* [b].



[a] Weibull parameters are b = 81,353.30 ± 50,962.54 with the low 95% confidence interval at 30,499.68 and the high 95% confidence interval at 27,5530.86, and n = -2.10 ± 0.19 with the low 95% confidence interval at -2.47 and the high 95% confidence interval at -1.81

[b] The wet weight of the iceberg lettuce after processing is the cumulative mass.

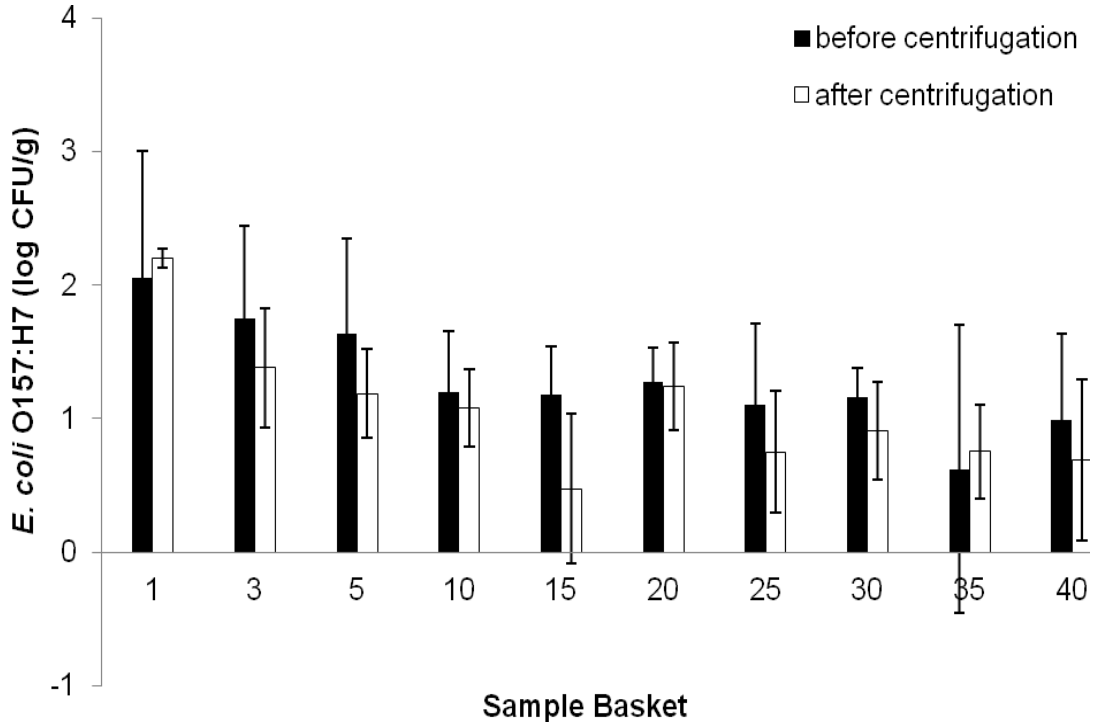
Figure 4.3: *Weibull model fitted to mean (± SD)* [a] E. coli *O157:H7 populations on iceberg lettuce after leafy green processing* [b].



[a] Weibull parameters are $b = 3.89 \pm 0.73$ with the low 95% confidence interval at 2.61 and the high 95% confidence interval at 5.58, and $n = -0.19 \pm 0.03$ with the low 95% confidence interval at -0.26 and the high 95% confidence interval at -0.13.

[b] The wet weight of the iceberg lettuce after processing is the cumulative mass.

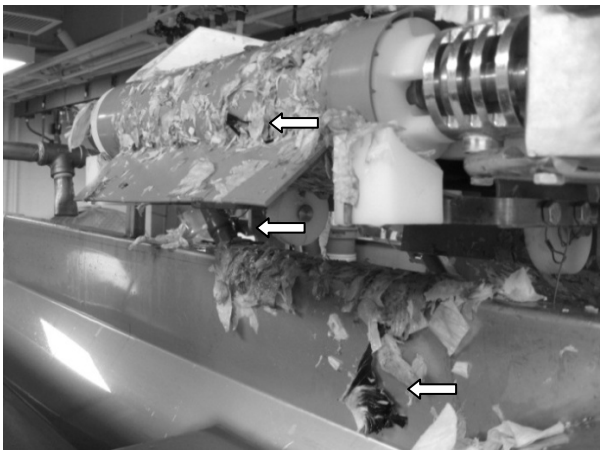
Figure 4.4: E. coli *O157:H7 populations (mean ± SD)* [a]*, on iceberg lettuce before and after centrifugal drying.*



[a] Means of the same sample baskets did not differ significantly ($P > 0.05$) before and after centrifugal drying.

Figure 4.5: *Radicchio remaining on equipment surfaces after 907 kg of uninoculated product was processed: (A) conveyer belt and flume tank and (B) blade and interior surface of the lettuce shredder.*

A



B

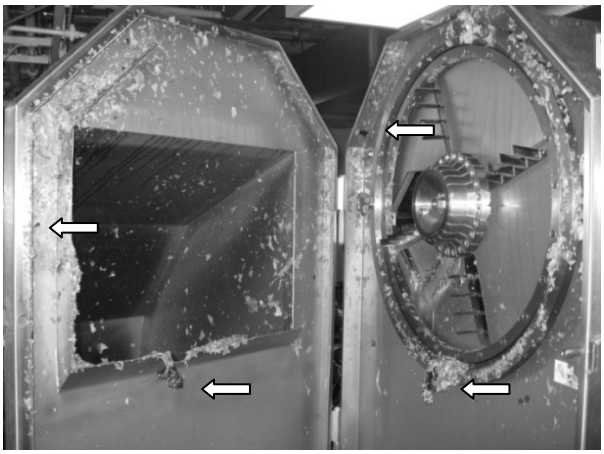
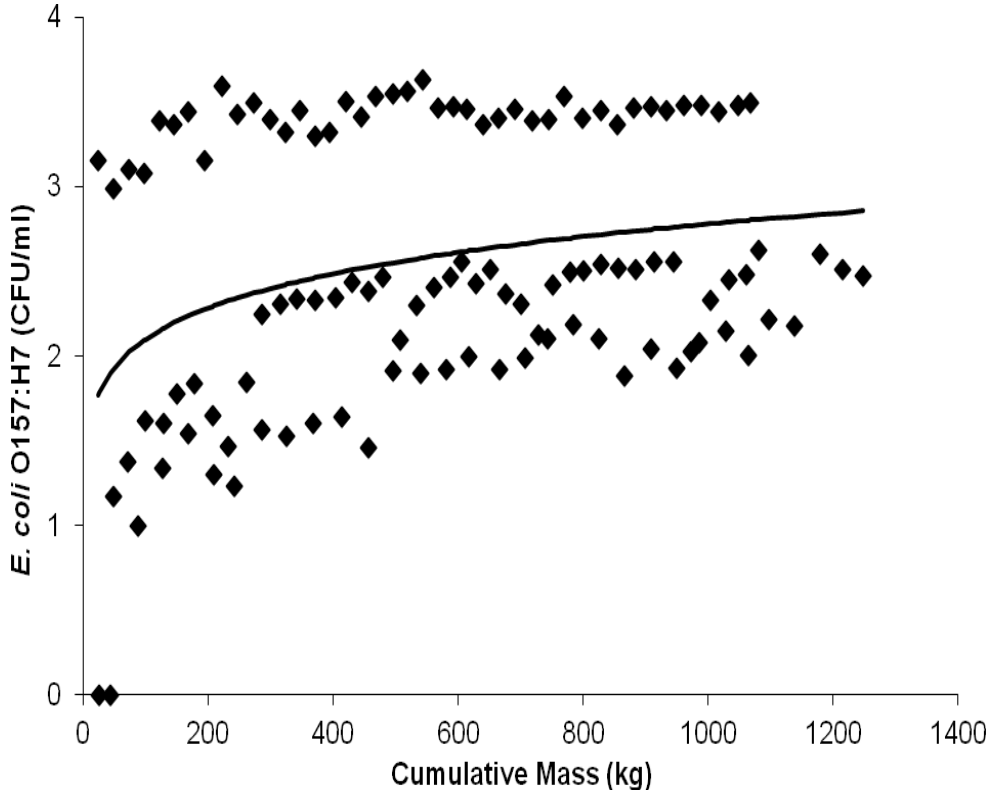


Figure 4.6: *Weibull model fitted to mean (± SD)* [a], E. coli *O157:H7 populations in 900 L of water during processing of leafy green inoculated CFU/g*



[a] Weibull parameters are $b = 1.20 \pm 0.28$ with the low 95% confidence interval at 0.75 and the high 95% confidence interval at 1.83, and $n = 0.21 \pm 0.04$ with the low 95% confidence interval at 0.05 and the high 95% confidence interval at 0.20.

4.7 TABLES

Table 4.1: *Weight (g) ± SD and Percent ± SD of Radicchio recovered from equipment surfaces after processing* [a]

Processing Equipment	Weight	Percent
Shredder	8.3 ± 5.1 $_{AB}$	1.5 ± 1.0
Conveyer Belt	9.8 ± 2.8 $_{A}$	1.7 ± 1.0
Flume Tank	3.5 ± 3.4 $_{AB}$	0.7 ± 0.6
Shaker Table	0.1 ± 0.1 $_{B}$	0.0 ± 0.0

[a] Means of not labeled with by the same letter are significantly different ($P \leq 0.05$).

Table 4.2: E. coli *O157:H7 populations (mean ± SD) recovered from equipment surfaces after leafy green processing* [a]

Processing Equipment	*E. coli* O157:H7 (log CFU/100 cm^2)	
Shredder	2.4 ± 0.4	A
Conveyer Belt	3.2 ± 0.7	A
Flume Tank	2.3 ± 0.5	A
Shaker Table	1.9 ± 0.4	A

[a] Means of not labeled with by the same letter are significantly different ($P \leq 0.05$).

CHAPTER 5:

Impact of Post-inoculation Hold Time on *Escherichia coli* O157:H7 Transfer during Commercial Production of Fresh-cut Leafy Greens

5.1 ABSTRACT

Leafy greens are prone to pathogen contamination in the field as well as during subsequent handling and processing, with the strength of bacterial attachment to the leaf surface being highly variable. This study assessed the impact of post-inoculation hold-times and temperatures on *Escherichia coli* O157:H7 transfer during pilot-scale lettuce processing that included a shredder, step conveyor, flume tank and shaker table. Triplicate batches (0.5 kg) of Radicchio were dip-inoculated with a 4-strain cocktail of avirulent, GFP-labeled, ampicillin-resistant *E. coli* O157:H7 (~10^5 CFU/g) and then processed either immediately or after storage for 1 d at 4°C or 5 d at 22°C. After processing 2.3 kg of uninoculated iceberg lettuce, inoculated Radicchio was processed, followed by 45.5 kg of uninoculated iceberg lettuce. Eight lettuce/Radicchio (25 g), 8 water (500 ml) and 50 equipment swab (100 cm^2) samples were collected during processing. After processing, all Radicchio pieces from eight 5-kg baskets of shredded lettuce, the equipment and the floor were weighed after which three iceberg lettuce (25 g) samples from each basket were examined for *E. coli* O157:H7 by direct plating or membrane filtration using trypticase soy agar containing 0.6% yeast extract and 100 ppm ampicillin. After processing, *E. coli* O157:H7 was found in all iceberg lettuce samples, with mean counts of 0.3, 0.0 and -0.4 log CFU/g for the 1 h-, 1 d- and 5 d-held product, respectively ($P > 0.05$). Scanning electron micrographs showed greater bacterial attachment for 5 d-held product. *E. coli* O157:H7 populations in the flume water did not differ ($P > 0.05$) for 1 h compared to 1 d and 5 d-held product with a similar trend seen for the equipment surface samples. Based on these findings, the point at which contamination occurs will impact the extent to which *E. coli* O157:H7 will detach from leafy greens during processing.

5.2 INTRODUCTION

The high number of foodborne outbreaks traced to fresh fruits and vegetables is partially attributed to current large-scale, centralized production and processing practices. Since 1990, 19 outbreaks have been specifically associated with the consumption of leafy greens (CSPI 2008). The problems associated with contamination of leafy greens became national news in the fall of 2006, when consumption of iceberg lettuce and pre-bagged baby spinach caused 3 large-scale *E. coli* O157:H7 outbreaks (CSPI 2009b, FDA 2006, FDA 2007, Jay and others 2007). While pathogen contamination likely originates on the farm, current harvesting and post-harvest processing practices can spread pathogens to previously uncontaminated product during field coring (Taormina and others 2009), cooling, shredding, washing, conveying drying and subsequent handling (Beuchat 1996; Beuchat and Ryu 1997; Gorny 2006), and increase the impact of an outbreak as evidenced by the recent multi-state outbreaks (Beuchat and Ryu 1997; Burnett and Beuchat 2001).

When present on the surface of leafy greens, *E. coli* O157:H7 can readily transfer to food contact surfaces on the processing equipment and to the wash water as demonstrated in Buchholz and others (2012b). In addition, both *E. coli* O157:H7 and the contaminated leafy greens will persist on equipment surfaces throughout the processing line and slough off over time to contaminate large quantities of uncontaminated product as shown in Buchholz and others (2012c and 2012d). Once contaminated, little can be done to reduce the microbial load on leafy greens with currently accepted commercial washing and sanitizing practices only able to generally decrease bacterial populations 1 to 2 log CFU/g (Sapers 2006). Bacterial attachment and biofilm formation on the surfaces of leafy greens make it nearly impossible to completely eliminate foodborne pathogens during conventional washing (Carmichael and others 1999, Sapers 2006).

Biofilms, defined as an aggregate of adherent bacterial cells within an exopolysaccharide matrix, provide a physical barrier against environmental stresses including chemicals sanitizers, temperature changes, desiccation and ultraviolet radiation (Annous and others 2009, Niemira and Cooke 2010, Ryu and Beuchat 2005, and Tarver 2009). Scanning electron microscopy (SEM) and confocal laser scanning microscopy have been frequently used to observe bacterial colonization and biofilm formation on fresh produce (Annous and others 2009, Carmichael and others 1999, Fett 2000, Keskinen and others 2009 and Morris and others 1997). One major concern to the produce industry is that pathogens, including *Salmonella* or *E. coli* O157:H7, can produce biofilms or become incorporated into biofilms of produced by background microflora. Using SEM, biofilms containing *E. coli* O157:H7 have been observed on Romaine and baby spinach leaves after 24 to 72 h of storage at 4°C for (Niemira and Cooke 2010) and on the stem ends lettuce leaves after submersion in an *E. coli* cocktail for 1 wk at 4°C (Annous and others 2009).

Increasing the time between dip inoculation and processing of lettuce from 1 h at 22^{o}C to 1 d at 4°C decreased the number of *E. coli* O157:H7 cells transferred to the water and equipment surfaces during processing (Buchholz and others 2012b). Lang and others (2004) have also shown that reduced (or equal to) numbers of *E. coli* O157:H7 and *Salmonella* were recovered from leafy greens held for a longer period of time. These findings suggest that longer refrigerated hold times before processing should decrease the potential spread of *E. coli* O157:H7 to uncontaminated product and lead to both fewer and smaller outbreaks.

This current study expands on previous work to determine how extending the time between the contamination event and post-harvest processing, and the production of a biofilm on the produce, effects the extent of bacterial transfer to previously uncontaminated produce during

processing. The objectives of this study were to: 1) assess the extent of *E. coli* O157:H7 attachment and biofilm formation on dip inoculated Radicchio leaves following three different time/temperature hold times and then 2) determine the impact of these three different post-inoculation time/temperature hold times on the quantitative transfer of *E. coli* O157:H7 to iceberg lettuce and the equipment used in commercial processing of leafy greens.

5.3 MATERIALS AND METHODS

5.3.1 Experimental Design. Radicchio was inoculated with *E. coli* O157:H7 at 10^5 CFU/g, held for 1 h at 22°C, 1 d at 4°C or 5 d at 22°C and then used to quantify transfer from equipment surfaces to water and uninoculated iceberg lettuce during simulated commercial processing. *E. coli* O157:H7 transfer was examined by processing 2.27 kg of uninoculated iceberg lettuce followed by 0.45 kg of inoculated Radicchio and then 45.5 kg of uninoculated iceberg lettuce with the red color of Radicchio allowing the separation of inoculated and uninoculated product before analysis. All experiments were conducted in triplicate.

5.3.2 Produce. Individually wrapped heads of iceberg lettuce (*Lactuca sativa* L.) (24 heads per case) and Radicchio (*Cichorium intybus*), (12 heads per case) were obtained from a local wholesaler (Stan Setas Produce Co., Lansing, MI). These products, which originated from California or Arizona depending on the growing season, were stored in a 4°C walk-in-cooler for a maximum of 5 days and then hand-cored immediately before use.

5.3.3 Bacterial strains. Four avirulent, GFP-labeled, ampicillin-resistant strains of *E. coli* O157:H7 (ATCC 43888, CV2b7, 6980-2, and 6982-2) were obtained from Dr. Michael Doyle at the Center for Food Safety, University of Georgia, Griffin, GA. Upon arrival, stock cultures of each strain were prepared in trypticase soy broth (Difco, Becton Dickinson, Sparks, MD) containing 0.6% yeast extract (Difco, Becton Dickinson) supplemented with 100 ppm ampicillin (ampicillin sodium salt, Sigma Life Science, St. Louis, MO) (TSBYE + amp) and 10% (v/v) glycerol (Sigma Chemical Company, St. Louis, MO) and stored at -80°C until needed. Working cultures were prepared by streaking each stock culture on trypticase soy agar (Difco, Becton Dickinson) containing 0.6% yeast extract and 100 ppm ampicillin (TSAYE + amp). After 18 - 24 h of incubation at 37°C, a single colony was transferred to 9 ml of TSBYE + amp and

similarly incubated. Thereafter, a 0.2 ml aliquot of each culture was transferred to 200 ml of TSBYE + amp, incubated for 18 – 20 h at 37°C and then combined in equal volumes to obtain the 4-strain cocktail.

5.3.4 Inoculation of Radicchio. An *E. coli* O157:H7 suspension containing 10^6 CFU/ml was obtained by adding 3 ml of the 4-strain cocktail to 3 L of tap water (~15^{o}C), which was used as the inoculum for Radicchio heads that were held 1 h or 1 d before processing. Similarly, an *E. coli* O157:H7 suspension containing 10^5 CFU/ml, which was obtained by adding 0.3 ml of the 4-strain cocktail to 3 L of tap water (~15^{o}C), served as the inoculum, for Radicchio heads that were held 5 d before processing. For inoculation, 0.6 kg of cored Radicchio heads (~2 heads) were immersed in the *E. coli* suspension for 15 min, drained, air-dried for 1 h at 22°C and then spun in a salad spinner (Oxo 32480 Good Grips Salad Spinner, Oxo, New York, NY) to remove residual inoculum. The inoculated heads were either processed immediately or after storage for 1 d at 4°C or 5 d at 22°C. Duplicate 25-g samples were aseptically collected to determine the initial inoculation level at the time of processing.

5.3.5 Scanning electron microscopy (SEM). Pieces of Radicchio were cut (1 x 1 cm), using a ethanol wiped razor blade, from upper half of the Radicchio leaf which had been held for 1 h, 1 d or 5 d before processing. Pieces were fixed at 4°C in 4% glutaraldehyde buffer with 0.1 M sodium phosphate at pH 7.4 for 1 to 2 h. After a quick rinse in the buffer solution, samples were dehydrated in an ethanol series (25%, 50%, 75% and 95%) for 10 to 15 min at each gradation followed by three final 10 min changes in 100% ethanol. They were then critical point dried in a Balzers Model 010 critical point dryer (Balzers Union Ltd., Balzers, Liechtenstein) using liquid carbon dioxide as the transitional fluid, mounted on aluminum stubs using Carbon suspension cement (SPI Supplies, West Chester, PA) and Carbon tape (Ted Pella, Inc., Redding,

CA), and sputter-coated with a thin layer of osmium (~10 nm thickness) in a NEOC-AT osmium coater (Meiwafosis Co. Ltd., Osaka, Japan). Samples were imaged using a JEOL JSM-7500F (cold field emission electron emitter) scanning electron microscope (JEOL Ltd., Tokyo, Japan), located at the Center for Advance Microscopy at Michigan State University.

5.3.6 Processing equipment. The pilot-scale size leafy green processing line that was used included a lettuce shredder, step conveyer, flume tank and shaker table. The commercial lettuce shredder (model TRS 2500 Urschel TranSlicer, Valparaiso, IN) was operated at a feed belt/slicing wheel speed of 198 m/min and 905 RPM, respectively, to obtain a shred size of approximately 5 x 5 cm. The step conveyor (Dorner model 736018 mc series, Hartland, WI) was fitted with a polyurethane step conveyer belt (ThermoDrive, Mol Industries, Grand Rapids, MI) that operated at 0.11 m/sec. A stainless steel, ~1000 L capacity, water recirculation tank was filled with 890 L of sanitizer-free Michigan State University tap water (~15^{o}C) since inclusion of chemical sanitizers leading to inactivation of *E. coli* O157:H7 would have confounded our ability to assess transfer during processing. This water tank was connected to a 3.6-m long stainless steel flume tank (Heinzen Manufacturing, Inc., Gilroy, CA) - equipped with two overhead spray jets (1 m from the start), by a 4.14 m long, 10 cm diameter hard plastic discharge hose and a centrifugal pump (model XB754FHA, Sterling Electric, Inc., Irvine, CA) that circulated the water at a rate of ~15 L/sec. The stainless steel shaker table for partial dewatering was operated by a 1 HP Baldor washdown motor (Baldor Electric Co., Ft. Smith, AR) at 1760 RPM. Water removed from the leafy greens during mechanical shaking passed through a fine mesh screen in the shaker table and was fed into the water holding tank by a water recirculation spout

5.3.7 Processing and collection of Radicchio and iceberg lettuce samples. Cored heads of uninoculated iceberg lettuce (2.3 kg), followed by 0.5 kg of Radicchio, were shredded, step-

conveyed to the flume tank, washed, partially dried on the shaker table and collected in one plastic mesh basket (United Plastics, Leomister, MA). After draining the water holding tank, flume tank and centrifugal pump hose, the water holding tank was refilled with ~890 L of tap water (~15^{o}C). The remaining 45.5 kg of uninoculated iceberg lettuce were then identically processed and sequentially collected in ~ 8 baskets. During processing, about eight 500-ml aliquots of water were collected from the water return spout on the shaker table. After processing, each of the ~8 baskets containing previously uninoculated lettuce was subdivided into three (~2 kg) layers (top, middle and bottom) with one 25-g iceberg lettuce sample aseptically removed from each layer (~24 samples) for *E. coli* O157:H7 quantification. In addition, all of the red colored Radicchio pieces in each basket and on the shredder, conveyor, flume tank, shaker table and floor were collected using sterilized tongs, weighed and then similarly assessed for numbers of *E. coli* O157:H7. Finally, 50 product contact areas (100 cm^2) on the equipment (14 shredder, 8 conveyer belt, 11 flume tank and 9 shaker table samples) previously identified using Glo Germ™ (Glo Germ Company, Moab, Utah) as described in Buchholz and others (2012b), were sampled using one-ply composite tissues (KimWipes, Kimberly-Clark Corp., Irving, TX) moistened in 1 ml of sterile 1% (w/v) phosphate buffer (8.5 g/L NaCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4, J.T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ) as described by Vorst and others (2004).

5.3.8 Microbiological analyses. All iceberg lettuce samples (25 g) were added to 100 ml of sterile 1% phosphate buffer in a Whirl-Pak™ filter bag (Nasco, Fort Atkinson, WI) and processed in a pulsifier (Pulsifier, Filtaflex Ltd., Almonte, Ontario, Canada) for 1 min. Radicchio samples were weighed in Whirl-Pak™ filter bags, diluted 1:10 in sterile 1% phosphate buffer, and then homogenized by stomaching (Stomacher 400 Circulator, Seward, Worthington,

UK) for 1 min at 260 rpm. Iceberg lettuce and Radicchio samples were either appropriately diluted and plated on TSBYE + amp or processed using a 0.45 μm membrane filter which was placed on TSAYE + amp to quantify *E. coli* O157:H7. The surface samples from each piece of equipment were composited, added to 100 ml of neutralizing buffer in a Whirl-Pak™ bag, homogenized by stomaching for 1 min. at 260 rpm and then plated identically to the product samples. 100 ml of each water sample were either appropriately diluted and plated on TSAYE + amp or processed by membrane filtration. Following 18 – 20 h of incubation at 37°C, all green fluorescing colonies as seen under ultraviolet light were counted as *E. coli* O157:H7.

5.3.9 Statistical analysis. *E. coli* O157:H7 counts were converted to log CFU per g, ml or 100 cm^2 and subjected to an ANOVA using JMP 8.0 (SAS Institute Inc., Cary, NC). For all tests, a P value of < 0.05 was considered significant. The Tukey-Kramer HSD test was used to determine if the numbers of *E. coli* O157:H7 transferred to the equipment surfaces, processing water and uninoculated product were influenced by the Radicchio's post-inoculation holding time.

5.4 RESULTS

5.4.1 Initial inoculum levels. Four-strain *E. coli* O157:H7 suspensions of 6.0 ± 0.2, 5.78 ± 0.1 and 4.8 ± 0.1 log CFU/ml were used to inoculate the heads of Radicchio to be held for 1 h, 1 d and 5 d, respectively, before processing. After a 15 min immersion followed by 1 h of drying and 1 min of centrifugal drying to remove excess inoculum, Radicchio to be processed immediately, held for 1 d or 5 d, respectively, contained *E. coli* O157:H7 populations of 5.5 ± 0.3, 5.9 ± 0.3 and 4.3 ± 0.2 log CFU/g. After 1 d of storage at 4°C, numbers decreased to 5.6 ± 0.3 CFU/g, while after 5 d of storage at 22°C the population increased to 5.4 ± 0.4 CFU/g. No significant differences ($P > 0.05$) were observed between the numbers of *E. coli* O157:H7 adhering to the three products at the time of processing.

5.4.2 Attachment and biofilm formation. The SEM images of Radicchio leaves held 1 h before processing showed only a few attached cells with no evidence of biofilm formation (Figure 5.1A), whereas a 1 d hold at 4°C led to initial signs of an extracellular matrix, with the cells organized in small aggregates mainly within the stomates and leaf crevices (Figure 5.1B). After 5 days of storage at 22°C to mimic pre-harvest contamination, larger cell aggregates (~5 to 20 cells) were enveloped in a dense extracellular matrix, with this material forming a web covering the entire leaf surface (Figure 5.1C).

5.4.3 Radicchio. Populations of *E. coli* O157:H7 on the 1 h-, 1 d- and 5 d-held Radicchio decreased 1.7, 1.3, and 2.1 log CFU/g after processing, respectively, with the counts on 1 d-held product being significantly higher than those on the 1 h- and 5 d-held product ($P < 0.05$) (Figure 5.2). After processing the inoculated Radicchio, 18.8, 20.8, and 18.0 % (w/w) remained in the processing line for the 1 h, 1 d and 5 d held Radicchio, respectively. As expected, the largest percentage of remaining inoculated Radicchio, 3.1, 3.4, and 2.5 %, was recovered in the first

aliquot of the collected iceberg lettuce after processing the Radicchio inoculated 1 h, 1 d and 5 d prior to processing (Figure 5.3). After 45.4 kg of iceberg lettuce were processed, there was 17.3, 16.1, and 15.85 % Radicchio was distributed throughout the processed lettuce during the 1 h, 1 d and 5 d attachment-time experiments, respectively.

5.4.4 Iceberg lettuce. After processing 1 h-, 1 d- and 5 d-held Radicchio followed by the 45.4 kg of iceberg lettuce, *E. coli* O157:H7 was respectively found in all iceberg lettuce samples collected from ~ 8 baskets at mean levels of 0.3 ± 0.6, 0.0 ± 0.6 and -0.4 ± 0.6 log CFU/g with the counts on the 1 h-held product being significantly higher than the 1 d held product, followed by the 5 d held product ($P < 0.05$). Mean *E. coli* O157:H7 counts in the final basket of product collected at the end of processing were 0.2 ± 0.4, -0.6 ± 0.2 and -0.8 ± 0.4 log CFU/g for the 1 h, 1 d and 5 d held Radicchio, respectively (Figure 5.4). No statistical difference was observed ($P > 0.05$) in the mean numbers of *E. coli* transferred to the iceberg lettuce after the uninoculated products were processed during the 1 h- (0.3 log CFU/g), 1 d- (0.0 log CFU/g) and 5 d- (-0.4 log CFU/g) attachment-time experiments, respectively. Maximum *E. coli* O157:H7 transfer of 1.5, 1.0 and 0.4 log CFU/g was observed on the iceberg lettuce after processing 3.8, 4.2 and 2.0 kg of previously uninoculated product that followed the Radicchio that had been processed 1 h, 1 d, and 5 d post-inoculation, respectively.

5.4.5 Flume water. After refilling the water holding tank with ~890 L of tap water (~15^{o}C), this water which was used to process the 45.4 kg of uninoculated lettuce, yielded *E. coli* O157:H7 populations of -1.2, 0.9 and -1.4 log CFU/ml at the end of processing for 1 h-, 1 d- and 5 d-held Radicchio, respectively (Figure 5.5) with no significant differences in terms of post-inoculation hold time ($P < 0.05$).

5.4.6 Processing equipment surfaces. Generally no significant differences were seen in the numbers of *E. coli* O157:H7 recovered from the shredder, step conveyor, flume tank or shaker table ($P < 0.05$), which ranged from 0.7 to 1.8, -0.1 to 1.2 and -0.6 to 0.8 log CFU/g, for Radicchio heads held 1 h, 1 d and 5 d before processing, respectively (Table 5.1). However, statistically higher ($P < 0.05$) *E. coli* populations were seen on the surface of the conveyer belt after processing the 1 h-held product compared to that, which was held for 5 d at 22°C. The other equipment surfaces did not differ significantly based on the post-processing attachment time of the inoculated product ($P < 0.05$) (Table 5.1).

In terms of the weight of Radicchio remaining on equipment after processing, the only significant difference ($P < 0.05$) was seen for the shredder when 1 d- (6.0 g) as opposed to 1 h-held product was processed with the remaining post-inoculation hold times not impacting the amount of Radicchio remaining on the equipment surfaces (Table 5.2). *E. coli* O157:H7 populations on the Radicchio did not differ significantly ($P < 0.05$) in terms of equipment location or holding time before processing (Table 5.2).

5.5 DISCUSSION

The incidence, growth, attachment and survival of foodborne pathogens on fresh and processed produce is well documented (Beuchat 1996; Beuchat and Bracket 1990; Beuchat and others 2004; Gleeson and O'Beirne 2005; Harris and others 2003), however, the extent of bacterial transfer and cross-contamination that occurs during leafy green processing remains poorly understood. Taormina and others (2009) at the University of Georgia demonstrated the ability for *E. coli* O157:H7 to transfer from inoculated coring blades (2.72 and 1.67 log CFU/g) to 10 and 5 consecutive heads of iceberg lettuce during field coring, respectively. In our previous work, approximately 90% of the *E. coli* O157:H7 population on inoculated lettuce was shed in sanitizer-free water with this pathogen contaminating the product contact surfaces of the shredder, conveyor, flume tank, shaker table and dewatering centrifuge during processing (Buchholz and others 2012b) and then transferring to large quantities of previously uncontaminated product during subsequent processing (Buchholz and others 2012c and 2012d).

This is the first study to look at the impact of microbial attachment and biofilm formation on cross-contamination from leafy greens contaminated in pre-harvest situations. In this study Radicchio inoculated at the 5 log CFU/g, representing a medium to high bacterial load, was used to visually track the spread of contaminated product during processing. This targeted inoculation level at the time of processing was based on previous work in Buchholz and others (2012b, 2012c, and 2012d), which showed that the data will have low variability between replicates and provide valuable information for future mathematical modeling and risk assessments. Higher inoculation levels can represent direct fecal contamination in the field from fecal matter, because cattle are a major source of this pathogen and can present a major contamination source of the environment (Williams and others 2008). While direct contact with produce is one source of

pre-harvest contamination, contaminated manure can also enter irrigation water to become a source for the spread of pathogens (Beuchat 2002; Ibekwe and others 2004). Uniform inoculation of the Radicchio by dipping was essential for determining the amount of cross-contamination and obtaining repeatable results. Lang and others (2004) previously reported greater recovery of *E. coli* O157:H7 from dip- as opposed to spray- or spot- inoculated lettuce when the same populations were initially applied.

To evaluate the extent of transfer from contaminated to uncontaminated leafy greens with certainty, inoculated and uninoculated products having the same surface characteristics must be differentiated from each other based on color, dye or physical markings (Annous and others 2001; Nou and Luo 2010; Zhang and others 2009). In one such example, Golden Delicious and uninoculated Fuji apples were used to assess the efficacy of washing with a commercial flatbed brush washer (Annous and others 2001). Similarly, Nou and Luo (2010) used inoculated "Lollo Rossa" and uninoculated green-leaf lettuce to evaluate a chlorine wash prior to cutting the mixed leaves. In our study Radicchio (red colored leaf chicory) acted as a surrogate for contaminated iceberg lettuce which allowed visual tracking of the inoculated product through the processing line. Although belonging to different genera, both Radicchio and iceberg lettuce, are members of the "leafy vegetable" category. Using Radicchio made it possible to evaluate the impact of microbial attachment and biofilm production on transfer during leafy green processing 5 days post-inoculation because the heads did not wilt or decompose as quickly during storage unlike other leafy greens.

Bacterial attachment and biofilm formation on the surface of leafy greens are two key factors that negatively impact the reduction of microorganisms during conventional washing (Carmichael and others 1999; Sapers 2006). When Lang and others (2004) assessed the impact

of different post-inoculation drying times on recovery of *E. coli* O157:H7 from iceberg lettuce and parsley, higher (or similar) populations were obtained from leafy greens that were dried for 2 h at 22°C as opposed to 2 h at 22°C followed by 22 h at 4°C. A similar trend was demonstrated in Buchholz and others (2012b), which showed that extending the time between the contamination event and processing from 1 h at 22°C to 1 h at 22°C followed by 1 d at 4°C decreased the removal and transfer of *E. coli* O157:H7 during iceberg lettuce processing.

In support of these findings, our results showed that Radicchio leaves held for 1 d at 22°C retained more of the *E. coli* O157:H7 inoculum after processing than did the heads inoculated 1 h prior to processing and held at 22°C. Holding leafy greens at refrigeration temperatures replicates a scenario where products become contaminated during harvest or storage prior to processing. A second possible contamination scenario involves leafy greens becoming contaminated in the field during growth, several days prior to processing. Unexpectedly, Radicchio stored for 5 d retained fewer *E. coli* O157:H7 cells after processing. This was especially surprising because bacterial aggregates and an extensive extracellular matrix developed on the Radicchio leaves as seen by SEM. These findings may be attributed to the methods used for microbiological analysis resulting in *E. coli* O157 not being released sufficiently into the buffer solution during stomaching as expected. This explanation is further justified by the counts for *E. coli* O157:H7 transferred from Radicchio to the iceberg lettuce. In this case, as expected, the uninoculated leafy greens that had been processed following the Radicchio held for 5 days at room temperature had lower levels of *E. coli* O157:H7, followed by those held for 1 day at refrigeration temperature and 1 h at room temperature. While these findings imply that refrigerating the product for 1 day post-harvest or a contamination event in the field taking place a few days before harvest will decrease the potential spread of *E. coli*

O157:H7 during processing and lead to both fewer and smaller outbreaks, the oral infectious dose for *E. coli* O157:H7 of less than 100 cells (Meng and others 2007) makes this an unreliable method to prevent an outbreak.

The high numbers of *E. coli* on iceberg lettuce collected after the inoculated batch of Radicchio was processed, indicates that cross-contamination of previously uninoculated product can occur through 1) residual water (approximately less than 2 L) from the flume tank, shaker table, centrifugal pump, hose or water recirculation tank, 2) direct contact with the inoculated Radicchio clinging to the shredder and conveyer belt surfaces, or 3) contact with the contaminated leafy greens/equipment surfaces throughout the processing line. In terms of transfer to the leafy greens, Buchholz and others (2012d) showed that *E. coli* O157:H7 was found throughout the entire batch of uninoculated iceberg (mean count of 0.3 CFU/g), and Romine lettuce (90.8 kg) (mean count of 0.7 log CFU/g) that was processed following 22.7 kg of leafy greens inoculated at ~4 log CFU/g. While the inoculation level in this current experiment was approximately 1 log higher and the ratio of inoculated to uninoculated products was larger, the same trend was apparent. Most of the transfer occurred to the first few baskets of initially uncontaminated product with a rapid decrease followed by a tailing effect. This study also mimicked the results described in Buchholz and others (2012c), which was a scaled up version of the current experiment using Radicchio held for 1 h at 22°C. Here we showed *E. coli* O157:H7 counts on the iceberg lettuce ranging from -0.1 to 3.0 log CFU/g (an overall average of 1.2 log CFU/g) after processing Radicchio inoculated at 5.9 log CFU/g. In our current experiment, *E. coli* O157:H7 transfer to iceberg lettuce from Radicchio held for 1 h before processing resulted in a slightly lower mean count of 0.3 log CFU/g and a lower maximum transfer level of log 1.5

CFU/g. These differences may be attributed to both a slightly lower initial inoculation level and a difference due to the scale of the experiments.

Commercial production of fresh-cut leafy greens entails a washing step to remove dirt and debris and reduce the microbial load with commercial sanitizer washing systems only capable of reducing microbial populations on the product by 90 to 99% (Burnett and Beuchat and 2001; Keskinen and others 2009; Sapers 2001; Weissinger and others 2000; Zhang and others 2009). Wash water is an ideal vehicle for the transfer of pathogens during processing as demonstrated by Buchanan and others (1999) in a study looking at cross-contamination during washing of apples. The goal of our work was to quantify the numbers *E. coli* O157:H7 transferred during leafy green processing; therefore chemical sanitizers could not be used in the flume tank. If sanitizers had been used, the numbers of *E. coli* O157:H7 would have most likely been reduced to non-detectable levels in the flume water, preventing quantification of cross-contamination to the uncontaminated lettuce. Therefore, the processing water used to wash inoculated product was drained with the water holding tank then refilled before processing uninoculated product. The data collected in our study demonstrates that even with low bacterial counts in the flume water, any product that is washed in that water will become immediately inoculated with *E. coli* O157:H7 via direct contact with the contaminated water and that an extended storage time after contamination will not reduce the likelihood that a pathogen will be spread through an entire batch of product. This aligns very well with the efficacy of normal produce washing practices for the removal of microorganisms and shows that the ineffectiveness of current processing/washing methods is clearly a major concern as supported by the three recent leafy green recalls involving *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* (FDA 2010a; FDA 2010b; FDA 2010c).

While hundreds of pieces of Radicchio were recovered from the surfaces of shredder, and step conveyor, few pieces were seen in the flume tank and on the shaker table after processing. Consequently, contaminated product can linger on processing equipment, eventually breaking free of the equipment surface and being carried downstream with uncontaminated product being processed, as evident by the numbers of small pieces of radicchio which were found even in the last baskets of iceberg lettuce collected. In a study examining the microbial changes of "Lollo Rosso" lettuce during processing, an increase of 1 log CFU/g was observed after shredding (Allende and others 2004), indicating that the shredder may be an important in-plant vehicle for spreading contamination to leafy greens during processing (Garg and others 1990; Stafford and others 2002). This type of transfer was the likely cause of an Australian *Salmonella* Bovismorbificans outbreak, in which 41 individuals became ill, when lettuce particulates were found behind the cutting wheel rim of the shredder used in the processing line (Stafford and others 2002).

Although *E. coli* counts on the Radicchio pieces were relatively high, very few *E. coli* O157:H7 cells transferred to the product contact surfaces. While these numbers may be low, only a few bacterial cells are needed to cross-contaminate large quantities of product. Our results indicated that microbial attachment and biofilm formation may play an important role in bacterial transfer from inoculated leaves to processing equipment surfaces. As expected more bacteria were recovered from the surface of the conveyer belt after processing the 1 h-held product compared to that, which was held for 5 d at 22 °C. Hence, 1 h may be insufficient for *E. coli* O157:H7 to properly attach to Radicchio leaves before coming in direct contact with the polyurethane conveyer belt. In previous work (Buchholz and others 2012b and 1012c) the shredder and conveyer belt generally yielded higher *E. coli* O157:H7 counts than the flume tank

or shaker table after the initial inoculated products were processed with the bacteria being retained even after processing uninoculated product, but in Buchholz and others (2012c) we did not observe any differences between bacterial counts remaining on the equipment surfaces. Following again a similar trend to the data seen in Buchholz and others (2012c), no significant differences were observed in *E. coli* populations recovered from the shredder, step conveyor, flume tank or shaker table within each post-inoculation holding time experiment. The different result in this experiment be attributed to the fact that rather than looking at 50 individual locations on the equipment, like in Buchholz and others (2012b and 2012d), we created a composite of the equipment swab samples and looked at mean transfer to the equipment.

This is the first report to assess the impact of microbial attachment and biofilm formation on *E. coli* O157:H7 cross-contamination during production of fresh-cut leafy greens. These results reveal that extending the time between a contamination event and post-harvest leafy green processing will reduce the ability to remove the attached bacteria from the surfaces of the leafy greens, but will decrease the potential spread of *E. coli* O157:H7 during those processing steps. Careful monitoring and cleaning is necessary to prevent spread of the contamination throughout a production lot.

5.6 FIGURES

Figure 5.1: *Scanning electron micrograph images showing attachment and biofilm formation* E. coli *and/or native microflora on Radicchio leaves after inoculation and holding for (A) 1 hour at 22°C, (B) 1 day at 4°C, and C) 5 days at 22°C.*

A

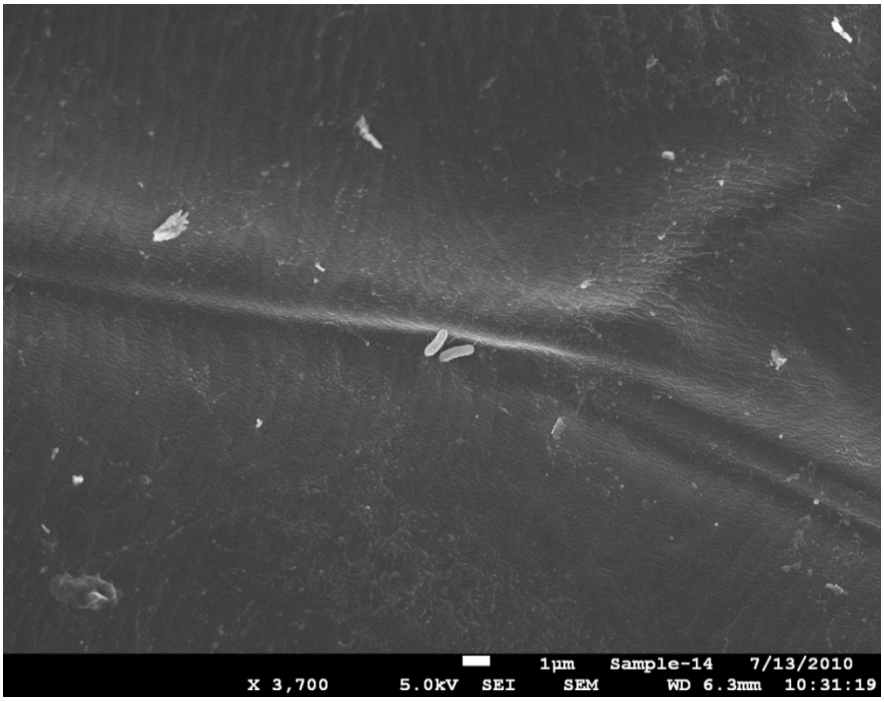
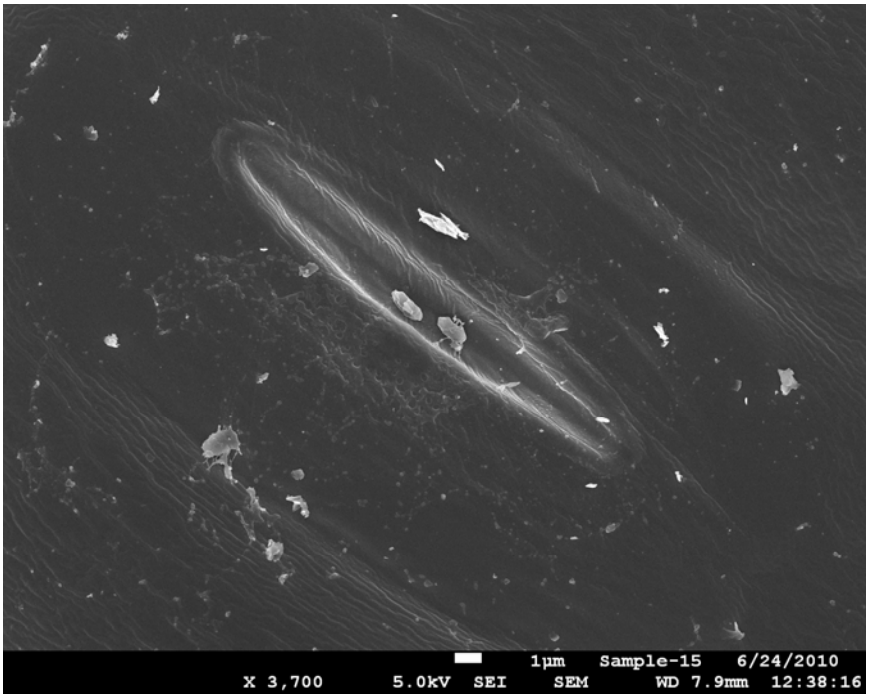


Figure 5.1 (cont'd)

B



C

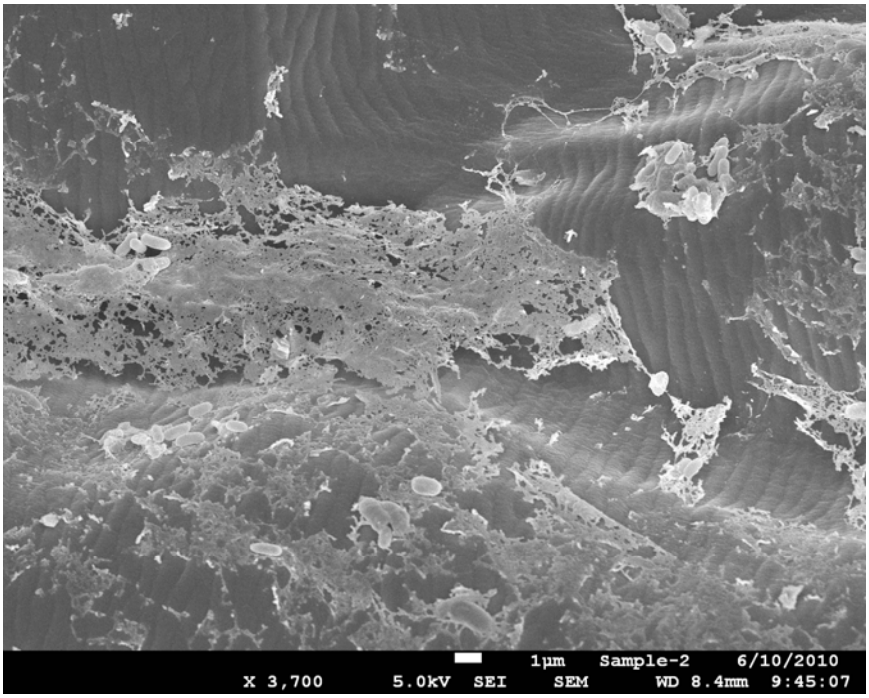


Figure 5.2: E. coli *O157:H7 populations (mean ± SD) on Radicchio after processing of iceberg lettuce*

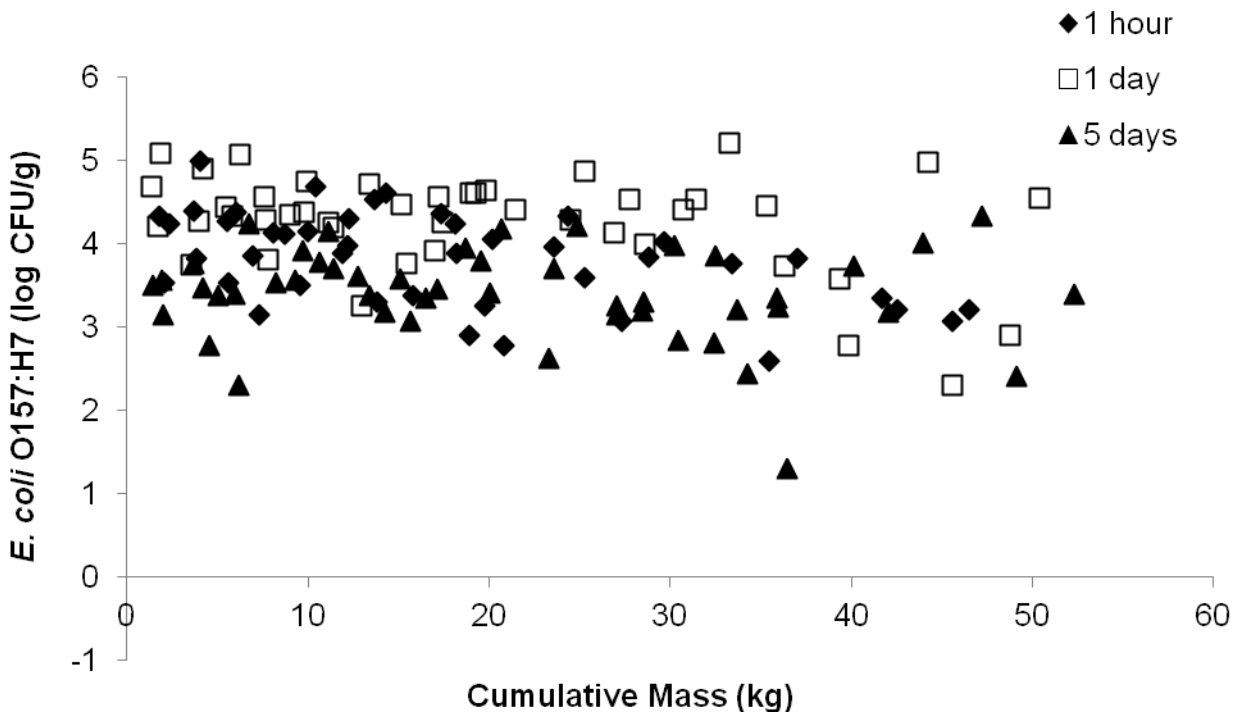


Figure 5.3: *Percentage by weight (± SD) of Radicchio recovered from the iceberg lettuce after processing*

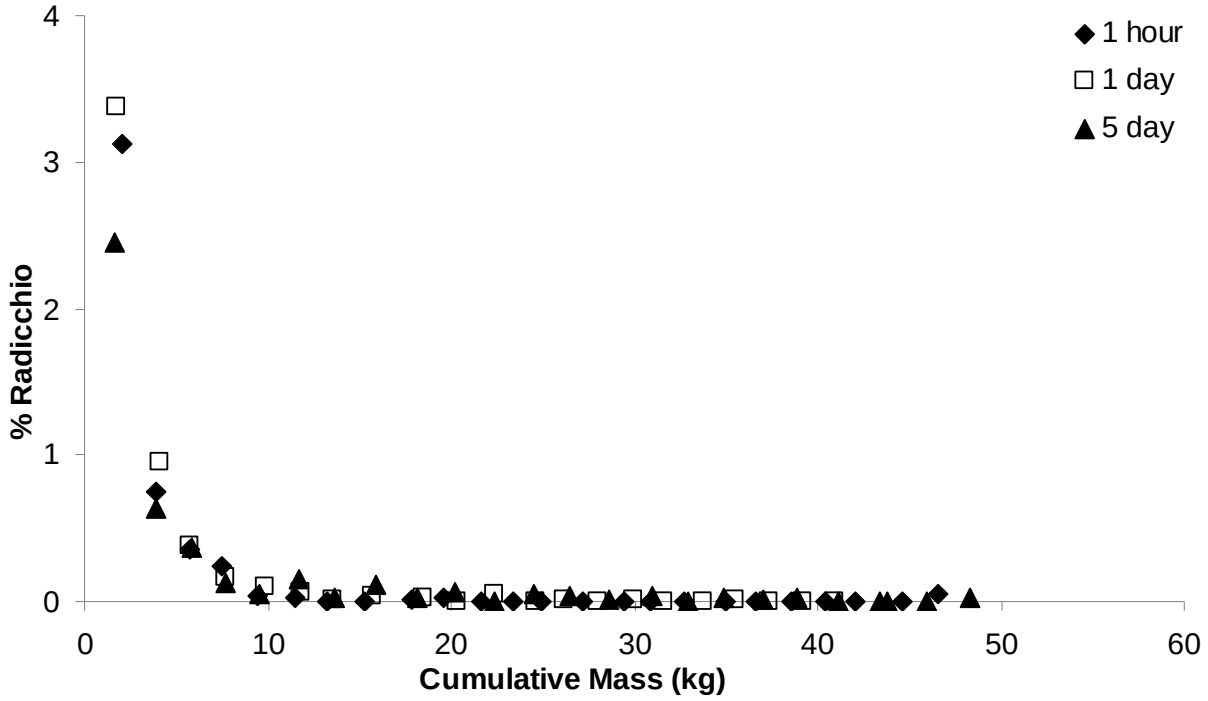


Figure 5.4: E. coli *O157:H7 populations (mean ± SD), on iceberg lettuce after processing.*

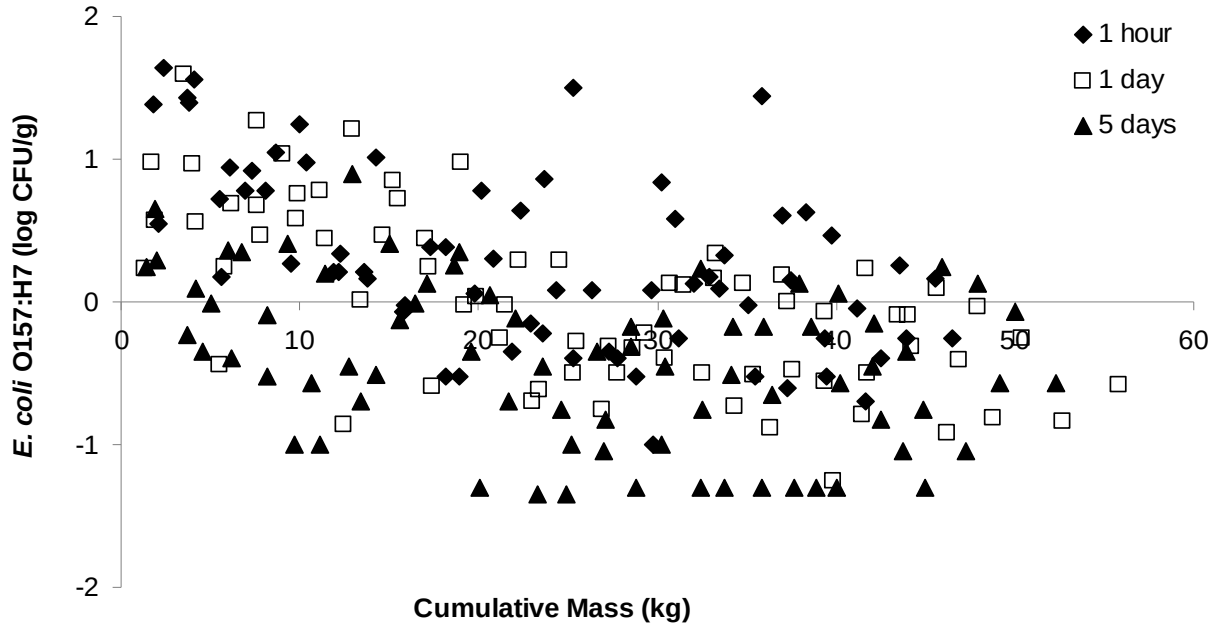
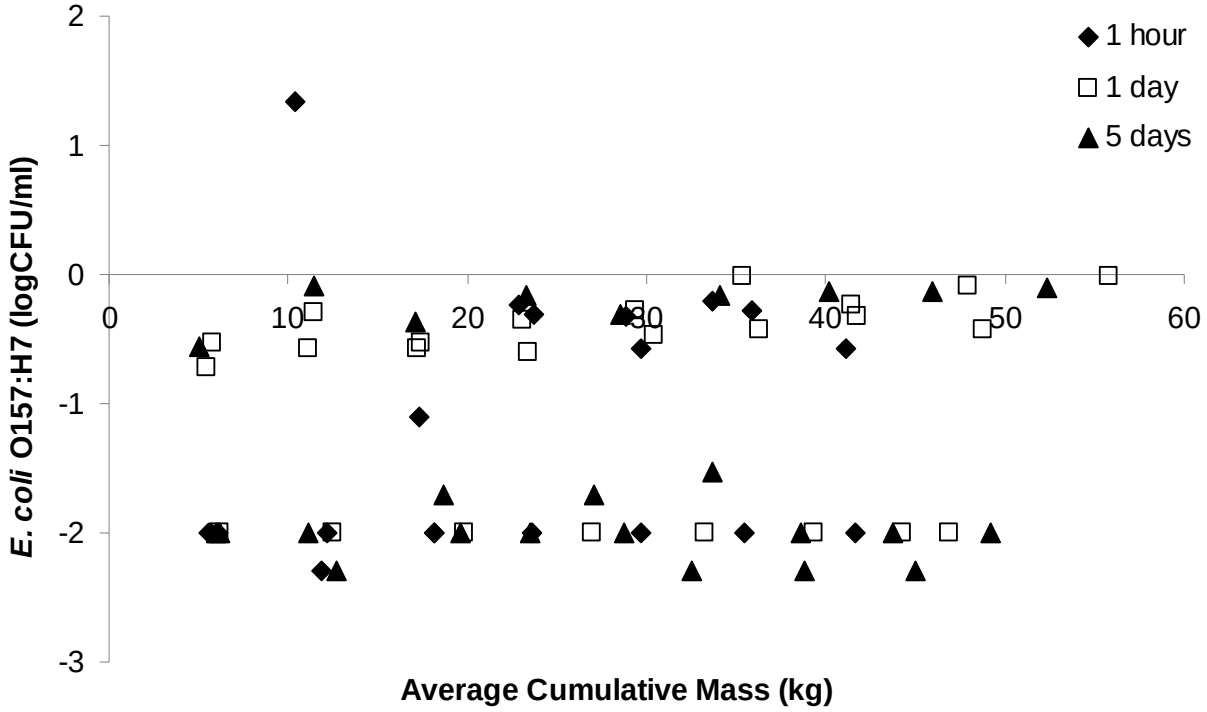


Figure 5.5: E. coli *O157:H7 populations (mean ± SD) in 890 L of recirculation water during processing of uninoculated iceberg lettuce*



5.7 TABLES

Table 5.1: E. coli *O157:H7 populations (mean ± SD) recovered from the processing equipment surfaces after leafy green processing* [a]

Product	Processing Equipment	*E. coli* O157:H7 recovered (log CFU/100 cm^2)			
Radicchio held at 22°C for 1 hour					
	Shredder	1.8	±	1.2	Aa
	Conveyer Belt	1.7	±	0.8	Aa
	Flume Tank	0.7	±	0.2	Aa
	Shaker Table	0.8	±	0.2	Aa
Radicchio held at 4°C for 1 day					
	Shredder	1.2	±	0.4	Aa
	Conveyer Belt	1.2	±	0.3	Aa
	Flume Tank	-0.1	±	0.9	Aa

Table 5.1 (cont'd)

	Shaker Table	0.2	±	0.7	Aa
Radicchio held at 22°C for 5 days					
	Shredder	0.8	±	0.5	Aa
	Conveyer Belt	0.8	±	0.5	Aa
	Flume Tank	-0.6	±	1.3	Aa
	Shaker Table	-0.1	±	0.8	Aa

[a] Means not labeled with the same capital letter are *E. coli* O157:H7 counts that differ significantly in terms of processing equipment location while means not labeled with the same lowercase letter differ significantly in terms of post-inoculation holding time ($P \leq 0.05$).

Table 5.2: E. coli *O157:H7 populations (mean ± SD), Weight ± SD and Percentage ± SD of Radicchio recovered from equipment surfaces after processing* [a]

Product	Processing Equipment	*E. coli* O157:H7 recovered (log CFU/100 cm^2)	Weight (g) of the Radicchio	Percent of the Radicchio
Radicchio held at 22°C for 1 hour				
	Shredder	4.6 ± 0.5 ^{Aa}	0.5 ± 0.7 ^{Ab}	0.4 ± 0.4
	Conveyer Belt	5.1 ± 0.5 ^{Aa}	0.8 ± 0.6 ^{Aa}	1.0 ± 0.9
	Flume Tank	3.6 ± 0.1 ^{Aa}	0.0 ± 0.0 ^{Aa}	0.0 ± 0.0
	Shaker Table	4.1 ± 0.6 ^{Aa}	1.1 ± 0.8 ^{Aa}	1.09 ± 0.3
Radicchio held at 4°C for 1 day				
	Shredder	4.6 ± 0.2 ^{Aa}	6.0 ± 3.0 ^{Aa}	6.0 ± 2.9
	Conveyer Belt	5.2 ± 0.3 ^{Aa}	1.4 ± 1.7 ^{Aa}	1.4 ± 2.0
	Flume Tank	4.3 ± 0.5 ^{Aa}	0.6 ± 0.7 ^{Aa}	0.4 ± 0.7

Table 5.2 (cont'd)

	Shaker Table	4.6	±	0.7	$_{Aa}$	1.1	±	0.4	$_{Aa}$	1.06	±	0.4
Radicchio held at 22°C for 5 days												
	Shredder	4.6	±	0.5	$_{Aa}$	3.6	±	2.0	$_{Aab}$	3.8	±	2.1
	Conveyer Belt	5.1	±	0.3	$_{Aa}$	0.9	±	0.5	$_{Aa}$	1.0	±	0.5
	Flume Tank	4.2	±	0.5	$_{Aa}$	1.0	±	0.8	$_{Aa}$	0.7	±	0.9
	Shaker Table	4.1	±	0.8	$_{Aa}$	0.5	±	0.2	$_{Aa}$	0.6	±	0.2

[a] Means not labeled with the same capital letter are *E. coli* O157:H7 counts that differ significantly in terms of processing equipment location while means not labeled with the same lowercase letter differ significantly in terms of post-inoculation holding time ($P \leq 0.05$).

CHAPTER 6:

Quantitative Transfer of *Escherichia coli* O157:H7 from Inoculated to Uninoculated Leafy Greens during Shaker Table Dewatering

6.1 ABSTRACT

Leafy greens are prone to cross-contamination during commercial shredding, conveying and flume washing with the potential for further spread of bacterial pathogens including *E. coli* O157:H7 via direct leaf-to-leaf contact during shaker table dewatering. Consequently, the goal of this study was to determine the rate of *E. coli* O157:H7 transfer between products during partial dewatering on a commercial leafy green shaker table. In this study, one (0.03 g) or eight (0.38 g each) Radicchio pieces were manually cut from a single leaf, dip-inoculated to contain (~10^4 CFU/g) of a 4-strain avirulent, GFP-labeled, ampicillin-resistant *E. coli* O157:H7 cocktail and dried for 1 h at 22°C. Thereafter, 300 or 297g of uninoculated iceberg lettuce was uniformly cut, wetted, briefly drained, and placed into each of seven square metal baskets (12'' x 7'' x 7'') mounted to the side of a commercial-scale shaker table, followed by the addition of one (0.03 g) or eight (0.38 g each) pieces of inoculated Radicchio to the baskets while shaking to obtain inoculated to uninoculated (w/w) ratios of 1:100 and 1:10,000, respectively The baskets were removed after 0 sec, 10 sec, 1 min, 5 min, 10 min, 30 min or 60 min. All shreds of Radicchio and two 25-g samples of only iceberg lettuce were then retrieved from the baskets. Sample homogenates were quantitatively examined for *E. coli* O157:H7 by direct plating w/o prior membrane filtration using trypticase soy agar containing 0.6% yeast extract and 100 ppm ampicillin as the growth medium. At the 1:10,000 ratio, *E. coli* O157:H7 transfer plateaued after ~22 min with average populations of 0.11 log CFU/g recovered from previously uninoculated iceberg lettuce. As expected, transfer leveled off faster for the 1:100 ratio (~0.5 min) with *E. coli* O157:H7 counts on the lettuce averaging ~2.52 log CFU/g. These findings confirm that cross-contamination can occur via leaf-to-leaf contact with *E. coli* O157:H7-contaminated product within a very short time frame after a contamination event.

6.2 INTRODUCTION

Between 1990 and 2008, 19 outbreaks have been specifically associated with the consumption of leafy greens (CSPI 2008b), many of which have been traced to produce grown in the Salinas Valley of California. The problems associated with contamination of leafy greens became national news in September of 2006, when consumption of pre-bagged baby spinach caused an *E. coli* O157:H7 outbreak in 26 states and Canada resulting in five deaths and 205 illnesses (Jay and others 2007). Three months later, two other *E. coli* O157:H7 outbreaks were traced to California-grown iceberg lettuce that was shredded and then purchased by two Mexican fast-food restaurant chains in the Midwest and Northeast (FDA 2006 and FDA 2007). These two outbreaks sickened a total of over 150 individuals (CSPI 2009a).

The high number of foodborne outbreaks traced to fresh fruits and vegetables is partially attributed to current large-scale, centralized production and processing practices. Contamination can occur at any point in the farm to table continuum. Many of the previous outbreaks have pointed to contamination on the farm from wild and domestic animal droppings, water runoff from livestock operations, green or improperly composted manure, dust, insects, contaminated irrigation water, and poor handling practices. While pathogen contamination likely originates on the farm, current harvesting and post-harvest processing practices can increase the impact and spread pathogens to previously uncontaminated product during field coring (Taormina and others 2009), cooling, shredding, washing, conveying drying and subsequent handling. (Beuchat 1996; Beuchat and Ryu 1997; Gorny 2006), as evidenced by the recent multi-state outbreaks (Beuchat and Ryu 1997; Burnett and Beuchat 2001). .

Once present on the surface of leafy greens *E. coli* O157:H7 can readily transfer to food contact surfaces on the processing equipment and to the wash water as demonstrated by

Buchholz and others (2012b). In addition, both *E. coli* O157:H7 and contaminated leafy greens can persist as transient residents on equipment surfaces throughout the processing line and slough off over time to contaminate large quantities of previously uncontaminated product during processing as shown by Buchholz and others (2012a, 2012c and 2012d). Our previous work investigated the different routes of bacterial transfer between contaminated products, equipment and water. This current study expands on previous work to determine if the extent of *E. coli* O157:H7 cross-contamination between inoculated and uninoculated leafy greens increases during partial dewatering following longer periods of time on the shaker table and a greater inoculated to uninoculated ratio.

6.3 MATERIALS AND METHODS

6.3.1 Experimental Design. In this study, Radicchio inoculated to contain 10^4 CFU/g *E. coli* O157:H7 was used to quantify leaf-to-leaf transfer. All experiments were conducted in triplicate. Transfer coefficients were determined for two different ratios of inoculated to uninoculated leafy greens - 1:100 and 1:10,000, with the red color of Radicchio allowing for easy differentiation between inoculated and uninoculated product.

6.3.2 Produce. Individually wrapped heads of iceberg lettuce (*Lactuca sativa* L.) (24 heads per case) and Radicchio (*Cichorium intybus*), (12 heads per case) were obtained from a local wholesaler (Stan Setas Produce Co., Lansing, MI). These products, which originated from California or Arizona depending on the growing season, were stored in a 4°C walk-in-cooler for a maximum of 5 days and then hand-cored immediately before use.

6.3.3 Bacterial strains. Four avirulent, GFP-labeled, ampicillin-resistant strains of *E. coli* O157:H7 (ATCC 43888, CV2b7, 6980-2, and 6982-2) were obtained from Dr. Michael Doyle at the Center for Food Safety, University of Georgia, Griffin, GA. Upon arrival, stock cultures of each strain were prepared in trypticase soy broth (Difco, Becton Dickinson, Sparks, MD) containing 0.6% yeast extract (Difco, Becton Dickinson) supplemented with 100 ppm ampicillin (ampicillin sodium salt, Sigma Life Science, St. Louis, MO) (TSBYE + amp) and 10% (v/v) glycerol (Sigma Chemical Company, St. Louis, MO) and stored at -80°C until needed. Working cultures were prepared by streaking each stock culture on trypticase soy agar (Difco, Becton Dickinson) containing 0.6% yeast extract and 100 ppm ampicillin (TSAYE + amp). After 18 - 24 h of incubation at 37°C, a single colony was transferred to 9 ml of TSBYE + amp and similarly incubated. Thereafter, a 0.2 ml aliquot of each culture was transferred to 200 ml of

TSBYE + amp, incubated for 18 – 20 h at 37°C and then combined in equal volumes to obtain the 4-strain cocktail.

6.3.4 Inoculation of Radicchio. An *E. coli* O157:H7 suspension containing 10^5 CFU/mL was obtained by adding 300 µL (quantities were determined based on the growth rates from Buchholz and others 2012) of the 4-strain cocktail to 3 L of chlorinated tap water (~ 1 ppm chlorine, ~15P^{o}PC) in a 121 L plastic container (Rubbermaid, Wooster, Ohio). One (0.03 g) or eight (0.38 g each) pieces were manually cut from a single Radicchio leaf, immersed in the *E. coli* suspension for 15 min., drained, and air-dried for 1 h at 22°C before processing. Two 25-g Radicchio samples were also inoculated and used to determine the average inoculation level at the time of processing.

6.3.5 Processing equipment. The stainless steel mechanical shaker table for partial leafy green dewatering was equipped with a 1 HP Baldor washdown duty motor (Baldor Electric Co., Ft. Smith, AR) that operated at 1760 RPM. Water removed from the leafy greens during mechanical shaking passed through a screen on the bottom of the shaker table and was directed to the water holding tank via a water recirculation spout on the shaker table. This shaker table is part of a pilot-scale size leafy green processing line that consists of a lettuce shredder, conveyer belt, flume tank, shaker table and centrifugal dryer. Six rectangular 30 cm x 18 cm x 18 cm metal boxes with perforated (1 cm x 1 cm opening) bottoms were positioned side by side on the shaker table above the metal screen and secured in place with 8" c-clamps (Husky, Home Depot, Atlanta, GA).

6.3.6 Radicchio and iceberg lettuce processing and sample collection. Cored iceberg lettuce heads were manually cut into 1" x 1" pieces totaling 300 or 297 g to yield inoculated to uninoculated (w/w) ratios of 1:100 and 1:10,000, respectively. The iceberg lettuce pieces were

wetted with tap water (~15P°PC), briefly drained and placed into the six metal boxes mounted to the shaker table. Either one (0.03 g) or eight (0.38 g each) pieces of inoculated Radicchio were added to the boxes while shaking to obtain inoculated to uninoculated (w/w) ratios of 1:100 and 1:10,000, respectively. The six boxes were sequentially removed from the shaker table after 10 sec, 1 min, 5 min, 10 min, 30 min or 60 min. All Radicchio pieces were carefully separated from the iceberg lettuce to prevent any additional inoculum from transferring to the iceberg lettuce. Two 25-g samples of the iceberg lettuce were then collected from each box for microbial analysis.

6.3.7 Microbiological analyses. Iceberg lettuce samples (25 g) were added to 100 ml of sterile 1% (w/v) phosphate buffer (8.5 g/L NaCl, 1.44 g/L NaR_2RHPOR_4R, and 0.24 g/L KHR_2RPOR_4R, J.T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ) in a Whirl-Pak™ filter bag (Nasco, Fort Atkinson, WI) and processed in a pulsifier (Pulsifier, Filtaflex Ltd., Almonte, Ontario, Canada) for 1 min. The Radicchio pieces were composited, diluted 1:5 in 0.1% phosphate buffer in Whirl-Pak™ bag and similarly pulsified. These iceberg lettuce and Radicchio samples were plated on TSAYE + amp or processed using 0.45 μm membrane filters (Millipore, Millipore Corporation, Billerica, MA), which were placed on 60-mm dia. Petri plates containing TSAYE + amp to quantify *E. coli* O157:H7. Following 18 - 20 h of incubation at 37°C, all green fluorescing colonies as seen under ultraviolet light (365 nm, Blak-Ray, Ultra-violet Product Inc. San Gabriel, CA) were counted as *E. coli* O157:H7.

6.3.9 Modeling of non-linear curves. JMP 8.0 software was used to conduct a non-linear regression analysis and to calculate the 95% confidence intervals for the curves fitted to the *E. coli* O157:H7 counts on the iceberg lettuce. The cumulative Weibull model used was as follows:

$$\log N/N_0 = -bt^n,$$

where b and n are the scale and shape parameters, respectively. (Chen 2007, Gonzalez and others 2009, Marfart and others 2002, Peleg and Cole 1998).

6.4 RESULTS

6.4.1 Radicchio. The four-strain *E. coli* O157:H7 suspension used to inoculate the Radicchio pieces for inoculated to uninoculated (w/w) ratios of 1:100 and 1:10,000 contained 5.6 ± 0.5 and 4.7± 0.4 log CFU/ml, respectively. After inoculation by immersion, the Radicchio pieces contained *E. coli* O157:H7 populations of 4.9 ± 0.2 (1:100 (w/w) ratio) and 4.2 ± 0.3 (1:10,000 (w/w) ratio) log CFU/g. Numbers of *E. coli* O157:H7 decreased to 4.5 ± 0.5 log CFU/g on Radicchio used in the 1:100 ratio study, while no decrease was observed on Radicchio after shaking in the 1:10,000 ratio study (4.2 ± 1.0 log CFU/g).

6.4.2 Iceberg lettuce. At the 1:10,000 ratio, *E. coli* O157:H7 transfer plateaued after ~22 min with average populations of 0.11 log CFU/g recovered from previously uninoculated iceberg lettuce (Figure 6.1). After shaking for 60 min., 32.4 ± 6.4 % of the original inoculum transferred to uninoculated iceberg lettuce for the 1:10,000 ratio. As expected, transfer leveled off faster for the 1:100 ratio (~0.5 min) with *E. coli* O157:H7 counts on the lettuce averaging ~2.5 log CFU/g, with 52.4 ± 30.8 % of the original inoculum transferred after 60 min of shaking (Figure 6.1).

6.5 DISCUSSION

Previous studies have demonstrated the potential for bacterial cross-contamination during field coring (Taormina and others 2009) and post-harvest processing of leafy greens (Buchholz and others 2012a, 2012b, 2012c and 2012d) and washing of produce (Buchanan and others 1999; Lopez-Galvez and others 2009; Lopez-Galvez and others 2010; Nou and Lou 2010). Bacterial transfer has also been demonstrated in various other food preparation scenarios, including between lettuce, chicken, and cutting boards (Chai and others 2008; Chen and others 2001; Fravalo and others 2009; Jiménez and others 2009; Ravishankar and others 2010), and cloths, hands, gloves, utensils and various food products (Chen and others 2001; Gill and Jones 2002; Montville and others 2001; Scott and Bloomfield 1990). Work in our own laboratory has shown that approximately 90% of the *E. coli* O157:H7 population on inoculated lettuce was shed in sanitizer-free water during washing of leafy greens in a flume tank with this pathogen contaminating product contact surfaces of the shredder, conveyor, flume tank, shaker table and dewatering centrifuge during processing (Buchholz and others 2012b) and then transferring to large quantities of previously uncontaminated product during subsequent processing (Buchholz and others 2012a, 2012b and 2012d).

During post-harvest processing, inoculated and uninoculated leafy greens will inevitably become commingled, thus complicating the assessment of transfer from contaminated product to previously uncontaminated product (Buchholz and others 2012a, 2012c, and 2012d). In this study *E. coli* O157:H7-inoculated Radicchio was used as a colored surrogate for iceberg lettuce to track the spread of contaminated product during shaker table dewatering. Various methods for differentiating product are available based on colored dyes, physical markings (Zhang and others 2009), and similar products of different colors with Annous and others (2001) having used both

inoculated Golden Delicious and uninoculated Fuji apples to assess the efficacy of a commercial flatbed brush washer (Annous and others 2001). Similarly, Nou and Luo (2010) used inoculated "Lollo Rossa" and uninoculated green-leaf lettuce leaves to evaluate the efficacy of a chlorine wash in the production of mixed salad greens. In our study, Radicchio - a red colored leaf chicory, was used as a surrogate for iceberg lettuce to allow us to differentiate the inoculated and uninoculated lettuce pieces. Although not a member of the same genus as lettuce, Radicchio is in the same family with both considered to be "leafy vegetables".

In this study Radicchio inoculated at ~5 log CFU/g, representing a medium to high bacterial load, was used as an indicator to visualize inoculated from uninoculated product. This targeted inoculation level at the time of processing was used to minimize variability between replicates for future mathematical modeling. While less common in a real world scenario than lower pathogen loads, these high inoculation levels represent direct contamination from fecal matter in a field from cattle (the main carriers of *E. coli* O157:H7) that are a well-documented source of contamination in the field (Williams and others 2008).

Cross-contamination between leafy greens has been previously demonstrated with similar findings. A study by Wachtel and Charkowski (2002) showed that mixing of one dry lettuce piece, inoculated to contain ~10^5 CFU of EHEC per piece, with a large volume of dry lettuce, followed by storage at 4°C or 25°C for 20 h led to the contamination of all uninoculated leaves tested. When fitted to our data, the nonlinear Weibull model described the initial quick increase in *E. coli* O157:H7 numbers transferred to the uninoculated leaves followed by a leveling off as partial dewatering on the shaker table continued. A confidence interval of 95% was calculated for the *E. coli* O157:H7 counts based on non-linear regression analysis. As expected, faster cross-contamination was seen using an inoculated to uninoculated ratio of 1:100 than 1:10,000

(w/w) due to the higher number of *E. coli* O157:H7 cells available for transfer. In summary, these findings confirm that cross-contamination can occur via leaf-to-leaf contact with *E. coli* O157:H7-contaminated product within a very short time frame after a contamination event.

6.6 FIGURES

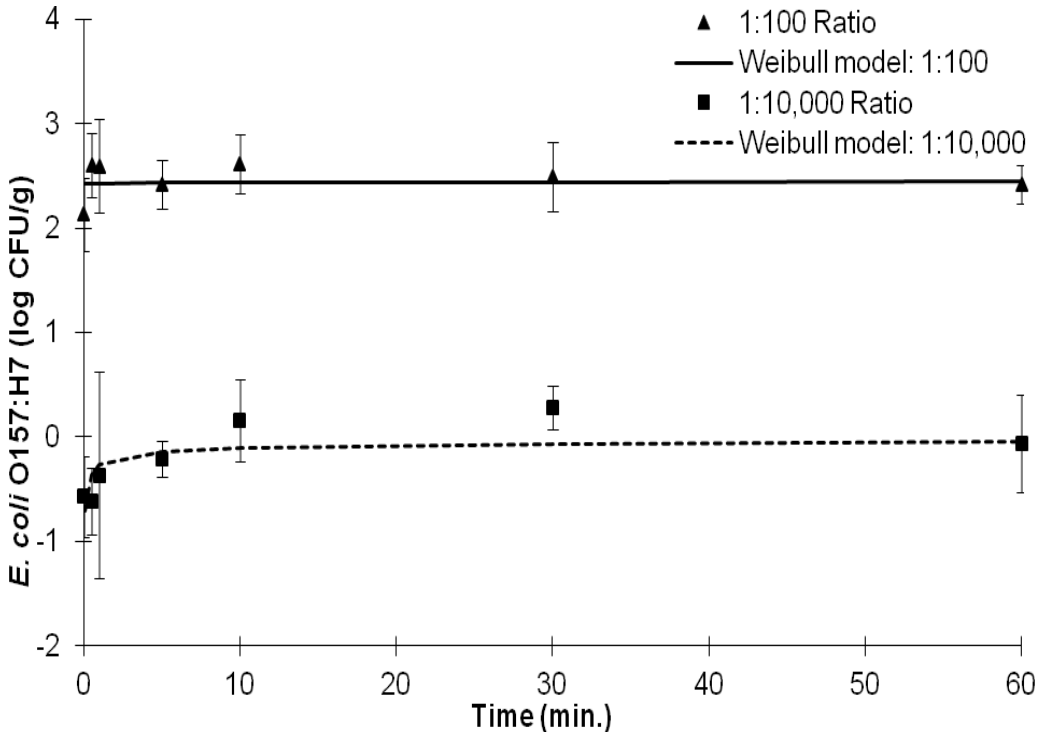


Figure 6.1: E. coli *O157:H7 populations (mean ± SD), on the iceberg lettuce after leafy green processing.*

6.7 TABLES

Table 6.1: *Weibull model parameters fitted to* E. coli *O157:H7 populations on iceberg lettuce.*

Ratio	Model Parameter	Value ± SE	Low CI 95%	High CI 95%
1:100	*b*	2.4 ± 0.1	2.3	2.6
	n	0.0 ± 0.0	-0.0	0.0
1:10,000	*b*	-0.3 ± 0.1	-0.4	-0.1
	n	-0.4 ± 0.1	-0.7	-0.2

CONCLUSIONS AND FUTURE RECOMMENDATIONS

Cross-contamination/multi-directional transfer of *Escherichia coli* O157:H7 during commercial shredding, conveying, fluming and drying of fresh-cut leafy greens has become a major public health concern, particularly because post-processing kill steps are currently not implemented in the industry. The first objective of the research was to quantify *E. coli* O157:H7 transfer to equipment during commercial production of fresh-cut leafy greens. These results indicated that during processing of iceberg lettuce, Romaine lettuce and baby spinach, approximately 90% of the inoculum was transferred to the sanitizer-free wash water, which generally agrees with the efficacy of normal produce washing practices for the removal of microorganisms. Very few *E. coli* O157:H7 cells transferred to the product contact surfaces compared to the water. However, in terms of the equipment surfaces, greatest transfer was seen to the shredder and conveyer belt, followed by the centrifugal dryer, flume tank and shaker table. Shred size does not appear to affect the numbers of *E. coli* O157:H7 transferred from contaminated leafy greens during processing; however, extending the time between contamination and processing will decrease the removal and transfer of this pathogen during production of fresh-cut leafy greens. Overall, ~29% of the remaining product inoculum was lost during centrifugal drying. Future research needs to be conducted to determine if routine microbial monitoring of the centrifugation water may be a useful strategy for minimizing and preventing the continued spread of bacterial pathogens to large quantities of product.

The second objective of this research was to quantify the transfer of *E. coli* O157:H7 from equipment surfaces to iceberg and Romaine lettuce during simulated commercial processing. These findings indicated that *E. coli* O157:H7 is capable of persisting on equipment surfaces even after 90.8 kg of clean uncontaminated product are processed. Initially, greatest *E.*

coli O157:H7 transfer was seen from inoculated lettuce to the shredder and conveyer belt, with all equipment surface populations decreasing only 90 - 99% after processing 90.8 kg of uncontaminated product. Such cross-contamination can occur even when *E. coli* O157:H7 is initially present in very low levels. This study supports the importance of sanitizers in wash waters, or other interventions, to adequately reduce the microbial load on the product and in flume water during commercial processing to prevent future product recalls and outbreaks. Future research may focus on determining the efficacy of various commercially available sanitizers against *E. coli* O157:H7 in wash water and to prevent cross-contamination during pilot-scale leafy green processing.

Based on the findings of the second objective, the third objective of this study was developed to track an *E. coli* O157:H7 inoculated batch of leafy greens through a processing line using Radicchio as visual marker. The results clearly indicate that *E. coli* O157:H7 persisted in the same processing line after 907.2 kg of clean uncontaminated iceberg lettuce had passed through. Hundreds of contaminated Radicchio shreds were recovered from the leafy green processing line with at least a few shreds remaining on each processing equipment piece. Radicchio build-up was most evident on the discharge chute and area surrounding the cutting wheel of the shredder, the front of the flume tank and the polyurethane conveyer belt, especially at the junction between the guard rails and the belt. These findings provide important insight into potential lapses in current cleaning/sanitizing practices and improved equipment designs. The results of objective 3 clearly showed that one small inoculated batch could contaminate subsequent large quantities of previously uncontaminated product during processing if an effective microbial intervention strategy is not implemented. This was demonstrated by the *E. coli* O157:H7 populations averaging 1 log CFU/g on the uninoculated product and the shreds of

Radicchio that were found in all but one bag of the ~38 bags of processed product. These data can later be used to validate a simulation model created to estimate the prevalence and percent of *E. coli* O157:H7 transferred during a cross-contamination event in fresh-cut bagged leafy greens under different scenarios. In the event of an outbreak, these findings could be used as a guide to help estimate the amount of product which may have become cross-contaminated during processing and would need to be recalled.

Building on results from the first objective, the fourth objective of this study determined the effect that bacterial attachment time may have on cross-contamination during processing. After processing, *E. coli* O157:H7 was found in all previously uncontaminated iceberg lettuce samples with mean counts highest for 1 h- followed by 1 d- and 5 d-held product. Scanning electron microscopy images showed greater bacterial attachment for 5 d-held product, with larger cells aggregates enveloped in a dense extracellular matrix of material which formed a web covering the entire leaf surface. *E. coli* O157:H7 populations in the flume water or on the equipment surface samples did not differ significantly for the three post-inoculation holding times. Based on these findings, the point at which contamination occurs will impact the extent of *E. coli* O157:H7 that will detach from leafy greens during processing. Future work may explore whether the contaminant's location on the leaf, the source of contaminant (e.g., animal feces vs. irrigation water), the region of the country the product was grown in, or the point in the growing season (early vs. late), may have an effect on bacterial transfer or the effectiveness of sanitizers frequently used during leafy green processing.

The final objective of this study was to quantify the *E. coli* O157:H7 transfer from inoculated leafy greens to uninoculated leafy greens during partial dewatering. This project was developed when it was determined that objectives 2, 3 and 4 alone could not be used to

determine the extent of cross-contamination from direct leaf-to-leaf contact likely to occur on the conveyer belt, shaker table, etc. As expected, faster cross-contamination was seen using an inoculated to uninoculated ratio of 1:100 than 1:10,000 (w/w) until a maximum population was reached due to the higher number of *E. coli* O157:H7 cells available for transfer. In summary, these findings confirm that cross-contamination can occur during shaker table dewatering within a very short time frame after a contamination event. Leaf-to-leaf contamination can be minimized by ensuring that all product moves through the leafy green processing line without delay.

Overall, this research shows that small numbers of *E. coli* O157:H7 in the water and on the equipment surfaces can lead to contamination of multiple batches of fresh-cut leafy greens in a processing facility with or without sanitizer. Flume tank wash water needs to contain a chemical sanitizer to minimize cross-contamination of leafy greens during post-harvest processing. A single shred of *E. coli* O157:H7-contaminated product can quickly spread *E. coli* O157:H7 to adjacent leaves via leaf-to-leaf contact. Monitoring the spent centrifugation water for microbial contaminants, including *E. coli* O157:H7, may be helpful in identifying potentially contaminated product. An extended the time between the contamination event and post-harvest leafy green processing will decrease the removal of *E. coli* O157:H7 from leaf surfaces during leafy green processing. Equipment designed to facilitate cleaning and sanitation and proper monitoring of the preventative controls are essential means to mitigate the extent of bacterial transfer and cross-contamination that can occur during commercial processing of leafy greens to prevent large-scale outbreaks.

APPENDICES

APPENDIX I

Mean *Escherichia coli* O157:H7 populations on equipment surfaces after processing

Table AI.1: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces after processing 22.7 kg of leafy greens inoculated at 6 log CFU/g (n=3)*

	Mean ± SD *E. coli* O157:H7 (log CFU/100 cm^2)		
Location on Processing Equipment	Baby Spinach	Romaine Lettuce	Iceberg Lettuce
Shredder			
1) front left, stainless steel	ND	4.69 ± 0.58	5.71 ± 0.39
1) front right, stainless steel	ND	5.24 ± 0.14	5.62 ± 0.22
2) left side, feed belt, plastic	ND	4.94 ± 1.14	5.34 ± 0.32
2) right side, feed belt, plastic	ND	4.33 ± 0.54	5.28 ± 0.34
3) front left feed belt guide, stainless steel	ND	5.13 ± 0.56	5.11 ± 0.92
3) front right feed belt guide, stainless steel	ND	5.71 ± 0.23	5.10 ± 0.34
3) end, left side, feed belt guide, stainless steel	ND	1.63 ± 1.08	4.82 ± 0.81
3) end, right side, feed belt guide, stainless steel	ND	1.63 ± 1.08	4.10 ± 1.07
4) below the cutting wheel, stainless steel	ND	4.23 ± 0.92	5.04 ± 0.72
5) cutting wheel, stainless steel	ND	4.58 ± 0.31	4.65 ± 0.31
6) discharge chute, stainless steel	ND	5.28 ± 0.52	6.07 ± 0.58

Table AI.1 (cont'd)

6) discharge chute, stainless steel	ND	5.45 ± 0.23	5.75 ± 0.44
6) discharge chute, stainless steel	ND	5.89 ± 0.69	5.60 ± 0.33
6) discharge chute, stainless steel	ND	5.70 ± 0.55	5.65 ± 0.56
Conveyer Belt			
1) conveyer belt, polyurethane	ND	5.57 ± 0.15	5.88 ± 0.72
1) conveyer belt, polyurethane	ND	5.23 ± 0.30	5.69 ± 0.42
2) bottom, left side, belt guide, plastic	ND	5.38 ± 0.34	6.02 ± 0.52
2) bottom, right side, belt guide, plastic	ND	4.75 ± 0.61	5.73 ± 0.40
2) center, left side, belt guide, plastic	ND	4.87 ± 0.34	5.36 ± 0.13
2) center, right side, belt guide, plastic	ND	4.06 ± 0.57	5.83 ± 0.59
1) top of the conveyer belt shelf, polyurethane	ND	5.37 ± 0.47	5.88 ± 0.32
1) bottom of the conveyer belt shelf, polyurethane	ND	4.21 ± 0.48	5.52 ± 0.13
Flume Tank			
1) slanted front, stainless steel	5.01 ± 0.51	5.31 ± 0.42	5.06 ± 0.19
2) left side, interior wall, 2' from start, stainless steel	3.66 ± 0.58	2.13 ± 0.24	3.75 ± 0.62

Table AI.1 (cont'd)

2) right side, interior wall, 2' from start, stainless steel	3.23 ± 0.12	2.02 ± 0.95	2.68 ± 0.26
2) left side, interior wall, 4' from start, stainless steel	2.89 ± 0.42	1.39 ± 0.68	2.61 ± 0.07
2) bottom, 4' from front, stainless steel	3.02 ± 0.65	3.64 ± 0.17	3.94 ± 0.29
2) right side, interior wall, 4' from start, stainless steel	2.27 ± 0.90	1.00 ± 0.00	2.44 ± 0.37
2) left side, interior wall, 6' from start, stainless steel	2.25 ± 0.35	1.94 ± 0.82	2.14 ± 0.27
2) right side, interior wall, 6' from start, stainless steel	1.97 ± 0.76	1.00 ± 0.00	2.00 ± 0.28
2) left interior wall, 8' from start, stainless steel	2.11 ± 0.53	1.00 ± 0.00	1.65 ± 0.44
2) bottom, 8' from start, stainless steel	2.41 ± 0.98	2.78 ± 0.56	3.08 ± 0.15
2) right interior wall, 8' from start, stainless steel	1.70 ± 0.42	1.78 ± 0.68	1.48 ± 0.28
Shaker table			
1) front, textured, stainless steel	3.08 ± 0.58	3.93 ± 0.26	3.67 ± 0.29
2) perforated dewatering screen, stainless steel	3.60 ± 0.68	4.42 ± 0.22	4.68 ± 0.01
3) 1' from start, textured, stainless steel	3.92 ± 0.26	4.59 ± 0.16	4.71 ± 0.05
3) left side, end, textured, stainless steel	3.81 ± 0.45	4.25 ± 0.21	4.59 ± 0.16
4) end (vertical portion), stainless steel	3.04 ± 0.49	3.60 ± 0.20	3.80 ± 0.49

Table AI.1 (cont'd)

3) right side, end, textured, stainless steel	3.67 ± 0.11	4.59 ± 0.08	4.53 ± 0.14
3) end (slanted portion), stainless steel	2.50 ± 1.29	2.44 ± 0.24	3.54 ± 0.39
5) left side, recirculation spout, stainless steel	1.75 ± 0.63	1.68 ± 0.61	2.76 ± 0.15
6) right side, recirculation spout, stainless steel	1.28 ± 0.17	1.00 ± 0.00	2.51 ± 0.39
Dewatering Centrifuge			
1) interior basket carrier, stainless steel	4.00 ± 0.31	4.95 ± 0.16	5.15 ± 0.63
1) interior basket carrier, stainless steel	4.06 ± 0.38	4.63 ± 0.49	5.14 ± 1.16
1) interior basket carrier, stainless steel	3.94 ± 0.23	4.95 ± 0.04	4.79 ± 0.24
1) interior basket carrier, stainless steel	4.30 ± 0.05	4.47 ± 0.18	4.69 ± 0.28
2) left side, interior wall, stainless steel	3.99 ± 0.22	4.05 ± 0.20	4.23 ± 0.55
2) right side, interior wall, stainless steel	3.99 ± 0.22	4.04 ± 0.28	4.21 ± 0.51
3) viewing window in the lid, plastic	2.62 ± 1.28	1.00 ± 0.00	2.10 ± 0.29
4) drain, stainless steel	5.22 ± 0.23	5.51 ± 0.10	5.95 ± 0.39

Table AI.2: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces after processing 22.7 kg of leafy greens inoculated at 4 log CFU/g (n=3)*

Location on Processing Equipment	Mean ± SD *E. coli* O157:H7 (log CFU/100 cm^2)				
	Baby Spinach	Romaine Lettuce	Iceberg Lettuce	Iceberg Lettuce (finely shredded)	Iceberg Lettuce (held 24 h before processing)
Shredder					
1) front left, stainless steel	ND	4.04 ± 0.19	3.71 ± 0.21	2.59 ± 0.53	1.66 ± 0.77
1) front right, stainless steel	ND	4.36 ± 0.07	3.93 ± 0.15	1.00 ± 0.75	2.35 ± 0.81
2) left side, feed belt, plastic	ND	4.24 ± 0.07	3.71 ± 0.39	0.77 ± 0.52	1.63 ± 0.79
2) right side, feed belt, plastic	ND	4.17 ± 0.17	3.62 ± 0.29	1.43 ± 0.72	1.67 ± 0.78
3) front left feed belt guide, stainless steel	ND	4.06 ± 0.44	3.89 ± 0.36	1.74 ± 0.65	1.48 ± 0.83

Table AI.2 (cont'd)

3) front right feed belt guide, stainless steel	ND	4.18 ± 0.28	4.06 ± 0.17	0.88 ± 0.11	2.59 ± 1.19
3) end, left side, feed belt guide, stainless steel	ND	4.10 ± 0.42	3.00 ± 0.80	1.13 ± 0.17	1.54 ± 0.57
3) end, right side, feed belt guide, stainless steel	ND	2.94 ± 0.54	2.54 ± 0.90	1.13 ± 0.21	1.52 ± 0.54
4) below the cutting wheel, stainless steel	ND	2.95 ± 0.93	3.26 ± 0.40	0.93 ± 0.13	2.20 ± 1.67
5) cutting wheel, stainless steel	ND	3.85 ± 0.14	2.27 ± 0.24	1.32 ± 1.09	2.33 ± 0.30
6) discharge chute, stainless steel	ND	4.21 ± 0.23	4.22 ± 0.34	1.56 ± 1.56	1.32 ± 0.28
6) discharge chute, stainless steel	ND	4.80 ± 0.14	3.80 ± 0.40	2.39 ± 0.83	3.08 ± 0.28
6) discharge chute, stainless steel	ND	4.52 ± 0.12	3.13 ± 1.22	2.74 ± 0.45	1.28 ± 0.17
6) discharge chute, stainless steel	ND	4.28 ± 0.63	3.57 ± 0.44	2.96 ± 0.61	2.81 ± 1.58
Conveyer Belt					
1) conveyer belt, polyurethane	ND	4.80 ± 0.07	3.82 ± 0.86	2.99 ± 0.41	2.11 ± 0.85

Table AI.2 (cont'd)

1) conveyer belt, polyurethane	ND	4.57 ± 0.11	4.22 ± 0.05	2.40 ± 0.77	2.80 ± 0.89
2) bottom, left side, belt guide, plastic	ND	4.40 ± 0.07	4.16 ± 0.32	3.02 ± 0.37	2.40 ± 0.53
2) bottom, right side, belt guide, plastic	ND	4.40 ± 0.31	4.10 ± 0.33	1.42 ± 1.49	2.35 ± 0.17
2) center, left side, belt guide, plastic	ND	4.40 ± 0.37	3.66 ± 0.26	1.59 ± 1.34	2.36 ± 0.90
2) center, right side, belt guide, plastic	ND	3.95 ± 0.12	3.73 ± 0.31	1.73 ± 1.33	3.03 ± 0.73
1) top of the conveyer belt shelf, polyurethane	ND	3.94 ± 0.11	4.30 ± 0.11	2.92 ± 0.70	2.90 ± 0.66
1) bottom of the conveyer belt shelf, polyurethane	ND	4.38 ± 0.23	3.73 ± 0.14	2.88 ± 0.34	2.74 ± 1.28
Flume Tank					
1) slanted front, stainless steel	2.88 ± 0.87	4.46 ± 0.32	3.27 ± 0.08	2.46 ± 0.14	2.64 ± 0.73

Table AI.2 (cont'd)

2) left side, interior wall, 2' from start, stainless steel	1.05	±	0.40	4.00	±	0.07	1.39	±	0.13	1.33	±	2.07	0.77	±	1.76
2) right side, interior wall, 2' from start, stainless steel	1.12	±	0.90	1.98	±	0.64	1.03	±	0.52	0.91	±	1.70	0.20	±	2.07
2) left side, interior wall, 4' from start, stainless steel	0.89	±	0.26	1.28	±	0.63	1.15	±	0.23	0.77	±	1.53	0.79	±	1.07
2) bottom, 4' from front, stainless steel	1.46	±	0.73	1.12	±	0.81	1.86	±	0.25	-0.35	±	1.13	2.19	±	1.53
2) right side, interior wall, 4' from start, stainless steel	0.96	±	0.55	2.74	±	0.17	0.66	±	0.59	2.14	±	1.70	1.34	±	2.43
2) left side, interior wall, 6' from start, stainless steel	0.10	±	0.17	0.56	±	0.43	1.50	±	0.14	1.45	±	2.13	1.12	±	2.51
2) right side, interior wall, 6' from start, stainless steel	0.17	±	1.17	1.42	±	0.43	1.04	±	0.43	1.41	±	2.10	0.14	±	1.13

Table AI.2 (cont'd)

2) left interior wall, 8' from start, stainless steel	0.78	±	0.88	1.16	±	0.44	0.44	±	0.42	1.70	±	2.35	-1.00	±	0.00
2) bottom, 8' from start, stainless steel	0.90	±	0.79	0.42	±	0.39	1.71	±	0.47	2.19	±	1.34	1.00	±	0.39
2) right interior wall, 8' from start, stainless steel	-0.21	±	1.67	2.18	±	0.11	0.65	±	0.33	1.00	±	1.74	-0.13	±	1.51
Shaker table															
1) front, textured, stainless steel	1.18	±	1.03	1.15	±	1.02	2.21	±	0.26	2.18	±	0.45	0.70	±	1.47
2) perforated dewatering screen, stainless steel	1.81	±	0.79	2.86	±	0.05	2.75	±	0.05	0.81	±	1.57	0.96	±	1.71
3) 1' from start, textured, stainless steel	1.80	±	0.83	3.23	±	0.15	2.70	±	0.09	1.84	±	0.27	1.17	±	1.89
3) left side, end, textured, stainless steel	1.81	±	0.14	3.23	±	0.12	3.05	±	0.07	2.82	±	0.32	1.55	±	2.24

Table AI.2 (cont'd)

4) end (vertical portion), stainless steel	1.43 ± 0.25	3.14 ± 0.05	2.30 ± 0.23	1.73 ± 0.06	1.39 ± 0.83
3) right side, end, textured, stainless steel	1.63 ± 0.14	2.81 ± 0.18	2.99 ± 0.17	3.44 ± 0.64	1.68 ± 2.33
3) end (slanted portion), stainless steel	0.93 ± 0.48	3.23 ± 0.21	1.97 ± 0.51	2.99 ± 1.57	1.33 ± 0.35
5) left side, recirculation spout, stainless steel	1.38 ± 1.07	1.84 ± 0.02	0.90 ± 0.63	2.12 ± 2.70	0.45 ± 1.61
6) right side, recirculation spout, stainless steel	0.67 ± 1.60	-0.22 ± 0.68	-0.03 ± 0.95	2.41 ± 2.95	0.07 ± 1.86
Dewatering Centrifuge					
1) interior basket carrier, stainless steel	2.29 ± 0.02	0.06 ± 0.10	2.52 ± 0.34	3.28 ± 1.52	3.25 ± 0.27
1) interior basket carrier, stainless steel	2.29 ± 0.35	3.03 ± 0.78	2.80 ± 0.16	2.96 ± 1.19	3.01 ± 0.45

Table AI.2 (cont'd)

1) interior basket carrier, stainless steel	2.16	±	0.14	3.06	±	0.41	2.78	±	0.19	3.14	±	0.88	3.10	±	0.10
1) interior basket carrier, stainless steel	2.35	±	0.44	3.34	±	0.61	2.85	±	0.19	3.33	±	0.66	2.73	±	1.08
2) left side, interior wall, stainless steel	1.69	±	0.28	2.97	±	0.31	2.02	±	0.42	3.38	±	0.40	2.53	±	0.09
2) right side, interior wall, stainless steel	1.73	±	0.13	3.21	±	0.16	2.11	±	0.28	2.90	±	1.07	2.23	±	0.74
3) viewing window in the lid, plastic	0.87	±	0.58	3.07	±	0.26	0.26	±	1.58	3.12	±	1.85	1.80	±	0.77
4) drain, stainless steel	3.08	±	0.25	0.92	±	0.82	3.87	±	0.06	3.53	±	0.68	3.48	±	0.33

Table AI.3: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces after processing 22.7 kg of leafy greens inoculated at 2 log CFU/g (n=5)*

	Mean ± SD *E. coli* O157:H7 (log CFU/100 cm^2)		
Location on Processing Equipment	Baby Spinach	Romaine Lettuce	Iceberg Lettuce
Shredder			
1) front left, stainless steel	ND	1.31 ± 0.59	1.53 ± 0.87
1) front right, stainless steel	ND	1.24 ± 0.58	1.69 ± 0.33
2) left side, feed belt, plastic	ND	0.80 ± 1.48	1.40 ± 0.67
2) right side, feed belt, plastic	ND	1.00 ± 0.70	1.16 ± 0.64
3) front left feed belt guide, stainless steel	ND	1.18 ± 0.87	1.06 ± 1.29
3) front right feed belt guide, stainless steel	ND	0.88 ± 0.55	1.95 ± 0.06
3) end, left side, feed belt guide, stainless steel	ND	-1.04 ± 0.08	-0.06 ± 1.22
3) end, right side, feed belt guide, stainless steel	ND	-1.04 ± 0.08	1.05 ± 0.76
4) below the cutting wheel, stainless steel	ND	0.17 ± 0.82	1.27 ± 0.60
5) cutting wheel, stainless steel	ND	0.86 ± 0.37	0.96 ± 0.55
6) discharge chute, stainless steel	ND	1.33 ± 0.29	1.91 ± 0.32

Table AI.3 (cont'd)

6) discharge chute, stainless steel	ND	1.79 ± 0.68	1.84 ± 0.25
6) discharge chute, stainless steel	ND	2.18 ± 0.65	1.58 ± 0.32
6) discharge chute, stainless steel	ND	2.06 ± 0.40	0.85 ± 0.85
Conveyer Belt			
1) conveyer belt, polyurethane	ND	1.71 ± 0.56	1.85 ± 0.40
1) conveyer belt, polyurethane	ND	1.50 ± 0.70	1.57 ± 0.46
2) bottom, left side, belt guide, plastic	ND	1.44 ± 1.04	1.92 ± 0.35
2) bottom, right side, belt guide, plastic	ND	1.28 ± 1.37	1.94 ± 0.60
2) center, left side, belt guide, plastic	ND	0.72 ± 0.44	1.30 ± 0.54
2) center, right side, belt guide, plastic	ND	0.62 ± 0.48	1.50 ± 0.47
1) top of the conveyer belt shelf, polyurethane	ND	0.61 ± 1.64	2.04 ± 0.38
1) bottom of the conveyer belt shelf, polyurethane	ND	0.42 ± 1.00	1.71 ± 0.60
Flume Tank			
1) slanted front, stainless steel	1.92 ± 1.01	0.27 ± 1.21	1.52 ± 0.21
2) left side, interior wall, 2' from start, stainless steel	-0.79 ± 0.87	-1.14 ± 0.08	-0.06 ± 1.21

Table AI.3 (cont'd)

2) right side, interior wall, 2' from start, stainless steel	-0.09 ± 1.09	-1.14 ± 0.08	-0.22 ± 1.27
2) left side, interior wall, 4' from start, stainless steel	-0.61 ± 0.79	-1.14 ± 0.08	-0.47 ± 0.64
2) bottom, 4' from front, stainless steel	-0.53 ± 0.95	-0.71 ± 0.64	0.13 ± 0.92
2) right side, interior wall, 4' from start, stainless steel	-1.18 ± 0.00	-1.14 ± 0.08	-0.15 ± 1.41
2) left side, interior wall, 6' from start, stainless steel	-1.18 ± 0.00	-1.14 ± 0.08	-0.41 ± 1.18
2) right side, interior wall, 6' from start, stainless steel	-0.66 ± 1.16	-1.14 ± 0.08	-0.70 ± 1.06
2) left interior wall, 8' from start, stainless steel	-0.63 ± 1.21	-1.14 ± 0.08	-0.80 ± 0.84
2) bottom, 8' from start, stainless steel	-0.55 ± 0.86	-0.91 ± 0.51	-0.09 ± 1.26
2) right interior wall, 8' from start, stainless steel	-0.84 ± 0.75	-1.14 ± 0.08	-1.18 ± 0.00
Shaker table			
1) front, textured, stainless steel	-0.29 ± 1.28	-0.79 ± 0.78	-0.08 ± 0.65
2) perforated dewatering screen, stainless steel	-0.36 ± 1.27	0.03 ± 0.80	0.52 ± 0.96
3) 1' from start, textured, stainless steel	-0.33 ± 1.33	0.22 ± 0.84	0.85 ± 0.49
3) left side, end, textured, stainless steel	0.01 ± 1.19	-0.27 ± 0.92	0.61 ± 1.01
4) end (vertical portion), stainless steel	-0.51 ± 0.98	-0.81 ± 0.72	-0.31 ± 0.80

Table AI.3 (cont'd)

3) right side, end, textured, stainless steel	0.46 ± 1.08	0.36 ± 0.97	0.35 ± 1.42
3) end (slanted portion), stainless steel	0.11 ± 1.32	-1.14 ± 0.08	0.15 ± 1.55
5) left side, recirculation spout, stainless steel	-0.76 ± 0.93	-0.85 ± 0.74	-0.32 ± 1.20
6) right side, recirculation spout, stainless steel	-1.18 ± 0.00	-1.14 ± 0.08	-0.14 ± 1.43
Dewatering Centrifuge			
1) interior basket carrier, stainless steel	0.74 ± 0.34	0.75 ± 1.08	1.30 ± 0.61
1) interior basket carrier, stainless steel	0.43 ± 0.99	0.53 ± 1.44	0.93 ± 0.54
1) interior basket carrier, stainless steel	0.63 ± 0.11	0.56 ± 1.11	0.88 ± 0.57
1) interior basket carrier, stainless steel	0.26 ± 0.87	0.44 ± 1.04	0.70 ± 1.11
2) left side, interior wall, stainless steel	-0.19 ± 0.94	-0.09 ± 0.92	0.30 ± 0.91
2) right side, interior wall, stainless steel	0.24 ± 0.89	-0.14 ± 0.80	-0.22 ± 0.88
3) viewing window in the lid, plastic	-0.94 ± 0.53	-1.00 ± 0.00	-0.10 ± 1.06
4) drain, stainless steel	0.80 ± 1.18	1.79 ± 0.59	1.71 ± 0.47

Table AI.4: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces after processing 22.7 kg of leafy greens inoculated at 6 log CFU/g followed by 90.8 kg of uninoculated leafy greens (n=3)*

Location on Processing Equipment	Mean ± SD *E. coli* O157:H7 (log CFU/100 cm^2)	
	Romaine Lettuce	Iceberg Lettuce
Shredder		
1) front left, stainless steel	3.30 ± 2.01	2.87 ± 0.39
1) front right, stainless steel	4.05 ± 0.89	3.74 ± 0.22
2) left side, feed belt, plastic	1.29 ± 0.51	2.60 ± 0.32
2) right side, feed belt, plastic	1.00 ± 0.00	3.93 ± 0.34
3) front left feed belt guide, stainless steel	1.45 ± 0.78	4.19 ± 0.92
3) front right feed belt guide, stainless steel	1.57 ± 0.99	4.84 ± 0.34
3) end, left side, feed belt guide, stainless steel	1.58 ± 0.51	3.28 ± 0.81
3) end, right side, feed belt guide, stainless steel	1.74 ± 0.69	3.31 ± 1.07
4) below the cutting wheel, stainless steel	3.51 ± 1.02	5.82 ± 0.72
5) cutting wheel, stainless steel	3.92 ± 0.59	5.20 ± 0.31
6) discharge chute, stainless steel	4.84 ± 0.81	4.53 ± 0.58

Table AI.4 (cont'd)

6) discharge chute, stainless steel	3.05	±	2.05	1.95	±	0.44
6) discharge chute, stainless steel	4.35	±	0.83	2.31	±	0.33
6) discharge chute, stainless steel	3.81	±	0.95	3.13	±	0.56
Conveyer Belt						
1) conveyer belt, polyurethane	4.59	±	0.77	2.71	±	0.72
1) conveyer belt, polyurethane	4.80	±	0.34	2.76	±	0.42
2) bottom, left side, belt guide, plastic	4.60	±	0.46	2.54	±	0.52
2) bottom, right side, belt guide, plastic	4.43	±	0.77	1.11	±	0.40
2) center, left side, belt guide, plastic	3.23	±	0.39	1.67	±	0.13
2) center, right side, belt guide, plastic	3.66	±	0.56	2.77	±	0.59
1) top of the conveyer belt shelf, polyurethane	5.20	±	0.18	1.61	±	0.32
1) bottom of the conveyer belt shelf, polyurethane	4.25	±	1.27	4.34	±	0.13
Flume Tank						
1) slanted front, stainless steel	4.19	±	0.79	3.80	±	0.19
2) left side, interior wall, 2' from start, stainless steel	1.69	±	0.14	2.80	±	0.62

Table AI.4 (cont'd)

2) right side, interior wall, 2' from start, stainless steel	0.85	±	1.60	1.98	±	0.26
2) left side, interior wall, 4' from start, stainless steel	0.30	±	1.17	4.50	±	0.07
2) bottom, 4' from front, stainless steel	1.61	±	0.33	3.98	±	0.29
2) right side, interior wall, 4' from start, stainless steel	-0.22	±	0.68	3.96	±	0.37
2) left side, interior wall, 6' from start, stainless steel	0.99	±	0.45	3.60	±	0.27
2) right side, interior wall, 6' from start, stainless steel	0.51	±	0.35	3.26	±	0.28
2) left interior wall, 8' from start, stainless steel	0.28	±	0.17	2.53	±	0.44
2) bottom, 8' from start, stainless steel	1.47	±	0.37	4.79	±	0.15
2) right interior wall, 8' from start, stainless steel	0.61	±	0.51	3.21	±	0.28
Shaker table						
1) front, textured, stainless steel	2.23	±	0.20	3.58	±	0.29
2) perforated dewatering screen, stainless steel	2.71	±	0.22	1.56	±	0.01
3) 1' from start, textured, stainless steel	2.68	±	0.24	1.08	±	0.05
3) left side, end, textured, stainless steel	2.39	±	0.41	0.59	±	0.16
4) end (vertical portion), stainless steel	2.60	±	0.46	1.22	±	0.49

Table AI.4 (cont'd)

3) right side, end, textured, stainless steel	2.93	±	0.16	-0.63	±	0.14
3) end (slanted portion), stainless steel	1.88	±	0.50	1.44	±	0.39
5) left side, recirculation spout, stainless steel	0.68	±	0.46	-0.68	±	0.15
6) right side, recirculation spout, stainless steel	0.24	±	1.15	-0.39	±	0.39
Dewatering Centrifuge						
1) interior basket carrier, stainless steel	3.30	±	2.01	1.01	±	0.63
1) interior basket carrier, stainless steel	4.05	±	0.89	0.82	±	1.16
1) interior basket carrier, stainless steel	1.29	±	0.51	1.43	±	0.24
1) interior basket carrier, stainless steel	1.00	±	0.00	2.47	±	0.28
2) left side, interior wall, stainless steel	1.45	±	0.78	2.24	±	0.55
2) right side, interior wall, stainless steel	1.57	±	0.99	2.08	±	0.51
3) viewing window in the lid, plastic	1.58	±	0.51	2.03	±	0.29
4) drain, stainless steel	1.74	±	0.69	2.26	±	0.39

Table AI.5: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces after processing 22.7 kg of leafy greens inoculated at 4 log CFU/g followed by 90.8 kg of uninoculated leafy greens (n=3)*

Location on Processing Equipment	Mean ± SD *E. coli* O157:H7 (log CFU/100 cm^2)	
	Romaine Lettuce	Iceberg Lettuce
Shredder		
1) front left, stainless steel	0.77 ± 0.59	0.77 ± 0.59
1) front right, stainless steel	-0.02 ± 0.90	-0.02 ± 0.90
2) left side, feed belt, plastic	0.34 ± 1.19	0.34 ± 1.19
2) right side, feed belt, plastic	1.60 ± 0.80	1.60 ± 0.80
3) front left feed belt guide, stainless steel	0.30 ± 1.22	0.30 ± 1.22
3) front right feed belt guide, stainless steel	-0.17 ± 1.43	-0.17 ± 1.43
3) end, left side, feed belt guide, stainless steel	-0.67 ± 0.58	-0.67 ± 0.58
3) end, right side, feed belt guide, stainless steel	-1.00 ± 0.00	-1.00 ± 0.00
4) below the cutting wheel, stainless steel	1.00 ± 0.63	1.00 ± 0.63
5) cutting wheel, stainless steel	1.00 ± 0.50	1.00 ± 0.50
6) discharge chute, stainless steel	2.08 ± 0.59	2.08 ± 0.59

Table AI.5 (cont'd)

6) discharge chute, stainless steel	1.42	±	0.37	1.42	±	0.37
6) discharge chute, stainless steel	-0.04	±	0.94	-0.04	±	0.94
6) discharge chute, stainless steel	-0.67	±	0.58	-0.67	±	0.58
Conveyer Belt						
1) conveyer belt, polyurethane	2.07	±	0.95	2.07	±	0.95
1) conveyer belt, polyurethane	1.61	±	0.85	1.61	±	0.85
2) bottom, left side, belt guide, plastic	1.88	±	0.33	1.88	±	0.33
2) bottom, right side, belt guide, plastic	1.52	±	0.23	1.52	±	0.23
2) center, left side, belt guide, plastic	0.81	±	0.53	0.81	±	0.53
2) center, right side, belt guide, plastic	-0.41	±	1.03	-0.41	±	1.03
1) top of the conveyer belt shelf, polyurethane	1.85	±	0.52	1.85	±	0.52
1) bottom of the conveyer belt shelf, polyurethane	1.88	±	0.97	1.88	±	0.97
Flume Tank						
1) slanted front, stainless steel	2.23	±	0.31	2.23	±	0.31
2) left side, interior wall, 2' from start, stainless steel	-0.29	±	0.78	-0.29	±	0.78

Table AI.5 (cont'd)

2) right side, interior wall, 2' from start, stainless steel	-0.78	±	0.68	-0.78	±	0.68
2) left side, interior wall, 4' from start, stainless steel	-0.68	±	0.85	-0.68	±	0.85
2) bottom, 4' from front, stainless steel	-0.11	±	1.02	-0.11	±	1.02
2) right side, interior wall, 4' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
2) left side, interior wall, 6' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
2) right side, interior wall, 6' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
2) left interior wall, 8' from start, stainless steel	-0.68	±	0.85	-0.68	±	0.85
2) bottom, 8' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
2) right interior wall, 8' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
Shaker table						
1) front, textured, stainless steel	0.10	±	0.17	0.10	±	0.17
2) perforated dewatering screen, stainless steel	0.64	±	0.19	0.64	±	0.19
3) 1' from start, textured, stainless steel	-0.09	±	0.95	-0.09	±	0.95
3) left side, end, textured, stainless steel	0.10	±	1.11	0.10	±	1.11
4) end (vertical portion), stainless steel	0.26	±	0.24	0.26	±	0.24

Table AI.5 (cont'd)

3) right side, end, textured, stainless steel	0.44	±	0.42	0.44	±	0.42
3) end (slanted portion), stainless steel	-0.68	±	0.85	-0.68	±	0.85
5) left side, recirculation spout, stainless steel	-1.18	±	0.00	-1.18	±	0.00
6) right side, recirculation spout, stainless steel	-1.18	±	0.00	-1.18	±	0.00
Dewatering Centrifuge						
1) interior basket carrier, stainless steel	0.77	±	0.59	0.77	±	0.59
1) interior basket carrier, stainless steel	-0.02	±	0.90	-0.02	±	0.90
1) interior basket carrier, stainless steel	0.34	±	1.19	0.34	±	1.19
1) interior basket carrier, stainless steel	1.60	±	0.80	1.60	±	0.80
2) left side, interior wall, stainless steel	0.30	±	1.22	0.30	±	1.22
2) right side, interior wall, stainless steel	-0.17	±	1.43	-0.17	±	1.43
3) viewing window in the lid, plastic	-0.67	±	0.58	-0.67	±	0.58
4) drain, stainless steel	-1.00	±	0.00	-1.00	±	0.00

Table AI.6: E. coli *O157:H7 populations (mean ± SD) on equipment surfaces after processing 22.7 kg of leafy greens inoculated at 2 log CFU/g followed by 90.8 kg of uninoculated leafy greens (n=5)*

Location on Processing Equipment	Mean ± SD *E. coli* O157:H7 (log CFU/100 cm^2)	
	Romaine Lettuce	Iceberg Lettuce
Shredder		
1) front left, stainless steel	-0.19 ± 0.99	-1.18 ± 0.00
1) front right, stainless steel	0.02 ± 1.13	-0.80 ± 0.84
2) left side, feed belt, plastic	-0.57 ± 0.87	-0.71 ± 0.64
2) right side, feed belt, plastic	-0.82 ± 0.80	-1.18 ± 0.00
3) front left feed belt guide, stainless steel	-0.17 ± 1.51	-1.18 ± 0.00
3) front right feed belt guide, stainless steel	-0.53 ± 0.90	-1.18 ± 0.00
3) end, left side, feed belt guide, stainless steel	-1.18 ± 0.00	-1.18 ± 0.00
3) end, right side, feed belt guide, stainless steel	-0.71 ± 0.64	-1.18 ± 0.00
4) below the cutting wheel, stainless steel	0.01 ± 1.24	-1.18 ± 0.00
5) cutting wheel, stainless steel	-0.85 ± 0.74	-1.18 ± 0.00
6) discharge chute, stainless steel	0.99 ± 1.28	-0.94 ± 0.53

Table AI.6 (cont'd)

6) discharge chute, stainless steel	1.01	±	0.98	-0.41	±	1.06
6) discharge chute, stainless steel	0.60	±	1.25	-0.94	±	0.53
6) discharge chute, stainless steel	-0.72	±	1.02	-1.18	±	0.00
Conveyer Belt						
1) conveyer belt, polyurethane	0.85	±	1.26	0.02	±	0.74
1) conveyer belt, polyurethane	1.04	±	0.16	-0.55	±	0.86
2) bottom, left side, belt guide, plastic	1.09	±	0.60	-0.76	±	0.93
2) bottom, right side, belt guide, plastic	0.96	±	0.36	-1.18	±	0.00
2) center, left side, belt guide, plastic	-0.88	±	0.66	-1.18	±	0.00
2) center, right side, belt guide, plastic	-0.40	±	1.07	-1.18	±	0.00
1) top of the conveyer belt shelf, polyurethane	0.18	±	0.92	-0.57	±	0.87
1) bottom of the conveyer belt shelf, polyurethane	-0.51	±	0.98	-1.18	±	0.00
Flume Tank						
1) slanted front, stainless steel	0.37	±	0.96	-0.71	±	0.64
2) left side, interior wall, 2' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00

Table AI.6 (cont'd)

2) right side, interior wall, 2' from start, stainless steel	-0.88	±	0.66	-1.18	±	0.00
2) left side, interior wall, 4' from start, stainless steel	-0.94	±	0.53	-1.18	±	0.00
2) bottom, 4' from front, stainless steel	-1.18	±	0.00	-1.18	±	0.00
2) right side, interior wall, 4' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
2) left side, interior wall, 6' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
2) right side, interior wall, 6' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
2) left interior wall, 8' from start, stainless steel	-0.88	±	0.66	-1.18	±	0.00
2) bottom, 8' from start, stainless steel	-0.88	±	0.66	-1.18	±	0.00
2) right interior wall, 8' from start, stainless steel	-1.18	±	0.00	-1.18	±	0.00
Shaker table						
1) front, textured, stainless steel	-0.53	±	0.90	-1.18	±	0.00
2) perforated dewatering screen, stainless steel	-0.53	±	0.95	-1.18	±	0.00
3) 1' from start, textured, stainless steel	-0.42	±	1.05	-1.18	±	0.00
3) left side, end, textured, stainless steel	-0.76	±	0.93	-0.94	±	0.53
4) end (vertical portion), stainless steel	-0.40	±	1.08	-0.94	±	0.53

Table AI.6 (cont'd)

3) right side, end, textured, stainless steel	-0.55	±	1.39	-1.18	±	0.00
3) end (slanted portion), stainless steel	-1.18	±	0.00	-1.18	±	0.00
5) left side, recirculation spout, stainless steel	-1.18	±	0.00	-1.18	±	0.00
6) right side, recirculation spout, stainless steel	-1.18	±	0.00	-1.18	±	0.00
Dewatering Centrifuge						
1) interior basket carrier, stainless steel	-0.19	±	0.99	-1.18	±	0.00
1) interior basket carrier, stainless steel	0.02	±	1.13	-0.80	±	0.84
1) interior basket carrier, stainless steel	-0.57	±	0.87	-0.71	±	0.64
1) interior basket carrier, stainless steel	-0.82	±	0.80	-1.18	±	0.00
2) left side, interior wall, stainless steel	-0.17	±	1.51	-1.18	±	0.00
2) right side, interior wall, stainless steel	-0.53	±	0.90	-1.18	±	0.00
3) viewing window in the lid, plastic	-1.18	±	0.00	-1.18	±	0.00
4) drain, stainless steel	-0.71	±	0.64	-1.18	±	0.00

APPENDIX II

Isolation and Identification of Gas-Producing Yeasts from Maraschino Cherries

Isolation and Identification of Gas-Producing Yeasts from Maraschino Cherries

By

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AII.1 ABSTRACT

Maraschino cherry production is a time-consuming and complex process with gas identified as an occasional quality defect. The objective of this study was to identify the specific yeasts responsible for gas production in jars of maraschino cherries. Thirty-eight samples of commercial maraschino cherry syrup were collected late in the production process and examined for yeasts by enriching in carbohydrate purple broth containing 1% high fructose corn syrup or corn syrup (pH 6.18 - 6.52), 50% aqueous cherry syrup (pH 3.66-3.81), and 50% cherry syrup diluted 1:1 in trypticase soy broth containing 0.6% yeast extract (Cherry:TSB-YE; pH 6.19 - 6.66). Yeasts were isolated on trypticase soy agar containing 0.6% yeast extract (TSA-YE), deMan-Rogosa-Sharpe agar (MRS), and potato dextrose agar (PDA) (48 h/28°C). Gas production from selected isolates was confirmed in the previous enrichment media using Durham tubes with each yeast isolate biochemically identified using API 20 C AUX test strips and through PCR. Eighteen of the 38 samples yielded gas-producing yeasts. Seventeen isolates were identified through PCR as *Zygosaccharomyces bailii* with one isolate identified as *Clavispora lusitaniae*. Mean D_{10}-values at 60 °C of these three yeasts ranged from 4.3 to 6.9 seconds, indicating adequacy of the current pasteurization treatment

AIII.2 INTRODUCTION

By definition, maraschino cherries are cherries that have been "dyed red, impregnated with sugar and packed in a sugar syrup flavored with oil of bitter almonds or a similar flavor" (FDA 1980). Maraschino cherry production begins with the bleaching of fresh sweet cherries in a brine solution containing 0.75 to 1.5% sulfur dioxide and 3000 to 5000 ppm calcium salts for 6 to 18 months. These bleached yellow cherries are then pitted, soaked in water to leach out the brine and impregnated with 40° Brix sugar syrup containing artificial colors and flavors (Nordlee and others 1985). After jarring cherries with or without pasteurization, the finished product is typically shelf-stable for a minimum of 3 years. While the microbiological safety of maraschino cherries has remained a non-issue, gas production of unknown origin is an occasional quality defect observed in jars of finished product.

AII.3 MATERIALS AND METHODS

AII.3.1 Sample collection. Twenty-four samples of commercial maraschino cherry syrup and three yellow cherry samples were collected in Whirl-Pak™ bags at different points during maraschino cherry production in a large-scale processing plant. These samples, along with eleven maraschino cherry jar samples (2 prior to pasteurization and 9 post-pasteurization) were transported to Michigan State University during a period from October, 2008 to May, 2009.

AII.3.2 Enrichment. Using Durham tubes for gas detection, 1 ml original sample aliquots were enriched in 12 ml of carbohydrate purple broth (Difco, Becton Dickinson, Sparks, MD) (CPB) + 1% high fructose corn syrup, CPB + 1% corn syrup (pH 6.18 - 6.52), 50% aqueous cherry syrup (provided by the maraschino cherry processor) diluted 1:1 in distilled water (pH 3.66 - 3.81), and 50% cherry syrup diluted 1:1 in trypticase soy broth (Difco, Becton Dickinson) + 0.6% yeast extract (Difco, Becton Dickinson) (Cherry:TSB-YE; pH 6.19 - 6.66). All tubes were incubated at 28 °C and visually examined for gas production during 30 days of incubation at 28 °C.

AII.3.4 Isolation. A 10 µl aliquot from enrichments producing gas was streaked to plates of trypticase soy agar (Difco, Becton Dickinson) + 0.6% yeast extract (TSA-YE), deMan-Rogosa-Sharpe agar (Oxoid, Oxoid Ltd, Cambridge, UK) (MRS), and potato dextrose agar (Difco, Becton Dickinson) (PDA) and incubated for 48 h at 28 °C. Three colonies per plate were streaked on the initial culture medium (TSA-YE, MSR or PDA) in duplicate to obtain pure isolates. These isolates were then inoculated into tubes of the same media to identify gas-producing isolates.

AII3.5 Biochemical identification of yeast isolates. All gas-producing isolates initially classified as yeasts based on morphology and Gram-staining were biochemically speciated in duplicate using API 20 C AUX test strips following the detailed usage instructions provided by bioMérieux, Inc. Presence of hyphae was confirmed through the method recommended by bioMérieux, Inc. Isolates were streaked on one quadrant of a corn meal agar (CMA) w/ 1% Tween 80 plate, in 2 - 3 parallel lines a few mm apart, an 'S' was then streaked over these lines in a dollar sign configuration. A glass cover slip was placed over the streaked lines and the plates were incubated at room temperature (22 ± 2 °C) for 3 - 7 days. Using the 40X objective placed directly on the glass cover slip, hyphae presence or absence was observed first at the crossroads of the streaks in the center and moving from the center toward the outer edge. One isolate from each species identified with the API 20 C AUX test strips was selected for confirmation by sequencing and further analysis.

AII3.6 Identification of yeast isolates through sequencing. Yeast colonies were grown for 1 day in YEPD (1% yeast extract, 2% peptone, 2% glucose) at 30 °C. The overnight liquid cultures (1.5 ml) were centrifuged at 14,000 rpm for 4 min and the yeast DNA was then extracted using DNA-Pure yeast genomic kit, from CPG Inc. Pure Biotech Product (Lincoln Park, NJ). DNA was amplified by PCR using the primers;

NL1= 5¹-ATA TCA ATA AGC GGA GGA AAA G-3¹

NL4=5¹-GGT CCG TGT TTC AAG ACG G-3¹

The PCR cycling parameters were as follows: 1 cycle at 94 °C for 5 minutes, 36 cycles of 1 minute at 94 °C, 1 minute at 52°C, and 2 minutes at 72 °C., 1 cycle for final elongation of 10 minutes at 72°C. The PCR product was loaded onto a 0.7% agarose gel

and run at 75 volts and after 30 minutes, the DNA band was cut from the gel and extracted using Qiaquick gel extraction kit (Valencia, CA). The samples were then sequenced using the NL1 and NL4 primers at the Biotechnology Resource Center (Cornell University) and the nucleotide sequence was searched for homology using the NCBI Blast data bank for identity.

AII.3.7 Gas confirmation during small-scale maraschino cherry production. Two lots of commercially brined and leached frozen yellow cherries were obtained from a large-scale maraschino processor. The small-scale (Figure AII.1) maraschino cherry production system comprised of 2 containers connected by the tube with a tap for flow control. Container 1 was placed ~24 inches lower than Container 2. The frozen yellow cherries were placed in Container 1 fill it with water to the top leaving no air space. The cherries defrosted with tap water ~3.5ml/min for a few hours and the temperature was monitored to determine when the products had thawed completely. The flow was halted and add the commercial sugar syrup (40 °Brix) was added to Container 2. The syrup was inoculated to have the same species of gas-producing yeasts identified with the API 20 C AUX test strips at a level of ~10^5 CFU/ml. Syrup was allowed to flow from Container 2 into Container 1 until the air in the system was completely removed from the outlet tube connected with the top of the Container 1. The tap was adjusted so the liquid in Container 1 would flow out at ~ 20 ml/hr. or 0.33 ml/min from the outlet tube. The outlet tube was used to determine if gas was being produced during the conversion. The °Brix in the outflow was monitored to determine when the maraschino cherries were finished – once the sugar concentration of the outlet syrup increased from 0 °Brix to 40 °Brix over 3 - 5

days at ambient temperature (22 ± 1 °C). The system can run continuously. The lab-scale experiment was done in duplicate.

AII.3.8 Survival curve for yeast isolates. Maraschino cherry syrup was diluted to achieve ratios of 1:7, 1:3, 1:1 and 3:1 commercial cherry syrup (OCCS) to distilled water (DW). 10 µl aliquots of the three yeast isolates (10^7 CFU/ml) were transferred into individual autoclaved test tubes with Durham tubes containing 12 ml of diluted or undiluted cherry syrup (Table AII.1). The tubes were observed twice daily for gas production. Using the same OCCS to DW ratios, 50 ml sterile centrifuge tubes were filled with 12 ml sterile cherry syrup spiked individually with 10 µl of the three yeast isolates to determine generation time at ambient temperature (22 ± 1 °C). Daily or every two days, a 100 µl aliquot was removed from each tube to determine yeast generation time. Samples were appropriately diluted in sterile 1% (w/v) phosphate buffer (8.5 g/L NaCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4, J.T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ) (PBS) and plated on PDA. Following 48 h of incubation at 28°C, all colonies resembling yeasts were counted to obtain the CFU/ml at that specific time point. Each experiment was replicated in duplicate.

AII.3.9 Thermal inactivation. Thermal resistance of three gas-producing yeasts was assessed in triplicate using a series of screw-capped 50 ml sterile centrifuge tubes containing 10 ml of autoclaved 40 °Brix syrup tempered to 55, 58 or 60 °C in a water bath. These submerged tubes were individually inoculated to contain ~10^6 CFU/ml of each gas-producing yeast, removed at five predetermined intervals and rapidly cooled to < 36 °C in a ice water bath. A 1 ml aliquot from each tube was appropriately diluted and

plated on PDA to quantify survivors. D_{10}-values and Z-values were calculated from the semi-log survivor curve.

AII3.10 Laboratory pasteurization. Maraschino cherry jars (225 ml) with 24 months of shelf life at ambient temperature, obtained from a large-scale cherry processor, were tempered in a water bath in duplicate until the core temperature of each jar reached either 60 or 71.1 °C. The lids of the jar samples were removed and each jar was individually inoculated with a 1 ml aliquot of one of three yeast isolate suspensions containing ~10^8/ml. New sterile lids replaced the old lids. The jars were removed from the water bath and placed immediately into ice water, dropping the internal temperature to < 36 °C within 40 seconds. Jars were stored at ambient temperature (22±1 °C), and checked daily for a popped lid, indicating gas in the sample.

AII.3.11 Statistical analysis. The generation time (hours) of yeast = {the incubation period (minutes)/[(log CFU/ml at end of incubation – log CFU/ml at beginning of incubation)/0.301]}/60. In order to correlate the syrup's sugar concentration or pH-value with the minimum generation time of the yeasts, the minimum generation time was converted to log (minimum generation time) and submitted to Spearman's correlation using SAS statistical software (Statistical Analysis System; SAS Institute Incorporation, Campus Drive Cary, NC, USA). The Student's t-test was applied to determine if there were significant differences between mean D-values of three yeasts. For all tests, $\alpha = 0.05$.

AII.4 RESULTS

AII.4.1 Isolation of gas-producing yeasts. Eighteen (Table AII.2) of the 38 samples yielded gas-producing yeasts based on cellular morphology. Yeasts that fit the selection criteria were round, white colonies with fringed edges, appearing on the streaked plates after 48h incubation at 28 °C, Gram-positive and were positive for Durham tube gas production. These same yeast isolates produced gas following re-inoculation into enrichment media with faster gas production seen in Cherry:TSB-YE compared to the other broths. Production of gas was visible one day after inoculation in Cherry:TSB-YE, two days after inoculation in CPB + 1% high fructose corn syrup or CPB + 1% corn syrup, and three days after inoculation in 50% aqueous cherry syrup diluted 1:1 in distilled water.

AII.4.2 Identification of yeast isolates. Gas-producing yeast isolates from sixteen samples were identified as *Candida krusei/inconspicua* (33.9% ID) with one isolate each of *Candida magnoliae* (62.4% ID) and *Candida lusitaniae/famata* (*C. lusitaniae* 74.6 % ID, *C. famata* 59.1% ID) by the API 20 C AUX test strips. One representative, Y1, was selected from the sixteen isolates identified as *Candida krusei/inconspicua*, and used in further testing to confirm gas during lab-scale maraschino cherry production, survivor curve development and thermal inactivation in cherry syrup. *C. magnoliae* and *C. lusitaniae/famata* labeled Y2 and Y3, respectively, also underwent further analysis.

Hyphae appeared on the outer edge of Y1 and Y2 colonies on PDA plates after ten days stored at ambient temperature (22 ± 1 °C). These findings indicate identifications may be different when using other media or longer incubation times for detection of hyphae/pseudo-hyphae than those recommended by bioMérieux, Inc. If the results of the

Y1 and Y2 hyphae test were positive, Y1 would have been identified as *Candida norvegensis* (ID 43.8 %) and Y2 as *Geotrichum capitatum* (ID 64.4 %). Percent confidence in the identification for the API 20 C AUX test strip results was small, implying that other test methods would be needed for further identification.

After comparing the yeast isolates nucleotide sequences to the NCBI Blast Database, Y1 and Y2 were both identified as *Zygosaccharomyces bailii*. Isolate Y3 was identified as *Clavispora lusitaniae*.

Y1 and Y2 only grew on PDA and MRS, with similar colony and cellular morphology, Gram reaction and the time needed for gas-production in maraschino cherry syrup. Y3 grew on all three plating media and produced larger colonies than the other yeast isolates. Gas production was slowest for Y3.

Of thirty-eight samples, Y1 was isolated from fourteen of twenty-three maraschino cherry syrup samples and two maraschino jar samples before pasteurization. Y2 was found in just one maraschino cherry syrup sample, and Y3 was isolated from one of three yellow cherry samples. Yeast was not found in any of the nine pasteurized maraschino cherry jar samples. The results indicated Y1 could survive in products during many stages of cherry production prior to pasteurization and was the most widely distributed yeast causing gas defects (Table AII.2).

AII.4.3 Gas confirmation during small-scale maraschino cherry production. Three yeast isolates were individually inoculated into 40 °Brix commercial cherry syrup (Figure AII.1A) to obtain 5.69 ± 0.34 log CFU/ml Y1, 6.41 ± 0.09 log CFU/ml Y2, and 6.93 ± 0.25 log CFU/ml Y3 in the syrup. During maraschino cherry production, gas was observed in the outlet tube of the container holding the cherries three days after the syrup

had been inoculated. These visual observations confirm that the yeasts originally isolated from the maraschino cherries and syrup are responsible for the observed gas defects.

AII.4.4 Survival curve for yeast isolates. Gas was visible in the Durham tubes six days after inoculation with yeasts (Table AII.3). The time until gas was observed for all three yeasts was the shortest in 21.8 °Brix syrup while the amount of gas production within 20 days was the longest in 31.5 °Brix syrup. Y1 could survive in 50% aqueous cherry syrup for at least 279 days during the trial in the lab. A positive correlation between the syrup's sugar concentration and log minimum generation time was determined through line regression and correlation analysis (Table AII.3). Figure AII.2 shows that the minimum generation time increases with the increase in sugar concentration indicating that the higher concentrations inhibit the growing of yeasts. The negative correlation between syrup pH and log minimum generation time determined through line regression and correlation analysis was not significant ($P > 0.05$, Table AII.3 and Figure AII.3).

The survival curves of the yeast isolates in five different sugar concentrations of maraschino cherry syrup at ambient temperature (22 ± 1 °C) are displayed in Figures AII.4, AII.5, and AII.6. While the biochemical tests resulted in different identifications, the similarities observed in the growth kinetics of Y1 and Y2 agree with the identifications by sequencing and indicate that these two isolates may be the same species.

AII.4.5 Thermal inactivation and laboratory pasteurization. Mean D_{10}-values at 60 °C for the yeast isolates ranged from 4.3 to 6.9 seconds (Table AII.4), suggesting they are unable to survive the post-bottling pasteurization step (160 °F, 5 minutes)

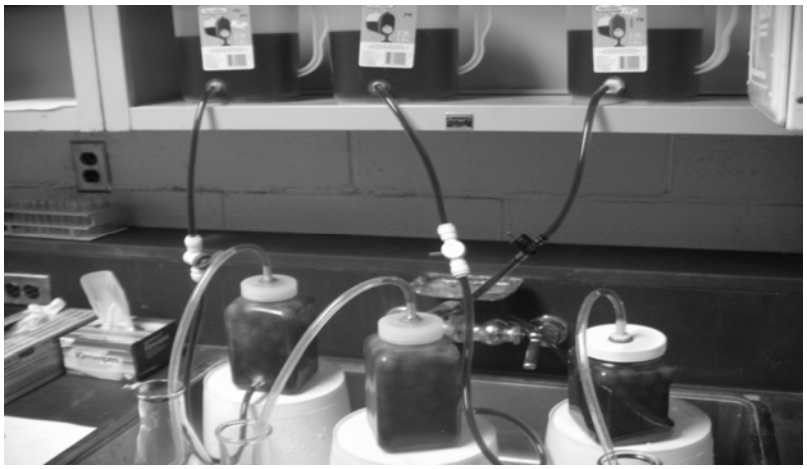
adopted by the maraschino cherry making plant. Although no significant difference ($P > 0.05$) among D_{10}-values of three yeasts at 60 °C was noted, the D_{10}-value of Y3 at 55 °C was higher than D_{10}-values of Y1 and Y2 ($P < 0.05$) indicating Y3 is more thermally resistance than the other two. The Z-value of these yeast isolates ranged from 3.7 to 4.7 °C (Table AII.4).

Following lab scale pasteurization, the lids of the maraschino cherry jars remained depressed even seven months later after pasteurizing. These findings establish that reaching the critical central temperature needed to decrease the yeast populations present in cherries during manufacture is one important step in producing a final product of high quality.

AII.5 FIGURES

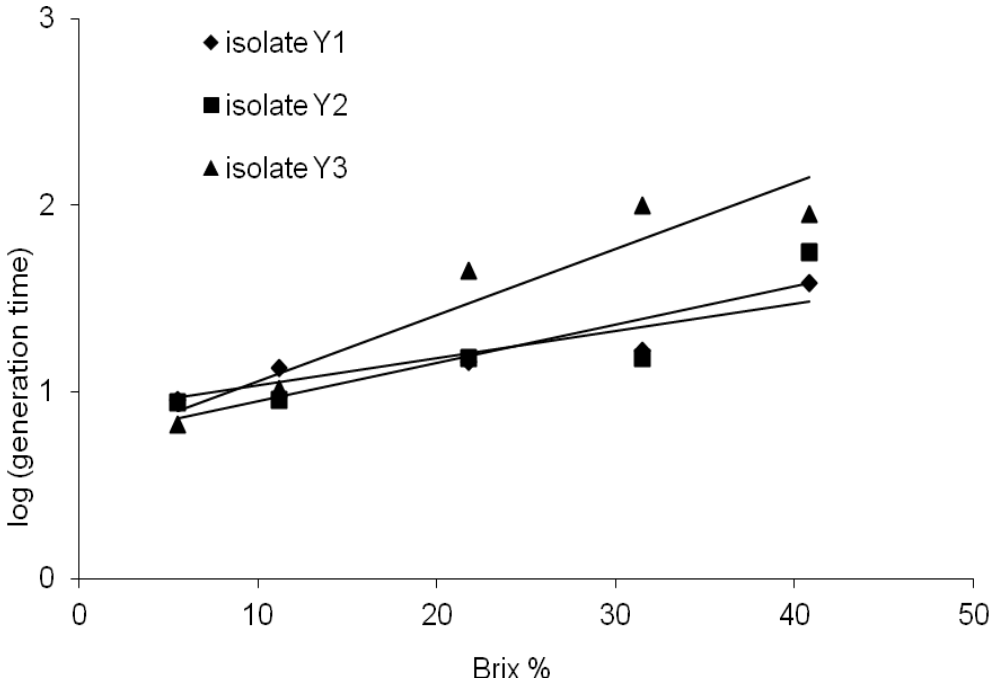
Figure AII.1: *Maraschino cherry production: (A) laboratory-scale set-up and (B) gas visible in the outlet tube*

A



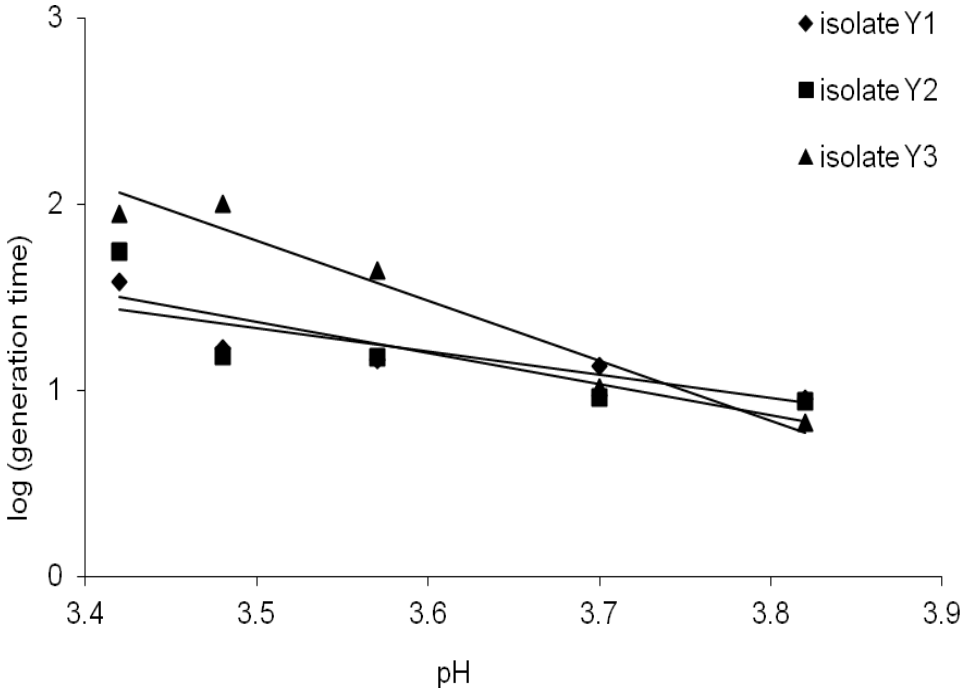
B

Figure AII.2: *The correlation between syrup concentration (°Brix) and generation time of yeast isolates* [a]



[a] The line was fit to Y1 data using the equation $y = 0.8892 + 0.0145x$ where $R^2 = 0.8369$. Y2 data was fit with the linear equation $y = 0.7486 + 0.0205x$ where $R^2 = 0.8135$. A line was fit to the Y3 data using the equation $y = 0.7067 + 0.0353x$ where $R^2 = 0.9015$.

Figure AII.3: *The correlation between pH of syrup and generation time of yeast isolates*



[a] The line was fit to Y1 data using the equation $y = 5.4869 - 1.1859x$ where $R^2 = 0.7122$. Y2 data was fit with the linear equation $y = 7.1695 + 1.6591x$ where $R^2 = 0.7684$. A line was fit to the Y3 data using the equation $y = 13.595 - 3.3673x$ where $R^2 = 0.9638$.

Figure AII.4: *The survival curve of isolate Y1 in different °Brix syrup at ambient temperature*

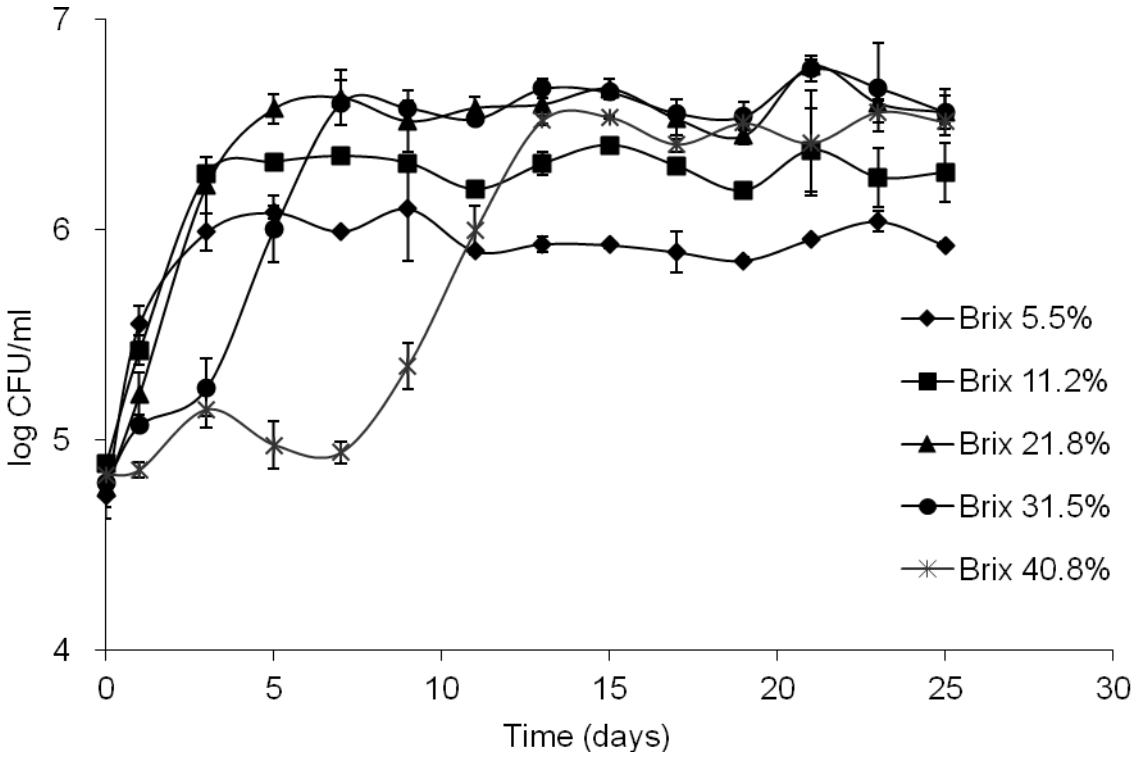


Figure AII.5: *The survival curve of isolate Y2 in different °Brix syrup at ambient temperature*

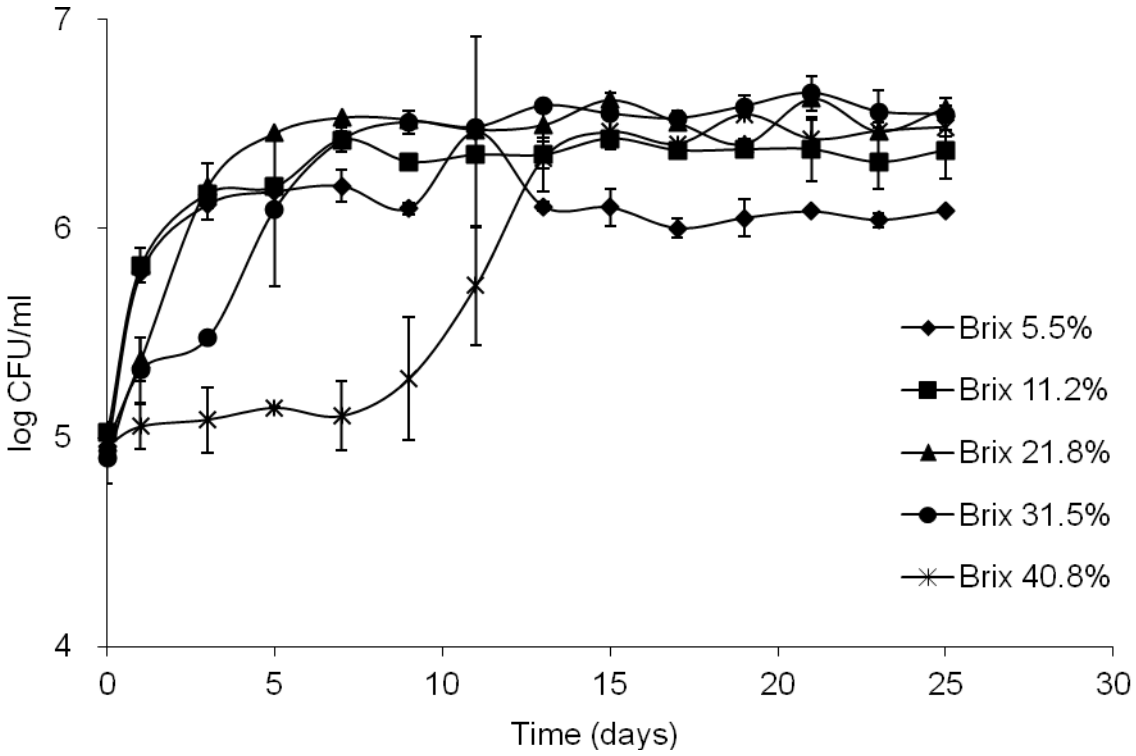
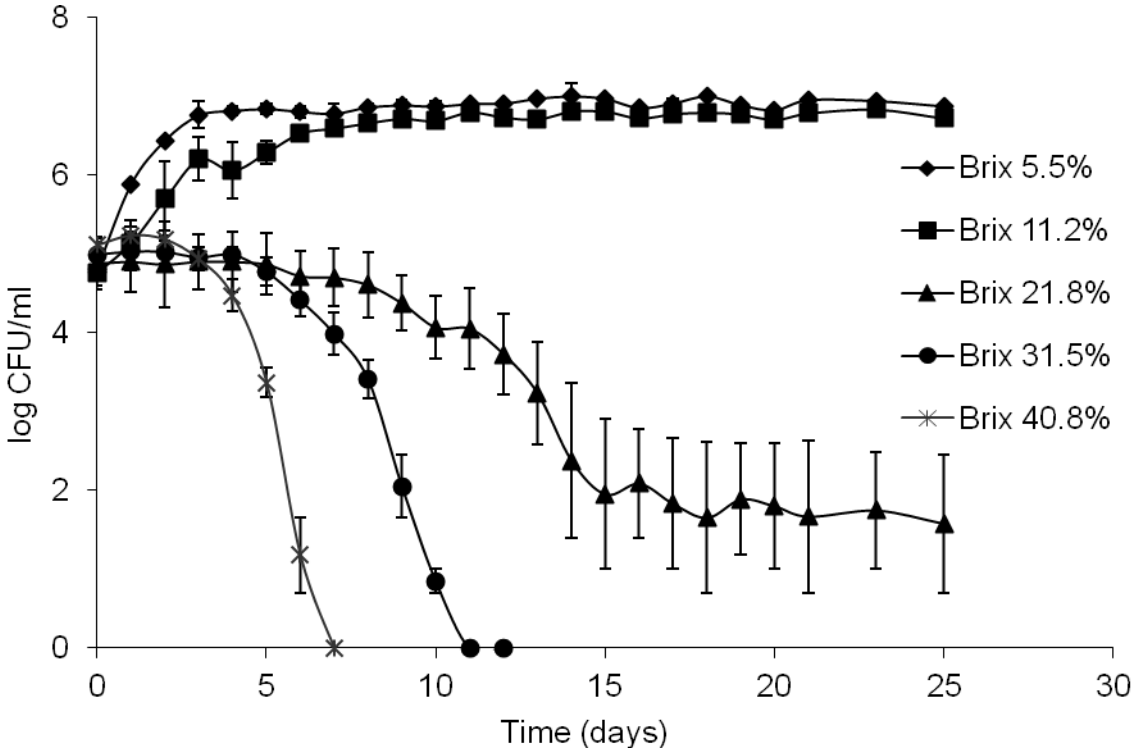


Figure AII.6: *The survival curve of isolate Y3 in different °Brix syrup at ambient temperature*



AII.6 TABLES

Table AII.1: *Sugar concentration (°Brix) and pH-value of cherry syrup*

Sample	OCCS:DW [a]	Sugar concentration (°Brix) [b]		pH	
		Before autoclaving	After autoclaving	Before autoclaving	After autoclaving
1	1:7	5.5	5.6	3.68	3.82
2	1:3	11.2	11.6	3.62	3.70
3	1:1	21.8	22.6	3.50	3.57
4	3:1	31.5	32.7	3.28	3.48
5	No dilution	40.8	41.7	3.32	3.42

[a] During the study, ambient temperature (mean ± SD) was 22.9 ± 0.8 °C and cherry syrup temperature (mean ± SD) was 21.5 ± 0.3 °C

[b] Sugar concentration and pH-value were measured by SPER Digital Refractometer 300017 (SPER Scientific, Scottsdale, AZ) (0 - 45 °Brix) and HANNA Microprocessor pH Meter (Hanna Instruments, Woonsocket, RI), respectively.

Table AII.2: *The number gas-producing yeast positive cherry samples*

Product	Samples collected	Samples positive for gas-producing yeasts
Yellow cherries	3	1
Maraschino cherry syrup	24	15
Cherry jars before pasteurization	2	2
Cherry jars after pasteurization	9	0
Total	*38*	*18*

Table AII.3: *Gas production and minimum generation time in sugar syrup at 22 ± 1 °C within 30 days (n = 2)*

Yeast isolate	°Brix	pH	Gas production [a]	Days until gas production	Minimum generation time (hours)
Y1	5.5	3.82	-	NA	9.06
	11.2	3.70	++	8 - 10	13.52
	21.8	3.57	+++	6 - 8	14.60
	31.5	3.48	++++	7 - 8	16.72
	40.8	3.42	+	14 - 15	20.71
Y2	5.5	3.82	-	NA	8.76
	11.2	3.70	++	8 - 10	9.11
	21.8	3.57	+++	6 - 7	15.14
	31.5	3.48	++++	7 - 8	15.33
	40.8	3.42	+	14 - 15	25.22
Y3	5.5	3.82	-	NA	6.70
	11.2	3.70	+-	9 - 10	10.40
	21.8	3.57	+-	8 - 9	44.45
	31.5	3.48	-	NA	100.00
	40.8	3.42	-	NA	89.50

[a] Gas production was rated on a scale where "++++" represented the greatest gas production followed by "+++", "++", "+" and "+-", and where "-" represented no visible gas production.

Table AII.4: *The D_{10}-values and Z-values of yeasts isolated from maraschino cherry syrup (n = 3)*

Yeast isolate	Temperature (°C)	D_{10}-value (sec.) [a]		Z-value (°C)	R^2
Y1	55	55.1	A	4.7	0.9296
	58	8.6	B		
	60	4.9	B		
Y2	55	64.7	A	4.3	0.9110
	58	20.7	C		
	60	4.3	B		
Y3	55	156.5	D	3.7	0.9949
	58	20.8	C		
	60	6.9	B		

[a] D_{10}-values not labeled with by the same letter are significantly different ($P \leq 0.05$).

APPENDIX III

Pulsed-Field Gel Electrophoresis as a Predictor of *Listeria monocytogenes* Biofilm Formation

Pulsed-Field Gel Electrophoresis as a Predictor of *Listeria monocytogenes* Biofilm Formation

By

Gordon R. Davidson, Annemarie L. Buchholz, Zhinong Yan, and Elliot T. Ryser

AIII.1 ABSTRACT

Persistence and spread of *Listeria monocytogenes* in food manufacturing and retail environments is greatly aided by the ability of this pathogen to form biofilms. While numerous *Listeria* isolates from environmental surveys have been serotyped and subjected to various strain-specific molecular typing methods, relatively few studies have characterized such strains in terms of biofilm formation, which could lead to improved *Listeria* intervention strategies. In this study, the relationship between biofilm formation, serotype and pulsed-field gel electrophoresis (PFGE) type was determined in a set of 30 *L. monocytogenes* environmental isolates from one delicatessen. All 30 *L. monocytogenes* isolates were serotyped using a PCR-based method with five different primer sets and then subjected to PFGE typing using the PulseNet protocol with restriction enzymes *Asc*I and *Apa*I. Biofilm formation was quantified using a standard microtiter plate assay with Modified Welshimer's Broth as the growth medium. After averaging the 9 optical density readings from triplicate wells for each of three replicates, each isolate was classified as a weak, medium or strong biofilm former. Overall, 16, 7, 6 and 1 *L. monocytogenes* isolate belonged to serotypes 1/2b(3b), 1/2a(3a), 4b(d,e) and 4a/c, respectively. A total of 12 PFGE types grouped into three distinct clusters. All 7 strong biofilm-forming strains belonged to serotype 1/2a(3a) and to two closely related

PFGE types containing a unique high kbp doublet using *Apa*I that were only 60.9% similar compared to the remaining 23 isolates. These 7 strong biofilm formers were isolated from multiple locations over 9 months. In contrast, 4 of 15 weak and 2 of 8 medium biofilm-forming strains persisted for 10 months, thereby reinforcing previous correlations between biofilm formation and persistence. These findings demonstrate that PFGE typing can be a predictor of *L. monocytogenes* biofilm formation. However, other factors in addition to biofilm formation are also clearly important in persistence.

AIII.2 INTRODUCTION

In 2002, consumption of *Listeria*-contaminated delicatessen meat was identified as the leading cause of listeriosis in the United States with a recent unpublished survey indicating a contamination rate 7 to 8-times higher for delicatessen- as opposed to manufacture-sliced products. Persistence and spread of *L. monocytogenes* in both retail and foodservice environments is greatly aided by the ability of this pathogen to form biofilms. Hence, 30 *L. monocytogenes* strains previously isolated from a commercial delicatessen and characterized by pulsed-field gel electrophoresis (PFGE) typing were now serotyped and assessed for biofilm formation (Yan and others 2008), which could lead to improved *Listeria* intervention strategies.

AIII.3 MATERIALS AND METHODS

AIII.3.1 Study design. Thirty delicatessen isolates previously identified as *L. monocytogens* were characterized according to serotype, PFGE type and biofilm-forming ability.

AIII.3.2 Serotyping. Five primer sets (D1, D2, GLT, FlaA and MAMA-C) were used to classify the strains into five serotype groups (1/2a(3a), 1/2b(3b), 1/2c(3c), 4b(d,e) and 4a/c) as described by Borucki and Call (2003).

AIII.3.3 DNA isolation and PFGE typing. DNA isolation and PFGE analysis followed the PulseNet protocol developed by CDC (Graves and Swaminathan 2000). Two rare restriction enzymes, *Apa*I and *Asc*I, were used. The PFGE types were obtained by combining both restriction enzyme patterns into one profile. The combined *Asc*I and *Apa*I restriction profiles were analyzed by automated cluster analysis using the DICE coefficient (tolerance of 1.5%) and unweighted pair group method with arithmetic averages (UPGMA). A similarity score value of 95% served as the cutoff.

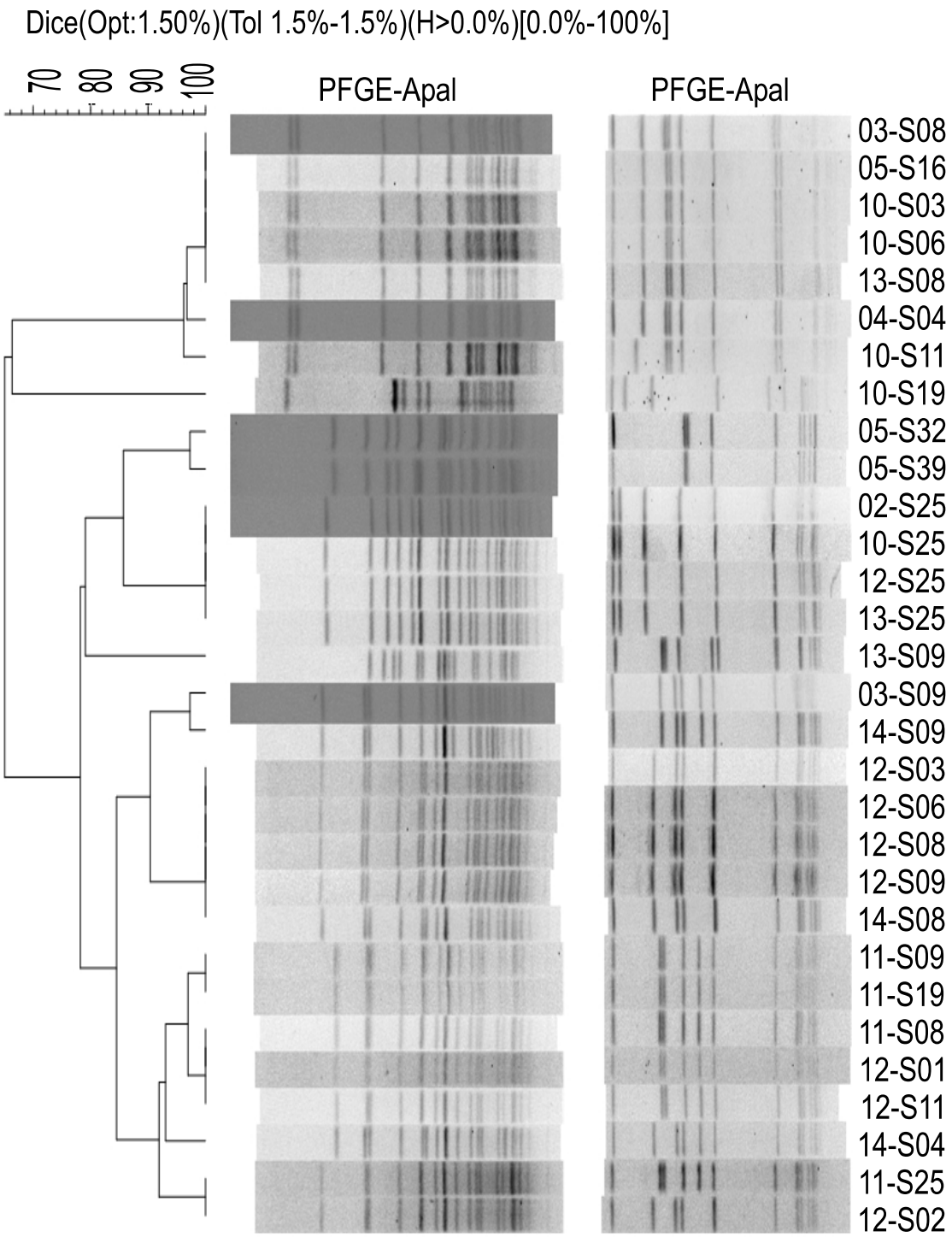
AIII.3.4 Biofilm Formation. Biofilm formation was quantified using a standard microtiter plate assay with Modified Welshimer's Broth inoculated at 10^2 CFU/ml serving as the growth medium (Djordjevic and others 2002). Assays were carried out at 22 ± 2°C for 4 d. Biofilms were stained with crystal violet which was resolubilized in glacial acetic acid. Optical densities were read at 570 nm using a spectrophotometer. After averaging the 9 optical density readings from triplicate wells for each of three replicates, each isolate was arbitrarily classified as a weak (OD < 0.50), medium (OD 0.51 – 0.70) or strong (OD > 0.70) biofilm former.

AIII.4 RESULTS

Overall, 16, 7, 6 and 1 *L. monocytogenes* isolate belonged to serotypes 1/2b(3b), 1/2a(3a), 4b(d,e) and 4a/c, respectively (Table AIII.1). A total of 12 PFGE types grouped into three distinct clusters (Figure AIII.1). Fifteen weak, 8 medium and 7 strong biofilm formers were identified (Table AIII.2). The 7 strong biofilm-forming strains belonged to serotype 1/2a(3a) and to two closely related PFGE types containing a unique high kbp doublet using *Apa*I. These strains were only 60.9% similar compared to the remaining 23 isolates. All 7 strong biofilm formers were isolated from multiple locations over 9 months. In contrast, 4 of 15 weak and 2 of 8 medium biofilm-forming strains persisted for 10 months, thereby reinforcing previous correlations between biofilm formation and persistence.

AIII.5 FIGURES

Figure AIII.1: L. monocytogenes *PFGE patterns obtained using ApaI and AscI*



AIII.6 TABLES

Table AIII.1: L. monocytogenes *serotypes*

Isolate	D1	D2	GLT	FlaA	MAMA-C	Division	Serotype
Control 1	+	-	-	NA	-	I	*4b*
Control 2	-	+	NA	+	NA	II	*1/2a*
Control 3	+	-	+	NA	NA	I	*1/2b*
Control 4	NA	NA	NA	NA	+	III	*4a*
Control 5	NA	NA	NA	NA	+	III	*4a*
2-S25	+	-	-	NA	-	I	4b(d/e)
10-S25	+	-	-	NA	-	I	4b(d/e)
12-S25	+	-	-	NA	-	I	4b(d/e)
13-S25	+	-	-	NA	-	I	4b(d/e)
5-S32	+	-	-	NA	-	I	4b(d/e)
5-S39	+	-	-	NA	-	I	4b(d/e)
12-S3	+	-	+	NA	NA	I	1/2b(3b)
12-S6	+	-	+	NA	NA	I	1/2b(3b)
14-S8	+	-	+	NA	NA	I	1/2b(3b)
12-S2	+	-	+	NA	NA	I	1/2b(3b)
14-S4	+	-	+	NA	NA	I	1/2b(3b)
3-S9	+	-	+	NA	NA	I	1/2b(3b)
14-S9	+	-	+	NA	NA	I	1/2b(3b)
12-S8	+	-	+	NA	NA	I	1/2b(3b)

Table AIII.1 (cont'd)

12-S9	+	-	+	NA	NA	I	1/2b(3b)
11-S8	+	-	+	NA	NA	I	1/2b(3b)
11-S9	+	-	+	NA	NA	I	1/2b(3b)
11-S19	+	-	+	NA	NA	I	1/2b(3b)
11-S25	+	-	+	NA	NA	I	1/2b(3b)
12-S1	+	-	+	NA	NA	I	1/2b(3b)
12-S11	+	-	+	NA	NA	I	1/2b(3b)
13-S9	+	-	+	NA	NA	I	1/2b(3b)
10-S19	+	-	-	NA	+	III	4a/c
4-S4	-	+	NA	-	NA	II	1/2c(3c)
5-S16	-	+	NA	+	NA	II	1/2a(3a)
10-S3	-	+	NA	+	NA	II	1/2a(3a)
10-S6	-	+	NA	+	NA	II	1/2a(3a)
10-S11	-	+	NA	+	NA	II	1/2a(3a)
3-S8	-	+	NA	+	NA	II	1/2a(3a)
13-S8	-	+	NA	+	NA	II	1/2a(3a)

Table AIII.2: L. monocytogenes *biofilm OD's, serotypes, and PFGE types*

Isolate	Sampling Area	Sampling Time	Mean OD		Serotype	PFGE
2-S25	Cheese case: Floor	8/2/2006	0.347	A	4b(d,e)	A
10-S25	Cheese case: Floor	3/27/2007	0.325	A	4b(d,e)	A
12-S25	Cheese case: Floor	6/5/2007	0.449	A	4b(d,e)	A
13-S25	Cheese case: Floor	6/26/2007	0.422	A	4b(d,e)	A
	Sandwich line: Slicer (Hobart) back			A		
5-S32	plate	11/1/2006	0.267		4b(d,e)	B
	Deli: Slicer (Bizerba) collection			A		
5-S39	area	11/1/2006	0.386		4b(d,e)	B
12-S3	Basement: Produce walk-in floor	6/5/2007	0.574	B	1/2b(3b)	C
12-S6	Basement: Drain under sink	6/5/2007	0.591	B	1/2b(3b)	C
14-S8	Kitchen: Cooler floor	7/24/2007	0.580	B	1/2b(3b)	C
12-S2	Basement: Catering walk-in entry	6/5/2007	0.383	A	1/2b(3b)	D
14-S4	Basement: Produce walk-in entry	7/24/2007	0.513	B	1/2b(3b)	E
3-S9	Kitchen: Cooler entry	9/6/2006	0.605	B	1/2b(3b)	F
14-S9	Kitchen: Cooler entry	7/24/2007	0.666	B	1/2b(3b)	F
12-S8	Kitchen: Cooler floor	6/5/2007	0.547	B	1/2b(3b)	G
12-S9	Kitchen: Cooler entry	6/5/2007	0.528	B	1/2b(3b)	G
11-S8	Kitchen: Cooler floor	5/1/2007	0.350	A	1/2b(3b)	H
11-S9	Kitchen: Cooler entry	5/1/2007	0.359	A	1/2b(3b)	H
11-S19	Sandwich line: Floor	5/1/2007	0.365	A	1/2b(3b)	H

Table AIII.2 (cont'd)

11-S25	Cheese case: Floor	5/1/2007	0.309	A	1/2b(3b)	H
12-S1	Basement: Catering walk-in floor	6/5/2007	0.350	A	1/2b(3b)	H
12-S11	Kitchen: Floor drain near cooler	6/5/2007	0.351	A	1/2b(3b)	H
13-S9	Kitchen: Cooler entry	6/26/2007	0.398	A	1/2b(3b)	I
10-S19	Sandwich line: Floor	3/27/2007	0.329	A	4a/c	J
4-S4	Basement: Produce walk-in entry	10/5/2006	0.883	C	1/2a(3a)	K
5-S16	Sandwich line: Counter top (board)	11/1/2006	0.938	C	1/2a(3a)	K
10-S3	Basement: Produce walk-in floor	3/27/2007	0.902	C	1/2a(3a)	K
10-S6	Basement: Drain under sink	3/27/2007	1.015	C	1/2a(3a)	K
10-S11	Kitchen: Floor drain near cooler	3/27/2007	0.991	C	1/2a(3a)	K
3-S8	Kitchen: Cooler floor	9/6/2006	0.793	C	1/2a(3a)	L
13-S8	Kitchen: Cooler floor	6/26/2007	0.850	C	1/2a(3a)	L

[a] The letters A, B, and C in the Mean OD column indicate the biofilm forming ability of the isolates where A is weak, B is medium and C is strong.

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