FATE OF *ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES*, AND *SALMONELLA* SPP. ON FRESH-CUT CELERY

By

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To my family, for being the source of inspiration and pride for me, and for supporting me throughout my academic life. And to my grandfather, Dr. Michael J. Begab, whose example demonstrated just how rich and fulfilling a career in science could be.

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FATE OF *ESCHERICHIA COLI* O157:H7, *LISTERIA MONOCYTOGENES,* AND *SALMONELLA* SPP. ON FRESH-CUT CELERY

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Escherichia coli O157:H7, *Listeria* monocytogenes, and *Salmonella* infections have been associated with the consumption of numerous fresh produce items. In 2010, an outbreak of listeriosis was traced back to fresh-cut celery, with contamination believed to have occurred during processing. While no *E. coli* O157:H7 or salmonellosis infections have been associated with celery, both pathogens have been isolated from celery sold at market. Little is known about the fate of these pathogens on celery, or the effect of consumer handling practices on the potential risk. The objective of this study was to determine the fate of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* on fresh-cut celery at refrigeration (4°C), abuse (12°C), and ambient temperatures (22°C) under different storage conditions. Fresh-cut celery samples were spot inoculated at 3 log CFU/g with a cocktail of one of the pathogens onto either cut or uncut surfaces and held at 4 ± 2°C, 12 ± 2°C, or 22 ± 2°C, and kept in either sealed bags or closed containers. Samples were enumerated following stomaching on selective and nonselective media after storage for 0, 1, 3, 5 and 7 days (when held at 4 ± 2°C and 12 ± 2°C), and after 0, 0.33, 0.71, 1, and 2 days (when held at 22 ± 2°C). At 4°C, each of the three pathogens populations declined by ca. 0.5-1.0 log CFU/g over 7 days. At 12°C, *E. coli* O157:H7 and *Salmonella* populations did not change, while *L. monocytogenes* populations increased over 7 days by ca. 0.5

log CFU/g. At 22°C, *E. coli* O157:H7 populations increased by ca. 1 log CFU/g, *Salmonella* populations increased by ca. 1.5 to 2.0 log CFU/g, and *L. monocytogenes* by ca. 0.3 log CFU/g, with the majority of growth in all three pathogens occurring after the first 0.71 days. Significant differences were observed between samples inoculated on cut versus uncut surfaces, but not between container types. This work indicates that *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. can grow on improperly stored fresh-cut celery, and demonstrates the importance of cooling and maintaining proper refrigeration during the distribution and handling of fresh-cut celery.

CHAPTER 1
INTRODUCTION

Americans have substantially increased their consumption of fresh produce. The U.S. Department of Agriculture (USDA) (2008) reported that between 1970 and 2008, the U.S. per capita consumption of fresh vegetables increased from 49 to 82 kg (approximately 67%). Data from the Produce Marketing Association (PMA) (2007) indicates retail sales of fresh produce increased from 32.5 to $56.3 billion between 1998 and 2006. In 2006, U.S. domestic celery sales grossed $920 million, 1.6% of the gross profit from produce sales (PMA, 2007). The U.S. is a net exporter of celery, shipping more the 2.5 million 100 lb. crates overseas in 2009 (Huntrods, 2010; Lucier and Jerardo, 2005). The U.S. also imports celery, especially during the winter months. In 2009, ca. 21,204 MT of fresh celery was imported from producers in Mexico (Huntrods, 2010).

In 1997, the 12 existing celery farms in Florida produced 4,114 acres of celery, the second largest crop in the U.S., after California (USDA, 1999). In 1992, the last year in which data was collected, Florida produced 315.4 million lbs. of celery, with an average yield of 41,500 lbs. per acre. The total value of the 1992 harvest was $39.1 million (USDA, 1999). Although not a major plate vegetable, versatility in both fresh and cooked forms, utility as a food ingredient, and nutritional properties have made celery a staple in the produce aisle. During 2009, U.S. per capita consumption of fresh celery was 6.1 lbs. (USDA, 2011; Huntrods, 2010).

The introduction of prepackaged fresh-cut products, including celery, over the past two decades has likely helped increase consumption of celery (Lucier and Jerardo, 2005). Celery is commonly consumed in two manners, either as an ingredient or as raw snack. Nearly the entire Florida crop of celery is destined for fresh market as full stalks, celery hearts, or fresh-cut

(USDA, 1999). The value-added segment of fresh-cut celery sticks is gaining in popularity and there is a growing market for new washed and ready to eat products (Freedonia Group, 2010).

Fruits and vegetables have gained notoriety in recent years as a vehicle of human disease (D'Aoust and Maurer, 2007). The U.S. Centers for Disease Control (CDC) estimates that in the 1990s, at least 12% of foodborne disease outbreaks were associated with the consumption of fresh produce (FDA, 2004). The raw nature of fresh-cut produce items contributes to its potential risk. Although the incidence of foodborne illness associated with the fresh produce is low in comparison to the total volume consumed per capita in the U.S., recent increases in consumption have coincided with an increase in reported outbreaks associated with their consumption (CAST, 2009). Because most vegetable crops are cultivated in an open, natural environment, they are vulnerable to contamination.

Escherichia coli O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. are among the top 6 disease-causing foodborne pathogens (CDC, 2011), and are found throughout the natural environment (Austin, 1991). Contamination can occur at any point in the supply chain from production through processing and distribution to preparation in the home or food service kitchen. Major processing cross-contamination points include cutting, trimming, washing, and cooling of produce (Doyle and Erickson, 2008). In the consumer kitchen, specific sources of produce contamination are often more difficult to trace, but are primarily believed to be the result of surface transfer from contact with contaminated meat or from improperly sanitized cutting utensils and cleaning surfaces, or contaminated wash water (Chai et al., 2008; Chen et al., 2001; De Wit et al., 1979; Doyle and Erickson, 2008; Kusumaningrum et al., 2003). It is important to note that consumer refrigerators differ in their ability to maintain a constant and uniform temperature. In a recent study of 200 home refrigerators, average temperatures fluctuated

between the top shelf (1.9°C), bottom shelf (3.3°C), and door (5.2°C) (Godwin, 2007). Temperatures also rose above the danger zone (4°C) for over 2 h per day in the top shelf (in 33%), middle shelf (in 45%), and door (in 80% of refrigerators).

Between 1973 and 1997, 190 produce-associated outbreaks involving 16,058 illnesses and 8 deaths were reported to the U.S. CDC (Sivapalasingam et al., 2004). Celery has been associated with three outbreaks of foodborne disease. The first was an outbreak of Typhoid fever in 1899, at a Massachusetts asylum which afflicted 40 inmates over two weeks (Morse, 1899). In 1991, a point-source outbreak of acute gastroenteritis at the U.S. Air Force Academy resulting from the consumption of chicken salad afflicted approximately 1,440 cadets, 105 of which required intravenous rehydration. The outbreak was originally attributed to *Salmonella*, however norovirus was eventually identified as the cause, due to the exposure of the celery component to non-potable water (Warner et al., 1991). Most recently in October, 2010, chopped celery was the confirmed cause of a listeriosis outbreak in Texas that sickened 6 to 10 people, 4 to 5 of whom died as a result (FDA, 2010).

To our knowledge, no studies have explored the fate of *E. coli* O157:H7 on celery. No *E. coli* O157:H7 outbreaks have been associated with fresh-cut celery; however, it has been isolated from 23 of 89 (27%) celery samples sold in Mexican markets (Zepeda-Lopez et al., 1995).

Listeria monocytogenes has been isolated from celery in several market surveys, and grows on asparagus, broccoli, and cauliflower stored at 4°C (Besser et al., 1999), and on lettuce at 5°C (Beuchat and Brackett, 1990; Steinbruegge et al., 1988). In one survey on the microbial safety of fresh-cut RTE celery and other vegetables, *L. monocytogenes* was detected on 8 of 120 (6.7%) fresh-cut celery samples stored at 10°C, but not (0 of 175 samples) on produce stored at

4°C (Odumeru et al., 1997). A Chilean survey detected *L. monocytogenes* on fresh-cut celery in supermarket-prepared salads in 2 of 13 (15.4%) samples taken (Cordano and Jacquet, 2009).

Several studies have detected *Salmonella* on celery, although its fate has not been determined. Garcia-Villanova et al. (1987) found 2 of 26 (8%) celery samples obtained at Spanish markets positive for *Salmonella* species. Viswanathan and Kaur (2001) sampled 120 fruits and vegetables procured at markets in Spain and found that out of eight celery samples, five (63%) were positive for *Salmonella* Typhi. Recently, in Mexico City, Quiroz-Santiago et al. (2009) confirmed 3 of 100 (3%) celery samples taken from a central supply station to be positive for *Salmonella* species.

Currently, very little is known about the behavior of *E. coli* O157:H7, *L. monocytogenes*, or *Salmonella* on fresh-cut celery, or the impact of consumer handling practices on the potential risk. Our objective was to determine the fate of these pathogens on fresh-cut celery under different storage conditions. We hypothesize that: (i) *E. coli* O157:H7 and *Salmonella* populations on fresh-cut celery will decrease at refrigeration temperatures and increase with increasing temperature throughout the range encountered in typical produce transportation and storage environments, (ii) *L. monocytogenes* will grow at typical storage temperatures, and grow optimally on temperature-abused fresh-cut celery, (iii) all three pathogen growth rates will be higher on cut than on uncut celery surface inoculation sites, and (iv) the type of storage container (bag or container) will affect the behavior of all three pathogens.

CHAPTER 2
LITERATURE REVIEW

Celery

Background

First cultivated about 3,000 years ago, celery (*Apium graveolens*) is a member of the parsley family native to brackish marshlands of southern Europe and North Africa that border the Mediterranean Sea (Ozores-Hampton et al., 2011). Celery is a cold season, biennial crop grown as an annual (Huntrods, 2010). A popular herb and vegetable in North America and Europe, celery varieties with fleshy stems are most popular, being served raw or diced and cooked as an important ingredient in many soups, sauces, stews, and gumbos. Modern cultivars are larger, more succulent, and less stringy than their ancestral wild varieties (Lucier and Jerardo, 2005). A stalk of celery (or head) is a modified leaf bundle, consisting of several individual fleshy stems (or petioles). Celery hearts are made by trimming off the outer petioles of a stalk, and are therefore composed of only the inner and most tender petioles (Lucier and Jerardo, 2005).

Although not a major plate vegetable, versatility in both fresh and cooked forms and nutritional properties of celery have made it a staple in the produce isle (Lucier and Jerardo, 2005). Long an important ingredient in vegetable platters, the introduction of prepackaged fresh-cut products over the past two decades has likely helped expand the reach of celery into the diet of consumers (Lucier and Jerardo, 2005). The leaves and roots are also used as flavoring herbs, most often in European cuisine. Celery seed and salt (ground celery seeds mixed with salt) are also important flavoring herbs used worldwide (Huntrods, 2010).

Nutritional Profile

Originally believed to be a sedative and cultured for its purported medicinal properties, use of celery as a food item was not recorded until 1623, in France (Huntrods, 2010). A low

calorie, high fiber food containing anti-inflammatory polysaccharide compounds, diets high in celery have been shown to improve serum lipid profiles (Ovodova et al., 2009; Tourkostani et al., 2009). The major bioactive antioxidants and polyphenolic flavonoid (typically glycosylated flavonoid malonates) constituents of celery include caffeic acid, p-courmaric acid, and ferulic acid, and apigenin, luteolin, chrysoeriol, and kaempferol, respectively (Lin et al., 2007; Yao et al., 2010). Celery is particularly high in luteolin, which in animals models and human cell culture studies inhibited angiogenesis, induced cancer cell apoptosis, and prevented carcinogenesis, as well as hyper-sensitize tumor cells to cytotoxic chemotherapeutic agents (Lopez-Lazaro, 2009). Luteolin can inhibit microglial, hippocampal production of IL-6, and thus may mitigiate neuro-inflammation and improve working memory, particularly in the elderly (Jang et al., 2010; Jang et al., 2008). Celery also contains 10, 4, 15, and 2% of the recommended daily allowance (RDA) of vitamin A, calcium, vitamin C, and iron, respectively and contains only 15 calories (mostly from sugars and polyols) per 110 g serving (FDA, 2009). The major sugar and polyol constituents of celery stalks include sucrose, glucose, fructose, and mannitol, the latter representing 33.5 to 39.3% of the total bioavailable carbohydrates (Ruperez and Toledano, 2003). Celery oil from ground seeds or roots is currently marketed as a dietary supplement, having demonstrated anti-rheumatic properties and hypolipidemic promotion of healthy blood pressure (Cheng et al., 2010; Huntrods, 2010; Lin et al., 2011).

Production

In the United States, the majority of celery is grown in Florida, California, and Michigan (Lucier and Jerardo, 2005). Over the last two decades, the industry has become increasingly concentrated in California, which, in 2005, accounted for 81% of the national celery acreage; up from 63% in 1992 (Lucier and Jerardo, 2005). The most common variety of celery in the United

States is the Pascal type (Huntrods, 2010). In Florida, common agricultural varieties include Floribelle-M9, June Belle, and Florida 683 (Ozores-Hampton et al., 2011).

Soil preparation is more important in celery than in most other crops (USDA, 1999). Celery in Florida is grown on heavily fertilized, muck soils. Fields are plowed, disked, and leveled, then alternately flooded and dried during the summer to manage nematodes, insects, and other parasites (USDA, 1999). After flooding, the plowing and leveling process is repeated, followed by a sub-soil mole-draining operation to aid in sub-surface irrigation prior to planting (Bertelson et al., 1994). Cutting transplantation is the primary method of planting, since it is difficult to obtain uniform stands from direct seeding (Masabni and Lillard, 2011). Seedlings are spaced in double rows about 6-12 inches apart in-row, with 18-40 inches between rows on 40 inch raised beds (Masabni and Lillard, 2011; Ozores-Hampton et al., 2011). Celery is grown from seed on mineral soils enriched with phosphorous oxide (P_2O_5), micronutrients, and 20 to 25% nitrogen, and potassium oxide (K_2O) before transplanting to histosol soil (Ozores-Hampton et al., 2011). The seeding mineral soil is side dressed in two applications, one 2 to 3 weeks after planting, and the second 6 to 8 weeks after planting. The histosol soils are broadcasted with a variety P_2O_5 and micronutrient fertilizers before planting. Cultivation time from transplanting to maturity is 75 to 90 days (Ozores-Hampton et al., 2011). The average population of celery crop per acre is 58,080. Several applications of potassium and nitrogen are made throughout the growth season (October to April) (Ozores-Hampton et al., 2011). A variety of herbicides, insecticides, and antibiotics are heavily relied upon by celery producers to help ensure a productive harvest (Ozores-Hampton et al., 2011).

Celery is more difficult to grow than most fruits and vegetables, requiring a longer growth season, large quantities of water on par with carrot crops, and cooler temperatures

(optimal 15.6-21.1°C) (Bertelson et al., 1994; Huntrods, 2010; Masabni and Lillard, 2011; USDA, 1999). One hundred percent of the celery acreage in Florida is irrigated. Because of its shallow root system, celery is very sensitive to changes in water level (Huntrods, 2010). If the specific microclimate requirements are not met, mature celery stalks can become dry and stringy (Huntrods, 2010). At the higher temperatures, celery crops becomes more susceptible to disease and insect damage, as well internal, physiological problems (USDA, 1999). In juvenile crops, if ambient temperature drop below 10°C for between 10 and 14 days, premature bolting can occur, leaving the stalk with no commercial value (USDA, 1999).

Harvest and Processing

Fresh celery must be harvested within a few days after reaching marketable size to avoid quality deterioration (Huntrods, 2010). Growers stagger planting to develop uniform lots that reach marketable size weekly (Huntrods, 2010). Harvested celery plants must be carefully handled and never stacked more than four stalks high within storage or shipping crates (Huntrods, 2010). Most Florida celery is harvested by hand, where labors trim, size, and wash in 1 ppm free Chlorine (Personal communication, May 2011), and pack the crop on site (USDA, 1999). Often, stalks are packed upright in crates and require constant storage and shipping temperature and humidity; ca. 32-34°F and 85-95% RH (Huntrods, 2010; Masabni and Lillard, 2011; Suslow and Cantwell, 2009). Cartons or crates of celery weigh 50 to 60 lbs (Masabni and Lillard, 2011). Harvested celery is brought to a hydrocooling facility and loaded onto refrigerated trucks for transport, usually to market.

About 8% of all U.S. celery is harvested for processing. Only celery destined for processing can be mechanically harvested (Lucier and Glaser, 2005). The market is dominated by a handful of large producers, which have matured an infrastructure that possesses the

processing and handling capabilities necessary to quickly deliver this relatively perishable crop to market (Huntrods, 2010).

Celery that that will be cut or diced may be machine harvested, then taken to a packinghouse, hydrocooled, and stored at 34-38ºF for not more than 48 h (Personal communication, May 2011). Cutting of celery sticks is often done by water jet, and dicing by machine using metal blades. Peracetic acid (PAA) may be used as a sanitizing agent in processing lines in either a flume or drenching processing. Processed celery is then packed into small (ca. 1.6 oz) bags or large (ca. 1,200 lb) bins for industrial use (Personal communication, May 2011). Within 24 h after processing, it is loaded onto temperature-controlled trucks for transport (Bertelson et al., 1994; Bewick, 1994). The entre process may take up to 3 days before export from the packinghouse.

Marketing of Celery in the United States

While most U.S. celery is sold in the fresh market, a portion is also used in prepared foods including soups, juices and premade entrees (Lucier and Jerardo, 2005). Established in 1957, there are three grades of celery based on uniformity, size, and defects (USDA, 1959). From highest quality to least, these grades are; U.S. Extra, U.S. #1, and U.S. #2 (Suslow and Cantwell, 2009; USDA, 1959) . However, celery is often sold as "ungraded" (Suslow and Cantwell, 2009).

Fresh and fresh-cut celery is primarily sold to retailer-wholesalers, terminal market brokers, wholesale handlers, and the military (Bertelson et al., 1994; Bewick, 1994). The majority of fresh celery sold is eventually consumed at home (76% of all market sales), while restaurant consumption account for about 14% of total per capita consumption (Lucier and Jerardo, 2005). Distributers have established only a small niche in the expanding fast-food

market, which represents only 4% of celery consumption in the U.S. (Lucier and Jerardo, 2005). Consumers eat >90% of processed celery products at home.

In 1997, in Florida, the 12 existing farms produced 4,114 acres of celery, the second largest crop in the U.S., after California (USDA, 1999). Nearly the entire Florida crop of celery is destined for fresh market sale as full stalks, celery hearts, or fresh-cut (USDA, 1999). The total cultivation cost—including labor, machinery, nutrients, insecticides, herbicides, and fungicides—of celery between 1993 and 1994 was $4,580 per acre, with pest management representing 42% of the cost (USDA, 1999). In 1992, the last year in which data was collected, Florida produced 315.4 million lbs. of celery, with an average yield of 41,500 lbs. per acre. The total value of the 1992 Florida harvest was $39.1 million (USDA, 1999).

The demographic profile of celery consumptions are skewed towards older populations, with U.S. citizens under 20 accounting for 30% of the total population, but only consuming 17% of the fresh celery sold at market (Lucier and Jerardo, 2005).

Economic Impact

A steady, ample supply from a relatively efficient industry has kept U.S. market prices low and limited the reach of foreign competitors into the American and Global markets (Lucier and Jerardo, 2005). In 2006, within the U.S., celery sales alone grossed $920 million, 1.6% of the gross profit from produce sales (PMA, 2007). Also according to the PMA (2007), about 50% of all North American households purchased celery 3 times or more per year in 2005. During 2009, U.S. consumers used a per capita average of 6.1 lbs. of fresh celery (USDA, 2011; Huntrods, 2010). This per capita consumption has held relatively uniform over the past 90 years, ranging from ca. 6.1 to 8.2 lbs/year, which peaked during the 1950s (USDA, 2000). Between 2003 and 2004, the average gross annual value of a celery farm in the U.S. was ca. $261 million (Lucier and Jerardo, 2005).

The United States is a net exporter of celery, having shipped more the 2.5 million cwt (100 lb. crates) overseas in 2009 (Brown et al., 2002; Huntrods, 2010; Lucier and Jerardo, 2005). Between 2002 and 2004, exported fresh celery totaled $48 million, while imported celery was valued at only $9 million (Lucier and Jerardo, 2005). Also during this time, 13% of the U.S. celery supply was exported annually. Canada is the largest single importer, having received 80% of total exports between 2002 and 2004, and about 93,970 metric tons (MT) in 2009 (Huntrods, 2010).

Despite being a major producer and exporter, the U.S. also imports celery. In 2009, ca. 21,204 MT of fresh celery was imported from producers in Mexico, a practice which increases during the winter months (Huntrods, 2010).

Unlike many storable commodities, fresh-market celery exhibits only a weak seasonal price pattern, reflecting the relatively consist season-to-season marketing (Lucier and Jerardo, 2005). Celery prices are known to follow a pronounced 3-year cycle, which may reflect recurring weather patterns (Lucier and Jerardo, 2005).

Price of a Recall to Industry

Over the last decade, the number—or at least the public profile—of food recalls has increased dramatically, with *Salmonella, L. monocytogenes,* and pathogenic *E. coli* having been associated with some of the largest scale recalls. Given the large number of variables involved, it is difficult to estimate the costs of these events to industry and shareholders with any accuracy, but catastrophic and devastating may in some cases be considered fair descriptors.

In one example, the well-publicized baby spinach recalled in 2006 attributed to *E. coli* O157:H7 cost Dole $350 million including lawsuits and refunds, not accounting for lost customers and business relationships (NRI, 2010). As a result of the *Salmonella*-related peanut recall of 2009, expert testimony from industrial insiders and government officials estimated that

the confirmed $1 billion loss in production and sales of peanuts could be just the beginning, as consumer demand for any product containing peanuts subsequently plummeted.

Among the multitude of costs incurred by food companies in the event of a recall include removal and disposal of both stored and distributed products, loss of trust and quite possibly business with large buyers, loss of consumer faith for the foreseeable future, often hefty lawsuits from those consumers struck ill from contaminated products, government mandated closure of processing plants, and possibly government-imposed penalties if it is found that codes have been violated (Associated Press, 2009).

Biological Hazards Associated with Fresh Produce

Fruits and vegetables have gained notoriety in recent years as a vehicle of human disease (D'Aoust and Maurer, 2007). The fact that fresh-cut produce items are often consumed raw contributes to the potential risk. Although the incidence of foodborne illness associated with fresh produce is low in comparison to the total volume consumed per capita in the United States, recent increases in consumption have coincided with an increase in reported outbreaks associated with their consumption (CAST, 2009). This increase in incidence is believed to have developed partially as a result of increases in global export of fresh produce from countries with tropical and subtropical climates (D'Aoust and Maurer, 2007). Production conditions in these countries do not always meet minimum standards and can facilitate contamination (D'Aoust and Maurer, 2007). Because fresh produce is often consumed raw, foodborne disease prevention efforts must focus on good sanitary practices throughout the supply chain.

Sources of Contamination on Fresh Produce

Escherichia coli O157:H7, *L. monocytogenes,* and *Salmonella* are found throughout the natural environment, and contamination can occur at any point in the supply chain from production through processing and distribution to preparation in the home or food service

kitchen. Because most produce is cultivated in an open, natural environment, it is highly vulnerable to contamination.

At the field production level, Good Agricultural Practices (GAPs) have been implemented by many producers to minimize the risk of microbial cross-contamination from various environmental sources/vectors including farm laborers, soil, fertilizers, spray and irrigation water, wild and domestic animals, and insects. Contamination may also arise during cultivation by infection through an open portal such as damaged plant tissues (e.g. herbivore damage) or scar tissue, internalization during embryogenesis, or from natural uptake of pathogens through the root system (Guo et al., 2001), though this likely is not a common occurrence. During further processing, several modes of produce cross-contamination have been identified. Major processing cross-contamination points include cutting, trimming, washing, and cooling of produce (Doyle and Erickson, 2008).

In a consumer kitchen, specific sources of produce contamination are often more difficult to trace, but are primarily believed to be the result of surface transfer from contact with contaminated meat, from improperly sanitized cutting and cleaning surfaces, or contaminated wash water (Chai et al., 2008; Chen et al., 2001; De Wit et al., 1979; Doyle and Erickson, 2008; Kusumaningrum et al., 2003).

High-Risk Consumer Handling Behaviors Associated with Fresh Produce

Fresh-cut produce may become contaminated in the kitchen through cross-contamination during final preparation or serving of the product. Due to the notorious difficulty in identifying a causal relationship between outbreaks and risky behaviors, the degree of risk associated with consumer behavior is an open question. For example, it has been shown that temperature abuse can increase the growth rate of pathogens in many fresh produce items (e.g. iceberg lettuce) (FDA, 2008; Koseki and Isobe, 2005), but it is not certain whether this behavior in the home has

contributed to illnesses associated with produce. Other risky consumer behaviors believed to contribute to produce-related outbreaks that have occurred in the home kitchen include inadvertent contact with raw meat (e.g. melon) (Harris et al., 2003), and from the contaminated hands of food handlers (Ayçiçek et al., 2004; De Roever, 1998; Iversen et al., 1987; Lues and Van Tonder, 2007; Shojaei et al., 2006). Reports also suggest that consumers often keep fresh-cut produce in the refrigerator well after the expiration date, and may regularly consume spoiled items (CDHS, 2007). It should also be noted that spreading of bacterial contaminants from outer organic surfaces (e.g. melon rind) can be facilitated by slicing (Chai et al., 2008), although this phenomenon has less bearing on consumer practices for already cut produce.

Much of the available data used to quantify consumer behavior in the home kitchen is obtained through national mail surveys. One such survey of 2,000 Americans that focused on consumer handling of fresh produce (but not necessarily fresh-cut) indicated that 6% of those surveyed seldom or never wash fresh produce before consumption (Li-Cohen and Bruhn, 2002). The same survey also showed that almost half of respondents did not consistently wash their hands before handling fresh produce. Further, 5% of respondents indicated that they did not use wash water, and 24% only wash produce with water after contact with meat products.

Li-Cohen and Bruhn (2007) conducted focus groups to observe first hand consumer practices related to fresh produce (not specifically fresh-cut). The results demonstrated a variety of frequent and potentially hazardous handling behaviors. Those who washed lettuce with water most often either rinsed leaves under running water or submerged them in a filled sink or washing basin. Most used plain water, although some added salt, vinegar or bleach. After washing, leaves were either shaken and directly served or left to drain in a colander, in the dish rack, or on paper towels or cloth. Few used a salad spinner, and some placed leaves in a

pillowcase and spun dry in a laundry dryer. Certain items including cut carrots and cut melons were not typically washed.

Bruhn and Li-Cohen (2007) also indicated that consumer refrigerator cleaning frequency ranged from once per week to once per year, and was dependent on whether the inside appeared dirty or stained. Common methods of cleaning the refrigerator included dish detergent or soap, bleach, baking soda, antibacterial soap, and cleaning solution.

It is important to note that consumer refrigerators differ in their ability to maintain a constant and uniform temperature. In a recent study of 200 home refrigerators, average temperatures fluctuated between the top shelf (1.9°C), bottom shelf (3.3°C), and door (5.2°C) (Godwin, 2007). Temperatures also rose above the danger zone (4°C) for over 2 h per day in the top shelf (in 33%), middle shelf (in 45%), and door (in 80% of refrigerators).

In-Home Hazard Intervention Strategies

Although washing of fresh-cut produce is one of the most common hazard mitigation strategies, there is limited data available to compare the relative effectiveness of many common washing methods employed in the home. Data published by Parnell and Harris (2003) on the efficacy of consumer washing methods of apples has been used to provide standard consumer guidance for washing fresh produce that involves rinsing and rubbing where possible under running water and drying with a clean towel. Parnell et al. (2005) studied the effects of washing cantaloupe and honeydew melons, which also provided critical information on the efficacy of washing smooth and netted rind melons. The latter study also demonstrated a potential for the transfer of pathogens from one site to another on the rind during washing with a volume of untreated (unchlorinated) water.

Escherichia coli

Biology of *Escherichia coli*

Escherichia coli was first discovered in 1885 by the German pediatrician and bacteriologist, Theodor Escherich (Feng et al., 2002). All *E. coli* can be classified into four groups based on ecological features and virulence potential: nonpathogenic mutualists; commensal opportunists (pathogenic under the right conditions); parasitic pathogens; and occasional symbionts (i.e. not common to gut microflora) (Gritsenko and Bukharin, 2000). Mutualistic *E. coli* can benefit their hosts by producing vitamin K_2 (Bentley and Meganathan, 1982), and by preventing the establishment of ingested pathogenic bacteria through spatial resource competition (Reid et al., 2001). *Escherichia coli* is one of the most well studied prokaryotic "model" organisms, and was one of the first organism to have its complete genome sequenced (Blattner et al., 1997).

As normal microbiota, *E. coli* is the primary facultative anaerobe in the human gut, assisting in the breakdown of polysaccharides (Salyers, 1985). *Escherichia coli* has been shown to colonize an infant's gastrointestinal tract within 40 h after birth, being delivered through food, water, or from individuals handling the child. Once in the colon, it adheres to the mucosa. Interestingly, enhanced food digestibility may not be the only mutualistic benefit from *E. coli* and other normal intestinal micrbiota to humans, and the hypothesis of a coevolutionary human adaptive immune system is well reasoned (Lee and Mazmanian, 2010). It is only when these commensal organisms acquire virulence genes through horizontal transfer that they become parasitic. In fact, Mutaflor, or *E. coli* Nissle 1917, is a probiotic proven in numerous clinically trials to be effective in alleviating symptoms of various irritable bowel diseases (IBDs) including irritable bowel syndrome, ulcerative colitis (UC), chronic constipation, Crohn's disease, and pouchitis, in addition to its utility as a bacterial prophylactic and immunological enhancer in

newborns (Hancock et al., 2010; Konturek et al., 2008; Konturek et al., 2009; Kruis et al., 1995; Midtvedt, 2009).

Escherichia coli are gram-negative, rod-shaped, non-sporulating, facultative anaerobes. These bacteria can thrive on a variety of substrates, and uses mixed-acid fermentation under anaerobic conditions, producing lactate, succinate, ethanol, acetate, and carbon dioxide (Meng et al., 2007). During cellular respiration, *E. coli* uses several respiratory pathways and a variety of redox pairs, including the oxidation of pyruvic acid, formic acid, amino acids and hydrogen, with reducible substrates including oxygen, nitrate, dimethyl sulfoxide and trimethylamine N-oxide (Ingledew and Poole, 1984). Some strains of *E. coli* possess peritrichous flagella, while others are immobile (Darnton et al., 2007).

A mesophile, *E. coli* optimal growth temperature is 35-37ºC, although some strains have been shown to survive temperatures up to 49ºC (Brock et al., 1994). Despite their mesophilic nature, *E. coli* are not always confined to the intestine, and can survive for months outside the body in many open environments (van Elsas et al., 2011), making them somewhat useful indicator organisms for fecal contamination (Duris et al., 2006; Feng et al., 2002). The minimum pH for growth of *E. coli* O157:H7 is 4.0 to 4.5 (Meng et al., 2007), although it can survive much lower pH. Studies have shown that hot acetic, citric, and lactic acid sprayed at concentrations up to 1.5% onto meat inoculated with *E. coli* were not sufficient to appreciably effect population size (Brackett et al., 1994). When inoculated at high concentrations, *E. coli* survives fermentation, drying, and storage of sausage at pH 4.5 for up to 2 months at 4ºC (Glass et al., 1992), in mayonnaise at pH 3.6 to 3.9 for up to 7 weeks at 5ºC and for 3 weeks at 20ºC (Zhao and Doyle, 1994), and in apple cider at pH 3.6 to 4 for up to 31 or 3 days at 8 or 25ºC, respectively (Zhao et al., 1993).

Enteric pathogens such as virulent *E. coli* must pass through the gastric acid pH barrier—with pH level as low as 1.5 to 2.5—to cause gastrointestinal disease. Specifically, *E. coli* O157:H7 has evolved several amino-acid-dependent systems that control the production of the decarboxylase isozymes GadA, GadB, and AdiA; pyridoxal phosphate-containing enzymes that replace the alpha-carboxyl groups of their amino acid substrates with H^+ recruited from the cytoplasm (Castanie-Cornet et al., 1999; De Biase et al., 1999; Hersh et al., 1996; Smith et al., 1992). Acid-resistance also provides cross-protection to *E. coli* O157:H7, increasing tolerance to heat, radiation, and antimicrobials (Meng et al., 2007).

Biochemically, MacConkey and Eosin Methylene Blue (EMB) agar are selective for *E. coli*. On MacConkey agar, deep red colonies are produced through the fermentation of lactose, which causes a drop in pH, leading to a darkening of the medium, and a pink halo of bile salts is confirmative for *E. coli*. On Levine EMB agar, *E. coli* produces colonies with a characteristic greenish-black metallic sheen. *E. coli* is also lysine-positive, and will grow on TSI slants with an A/AG+H_2S- profile. Additionally, IMViC is {+ + - -}, as *E. coli* is indole-positive (red ring), methyl red-positive (bright red), Voges-Proskauer-negative, and citrate-negative.

Nomenclature and Classification of Pathogenic *Escherichia coli*

Escherichia coli is a gamma-proteobacteria of the Enterobacteriaceae family. Although not necessarily based on evolutionary relatedness, the serotype subdivision system used is based on three major surface antigens, including the O antigen (part of the lipopolysaccharide layer), the H antigen (a flagellin), and the K antigen (capsular) (Orskov et al., 1977). As of November 2005, 173 O antigens, 56 H antigens, and 103 K antigens have been identified (Stenutz et al., 2006). Established nomenclature dictates that it is necessary only to determine the O and H antigens to serotype strains of diarrheagenic *E. coli*, with the O antigen identifying serogroup and the H antigen identifying serotype (Meng et al., 2007). Although certain serogroups often fall

into one category of diarrheagenic *E. coli*, some—such as O55, O111, O126, and O128—appear in more than one category (Meng et al., 2007). Further, diarrheagenic *E. coli* isolates are categorized into six pathotypes based on virulence factors, pathogenic biomechanics, associated clinical syndromes, as well as O:H serotypes. These pathotypes include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC; Shiga toxin producing *E. coli* STEC). Of these groups, the pathogens belonging to STEC cause the most frequent and severe illness (Meng et al., 2007).

The first *E. coli* pathotype to be described, EPEC is featured by severe diarrhea in infants, especially in developing countries (Meng et al., 2007). The major serogroups associated with this pathotype include O55, O86, O111ab, O119, O125ac, O126, O127, O128ab, and O142. EPEC induce attaching and effacing (A/E) lesions, and can invade epithelial cells (Nataro and Kaper, 1998).

ETEC are also a major cause of infantile diarrhea in developing countries, and are the most frequent agents causing travelers' diarrhea (Meng et al., 2007). *Escherichia coli* of this pathotype colonize the proximal small intestine by fimbrial colonization factors (CFA) and produce both heat-stable and heat-labile enterotoxins. Serogroups members include O6, O8, O15, O20, O25, O27, O63, O78, O85, O115, O128ac, O148, O159, and O167 (Nataro and Kaper, 1998).

EIEC is featured by nonbloody diarrhea and dysentery similar to that caused by *Shigella* species. With infection principally focused in the colon, EIEC invade and proliferate in epithelial cells, destroying host cells in the process. Serogroup members include O28ac, O29,

O112, O124, O126, O136, O143, O144, O152, O162, and O167, with O124 being most common (Nataro and Kaper, 1998).

The epidemiological profile of DAEC is age-related, manifesting in young children under 5 years old, and over 1 year old, with relative risk increases with age. Features are typically mild and include diarrhea with or without fecal leukocytes. Pathogenically, DAEC strains attach to host cells via a diffuse-adherent pattern invitro. They do not invade epithelial cells, synthesize heat-stable or heat-labile enterotoxins, or Shiga toxin, and do not possess EPEC adherence factors. Serogroup members include O1, O2, 021, and O75 (Meng et al., 2007; Nataro and Kaper, 1998).

EAEC manifests in infants and children, and is characterized by an aggressive and biomechanically unique pathogenic pattern of adherence. In vitro, EAEC adhere on the surface of HEp-2 cells in a stacked brick appearance (Meng et al., 2007). Serotype members include O3, O15, 044, 077, O86, O92, O111, and O127 (Meng et al., 2007; Nataro and Kaper, 1998).

STEC were first recognized as foodborne pathogens in 1982, when *E. coli* O157:H7 was the determined cause of two outbreaks of hemorrhagic colitis, a clinical feature unique to this pathotype. *Escherichia coli* O157:H7 has since been associated with many foodborne outbreaks and is the predominate STEC-associated disease (Meng et al., 2007). Other serogroup members include O26, O45, O103, O111, O121, O145 and the sorbitol-fermenting O157:NM serotype. The pathogenic profile of STEC includes the production of Shiga toxins (Stx), or Shiga-like verotoxins. VT is so named due to its cytotoxicity to African green monkey kidney (Vero) cells, and Shiga toxins (Stx) due to their similarity with the Shiga toxin produced by *Shigella dysenteriae* type 1 (Obrig, 1997). Stx and VT are structurally and functionally identical (Hoffmann et al., 2010; Takao et al., 1988).

To test for shiga toxin production, *E. coli* isolates can be exposed to mammalian tissue culture (Paton and Paton, 1998). Other methods for the detection of *E. coli* O157:H7 include real-time PCR, ELISA tests, colony immunoblots with verotoxin antibodies, direct fluorescence microscopy enzyme bioassay, and immunomagnetic capture techniques (De Boer and Heuvelink, 2000; Fedio et al., 2007; Lauer et al., 2009; Sharma et al., 2008; Song and Kwon, 2009).

Escherichia coli O157:H7 strains are distinguished by an inability to grow at temperatures ≥44.5ºC, ferment sorbitol within 24 h, or produce Beta-glucuronidase through hydrolysis of 4-methlyumbelliferyl-D-glucuronide (MUG), and the possession of the locus of enterocyte effacement (LEE) pathogenicity island, as well as a 60-MDa plasmid (Meng et al., 2007).

Evidence suggests that only 20% of the genome is common to all *E. coli* strains (Lukjancenko et al., 2010). Different strains of *E. coli* are often host-specific, which can make the original source of fecal contamination from environmental samples traceable. For example, strain-typing *E. coli* sampled from a water source may allow researchers to make assumptions about whether the original source of contamination was human, another mammal, or bird species (Feng et al., 2002).

Clinical Features of EHECs

Although the precise infectious dose of *E. coli* O157:H7 is unknown, it has been estimated at <100 cells, and possibly as little as 10 cells in high-risk populations (Meng et al., 2007). Food poisoning from *E. coli* O157:H7 is usually caused by eating unwashed vegetables or undercooked meat, although there are many other potential sources (Rangel et al., 2005). Ingestion of the bacteria is typically followed by a 3 to 4 day incubation period, but can range from 2 to 12 days, during which time colonization in the large intestine occurs (Meng et al., 2007). Illness typically onsets with nonbloody diarrhea and severe abdominal cramping for 1 to

2 days, progressing in the 2nd or 3rd day to bloody diarrhea that last from 4 to 10 days (Besser et al., 1999; Tarr et al., 2005). Symptoms usually resolve after 1 week. While most often mild, the severity of illness caused by pathogenic *E. coli* varies considerably. Virulent strains of *E. coli* typically cause gastroenteritis and urinary tract infection, which are unpleasant for adults, but can often lead to serious conditions such as hemolytic-uremic syndrome (HUS) and neonatal meningitis in children, particularly those of the developing world (Nataro and Kaper, 1998). Epidemiological studies suggest that the highest age-specific incidence of *E. coli* O157:H7 infection occurs in children between 2 and 10 years old (Meng et al., 2007).

The more virulent strains such as *E. coli* O157:H7, O121 and O104:H21 produce potentially lethal toxins and can cause serious illness or death in the elderly, very young, and immunocompromised populations (Nataro and Kaper, 1998). About 6% of all *E. coli* O157:H7 cases progress to HUS (most frequent in children), 75% of which require blood transfusions, and 50% requiring dialysis (Karmali et al., 2004; Meng et al., 2007). HUS is the primary cause of renal failure in children. Peritonitis, which can be fatal without intervention, can manifest when virulent *E. coli* penetrate the intestinal epithelium through a pre-existing gap in the barrier—such as an ulcer, ruptured appendix, or surgery—and enter the abdominal cavity.

The precise role of Stxs in colonic diseases, HUS, and neurological disorders has not been fully elucidated, although the presence of Stxs in neurons has been verified by immunoelectron microscopy (Meng et al., 2007). In HUS, it has been found that endothelial dysfunction is the triggering event, and that bloodborne Stxs invade the renal microvasculature causing thrombogenesis (Zoja et al., 2010). Histopathic examinations have also revealed structural alterations in the glomeruli of HUS patients, which suggests that subepithelial cell can also serve as target sites of opportunity for STEC attack (Meng et al., 2007).

Escherichia coli are extremely sensitive to some antibiotics including streptomycin and gentamicin. However, caution is always warranted since *E. coli* is known to quickly acquire multidrug resistance (Amaya et al., 2011; Aslani et al., 2011; Cuevas et al., 2011).

The American Gastroenterological Association Foundation (AGAF) recommends that all clinical stool specimens be routinely tested for *E. coli* O157:H7 (Tarr, 1995).

Virulence Factors and Pathogenesis of EHECs

EHEC have evolved the ability to cause disease by adhering to host intestinal epithelium cell membranes with A/E lesions and then producing and secreting one or more Stxs. All A/E lesion-associated proteins known—which include a type III secretion system (TTSS), an outer membrane adhesin (intimin), and translocating intimin receptors (Tir) and other effector proteins—are encoded on the LEE pathogenicity island (Meng et al., 2007).

The TTSS is a complex "needle and thread" structure assembled by the products of approximately 20 different genes, and is used by STEC to secrete virulence factors from the bacterium into a host cell (Garmendia et al., 2005). Following effector translocation, the TTSS complex is disengaged to allow closer bacterial attachment through intimin-Tir interactions (Garmendia et al., 2005). Transmembrane intimin receptor (Tir) forms an extracellular loop that interacts with intimin in a reticular array between host and bacterium.

STEC serotypes contain a unique 60-MDa plasmid (pO157) that is thought to play a role in pathogenesis (Meng et al., 2007). The pO157 is composed of putative virulence genes with 7 insertion sequences located directly upstream. These genes including those encoding for the adhesin ToxB, EHEC-hemolysin, the serine protease EspP, a catalase-peroxidase encoded by *katP* (role in defense from oxidative attacks), and the metalloprotease StcE (Meng et al., 2007).

STEC can express both the heat-stable Stx2 and the heat-labile Stx1 enterotoxins (Rasooly and Do, 2010). Stx holotoxins are structurally and functionally similar to cholera

toxin, with the B subunits assisting in host intestinal epithelium cell adhesion and entry while the A subunit is cleaved by trypsin into an enzymatic A1 fragment, which is released into the cytosol causing cell death and preventing host epithelial cell water uptake, leading to diarrhea (Tauschek et al., 2002).

Rather than transfer via the TTSS involved in A/E lesion formation, Stxs are translocated via a Type II secretion pathway (Tauschek et al., 2002). B subunit receptor binding induces membrane invagination and the holotoxin is endocytosed and endosomally transferred via the trans-Golgi network and endoplasmic reticulum (Sandvig et al., 2010; Utskarpen et al., 2010). It is during this trafficking that the A subunit is released to the cytosol.

Once within the host cell cytosol, the A subunit acts toxicokinetically by inhibiting protein synthesis at translation (Sandvig, 2001). The cytosolic A subunit undergoes partial proteolysis by host cell proteases and is split into a 27 kDa active *N*-glycosidase (A1) bridged to the remaining 4 kDa fragment (A2) in a disulfide bond. The A1 target site of action is the 28S rRNA of the 60S ribosomal subunit. A1 cleaves the *N*-glycoside bond in one adenosine position, thereby inhibiting elongation factor-dependent binding to ribosomes by aminoacyl-bound tRNA (Sandvig and VanDeurs, 1996).

Epidemiology of *Escherichia coli* O157:H7; Focus on Fresh Produce

In most non-O157 STEC illness outbreaks, the modes of transmission are unknown, and only a few have been positively traced back to a food and/or water source (Brooks et al., 2005; Johnson et al., 1996). In contrast, *E. coli* O157:H7 has many times been identified as the cause of disease in outbreaks of severe illness worldwide. In 1996 in Japan, more than 11,000 cases of *E. coli* O157:H7 illness were reported (Meng et al., 2007). In the United States, between 1982 and 2002, 350 outbreaks and 8,598 cases of *E. coli* O157:H7 were documented (Rangel et al., 2005). Of these outbreak-related cases, 61% were foodborne. Also of these, 1,493 (17.4%)

resulted in hospitalization. The total number of outbreaks associated with *E. coli* increased dramatically from 1982, peaking during 2000.

A variety of foods have been implicated as the vehicle of transmission in *E. coli* O157:H7 cases including ground meat, roast beef, cooked meats, jerky, salami, raw milk, pasteurized milk, yogurt, cheese, ice cream bars, lettuce, unpasteurized apple cider or juice, cantaloupe, potatoes, radish sprouts, alfalfa sprouts, lettuce, spinach, fruit salad, vegetable salad, and cake (Meng et al., 2007; Patel et al., 2010). The first outbreak of *E. coli* O157:H7 infection related to fresh produce was reported in 1991 (Rangel et al., 2005). From 1982 to 2002, in cases where a source was determined, 21% of reported cases were produce-associated. Annual produce-associated outbreak size averaged 20, with a range from 2 to 736 incidences per outbreak (Rangel et al., 2005). In 1996 in Japan, the cause of multiple outbreaks of *E. coli* O157:H7 infection involving 11,826 cases was traced back to white radish sprouts (Meng et al., 2007).

Of all *E. coli* O157:H7 outbreaks reported on in the 1982 to 2002 study, 29% occurred in communities, 28% in restaurants, and 16% in schools (Rangel et al., 2005). According to both passive and active (i.e. Foodnet) investigations, outbreaks of *E. coli* O157:H7 are seasonal, peaking during the warmest months (Rangel et al., 2005). This trend may be the result of an increased survival and prevalence of the pathogen due to the increased ambient temperature, the increase in "cook-out" frequency by consumers, or, most likely, a combination of both (Meng et al., 2007).

Ecological Dissemination and Spread of EHECs

Pathogenic *E. coli* are carried in the gastrointestinal tracts of many mammals and other thermoregulators including humans, cattle and other livestock, domestic pets, rodents, birds, and other wildlife, and can be distributed to soil and water through fecal shedding and the agricultural application of manure (Beutin et al., 1993; Galiero et al., 2005; Meng et al., 2007;

Nielsen et al., 2004). Although the prevalence of STEC is generally highest in sheep (Kudva et al., 1996), in the human food chain, cattle are believed to be the primary source and ground beef is implicated in the majority of outbreaks (Meng et al., 2007). In 2002, a USDA national study found that 38.5% of dairy farms had at least 1 cow positive for fecal *E. coli* O157:H7, and that overall, 4.3% of individual cows were shedders of *E. coli* O157:H7 (Meng et al., 2007). Because *E. coli* O157:H7 is primarily a human pathogen, animals that carriers are not typically ill. However, evidence exists that *E. coli* O157:H7 can cause diarrhea and A/E lesions in neonatal calves (Naylor et al., 2003). Studies have shown that STEC are present in up to 60% of bovine herds, but in most cases, prevalence rates range from 10 to 25% (Meng et al., 2007).

In human patients, fecal shedding of *E. coli* O157:H7 usually lasts no more than 21 days following the onset of symptoms (Meng et al., 2007). The fecal carriage and dissemination of *E. coli* O157:H7 directly (from person-to-person), or indirectly (from person-to-food-to-person), has been repeatedly implicated in outbreak scenarios (Meng et al., 2007). Contributing to the significance of person-to-person transfer of *E. coli* O157:H7 is its extraordinarily low infectious dose. It should be noted that asymptomatic long-term carriage of pathogenic *E. coli* has not been observed (Meng et al., 2007). Interestingly, studies of dairy farm families have reveal elevated antibody titers against surface antigens of *E. coli* O157:H7, although there was no evidence of fecal contamination (Meng et al., 2007).

Listeria monocytogenes

Biology of *Listeria monocytogenes*

Listeria monocytogenes was discovered in 1926 by both E.G.D. Murray and James Pirie, independently, and named after Lord Joseph Lister (Rocourt, 1996). Between 1930 and 1950, only a few cases of listeriosis were reported. However, in the past 30 years following a 1981 outbreak in Nova Scotia that was traced back to contaminated coleslaw (Schlech et al., 1983),

listeriosis has emerged as a foodborne pathogen of major public health concern. The 1981 event was the first definitively documented case of foodborne transmission of listeriosis, confirmed through the use of a case-control study in combination with strain typing (Schlech et al., 1983). In the time since the Nova Scotia outbreak, there have been hundreds of cases of listeriosis reported annually (Rocourt and Jacquet, 1992).

Listeria monocytogenes is a gram-positive, non-spore forming, rod-shaped, facultative anaerobe with motility via peritrichous flagella at <30ºC. However, the bacteria do not synthesize flagellar protein at ≥37ºC (Grundling et al., 2004). When viewed under a light microscope, *L. monocytogenes* exhibits a characteristic tumbling motility (Farber and Losos, 1988). An intracellular pathogen, *L. monocytogenes* moves within eukaryotic cells at body temperature by polymerization of actin filaments (known as "comet tails" or "actin rockets"). Several *Listeria* spp., including *L. monocytogenes* are catalase-positive and oxidase-negative. The further biochemical identification of *L. monocytogenes* is limited, but includes hemolysis via the CAMP-test to differentiate from non-pathogenic *Listeria* species, and acid production from D-xylose (Swaminathan et al., 2007).

To a greater extent than most other foodborne pathogens, *L. monocytogenes* is widely distributed in the natural environment, resistant to low pH and high NaCl environments, is microaerobic, and psychrotrophic. *Listeria monocytogenes* can grow in moderate salt environments (6.5% sodium chloride), can grow in the presence of up to 12% NaCl, and will survive for long periods under higher salinity (Swaminathan et al., 2007). *Listeria monocytogenes* is particularly problematic due to its extreme environmental durability, survival, and ubiquity, and its ability to grow at low temperatures (Swaminathan et al., 2007). With a growth temperature range of 2 to 45°C, the average generation times of 39 different *L.*

monocytogenes strains were 43, 6.6, and 1.1 h, at 4, 10, and 37°C, respectively (Barbosa et al., 1994). Respective lag times were 151, 48, and 7.3 h. *Listeria monocytogenes* does not survive over 50°C, and is preserved or moderately inactivated at temperatures below 0°C (Swaminathan et al., 2007). In one study, the *ltrB* gene associated with low temperature growth was found to be unique to serotype 4b strains (Zheng and Kathariou, 1997).

The pH range for the growth of *L. monocytogenes* is a function of temperature and acid type (Swaminathan et al., 2007). Although previously thought to have a pH growth range between 5.6 and 9.6, on media, *L. monocytogenes* will grow at values as low as pH 4.4. Below pH 4.3, *Listeria* spp. can survive, but not multiply. When exposed to 0.1% acetic, citric, and lactic acids in TSB, growth is inhibited, and inhibition is inversely proportional to temperature (Swaminathan et al., 2007). The type of acid used influences the fate of *L. monocytogenes*, with lactic and citric acids being less bacteriocidal than acetic acid and equivalent pH values (Swaminathan et al., 2007).

Generally, the minimum water activity (a_w) for growth of most strains of *L. monocytogenes* is 0.93, with an optimum a_w >0.97. However, some strains with grow at a_w values as low as 0.9, and most strains will survive at a_w 0.83 (Shahamat et al., 1980). Of importance to food manufacturers who use low a_w to preserve their products, the thermal kill temperature of *L. monocytogenes* has an inverse relationship with a_w (Sumner et al., 1991).

The genome of virulent *L. monocytogenes* strains encodes a large number of surface, secretory, transport system, and regulatory proteins. This suggests a high degree of adaptability, and is in keeping with the wide variety of environmental survivability of *L. monocytogenes* (Glaser et al., 2001). The vast array of proteins at the disposal of *L. monocytogenes* also appears

to be a critical feature in the evolution of virulence and unique infectious process (Doumith et al., 2004).

Phylogenics and Subtyping of *Listeria monocytogenes*

The genus *Listeria* includes six species; *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi* (Swaminathan et al., 2007). While both *L. ivanovii* and *L. monocytogenes* are pathogenic to some animals, *L. monocytogenes* is the only Listeriae species considered pathogenic to humans. While there are 13 serotypes of *L. monocytogenes* that are all pathogenic, more than 90% of human cases (in which type has been identified) belong to serotype 1/2a, 1/2b, or 4b (Salova et al., 2005; Seeliger, 1986). Since 2005, multiplex PCR-based methods have been established for identifying serotype between different *L. monocytogenes* isolates (Doumith et al., 2005). In recent years, several molecular typing methods have been successfully used to identify specific strains within a given *L. monocytogenes* serotype. These molecular tools include multilocus enzyme electrophoresis, ribotyping, DNA microrestriction, DNA macrorestriction, and random amplification of polymorphic DNA (Graves et al., 1999). In the United States, PulseNet, a division of the U.S. Center for Disease Control and Prevention (CDC) is a network of laboratories that routinely uses highly standardized molecular methods to subtype foodborne pathogens in order to rapidly detect disease clusters that may have a common source. Since 1999, PulseNet has used pulsed field gel electrophoresis (PFGE) to subtype *L. monocytogenes*, and their exact protocol has been available since 2006 (Graves and Swaminathan, 2006).

Worldwide, all of the major foodborne outbreaks of listeriosis that occurred during 1980s, and 33 to 50% of sporadic cases, were caused by serotype 4b. Intriguingly, also during this period, most isolates recovered from food were strains of 1/2a and 1/2b serotypes (Swaminathan et al., 2007). This observation suggests that serotype 4b may be the most virulent

of the three. DNA from several strains of serotype 4b associated with different outbreaks have demonstrated a host-mediated resistance to Sau3AI and other restriction enzymes, some of which are likely associated with virulence factor genes (Zheng and Kathariou, 1997), and a presumed restriction modification system (85M, 85R, and 85S) specific to these strains has been identified (Zhang et al., 2003).

Wiedmann et al. (1997) defined three distinct lineages for *L. monocytogenes*, with different pathogenic potential. Lineage I isolates included serotypes 1/2b and 4b, and were later confirmed as the most predominate human pathogen of the three, through meta-analysis of studies examining clinical isolates of *L. monocytogenes*, also from the Wiedmann laboratory (Gaillard et al., 1991; Gray et al., 2004; Jeffers et al., 2001; Norton et al., 2001; Swaminathan et al., 2007). Lineage II isolates were of serotype 1/2a and 1/2c, and lineage III contained only animal isolates.

Clinical Features of Listeriosis

Although the number of annual cases is fairly low, listeriosis is a major public health concern due to the severity of the disease, which can cause meningitis, septicemia, and fetal abortion, resulting in a high fatality rate (20-30% of cases). These and other severe disease manifestations are often preceded by influenza-like symptoms including persistent fever, and gastrointestinal disturbances such as nausea, vomiting, and diarrhea (Gray, 1962). The onset time from early symptoms to more serious listeric pathology ranges from a few days up to 5 weeks (Swaminathan et al., 2007). Listeriosis mainly presents in individuals pre-conditioned with impaired T-cell-mediated immunity (Swaminathan et al., 2007). The most severely immunocompromised patients include those with predisposing statuses such as malignancy, organ post-transplant, immunosuppressive therapy, human immunodeficiency virus (HIV), and the more advanced elderly. Listeriosis is 300 times more likely to occur in AIDS patients than in

the general population (Schuchat et al., 1991). High-risk populations also include pregnant women, neonates, immunocompromised adults, and the elderly.

The infectious dose and course of disease caused by *L. monocytogenes* is a function of host immunological status, in combination with microbial virulence factors (Swaminathan et al., 2007). The dose necessary for gastrointestinal listeriosis to afflict low-risk populations is believed to very high. In five outbreak investigations involving the gastrointestinal listeriosis (non-bacteremic) infection of low-risk persons, ingested food contamination levels ranged from 1.9×10^5 to 1.6×10^9 CFU (Swaminathan et al., 2007). Incubation time for the enteric form of listeriosis is short, typically between 18 and 27 h (Swaminathan et al., 2007). Whether the *L. monocytogenes* strains that cause gastrointestinal listeriosis in low-risk populations possess additional virulence factors similar to common enteric pathogens is not known.

Animal exposure studies suggest that severity of disease features is inversely proportional to dosage level (Farber et al., 1991). However, animal models do not aid in the accurate determination of the minimum infective dose in humans. Based on observational data obtained during outbreak and sporadic case investigations, it appears that the infective dose for listeriosis in at risk populations is ≥100 CFU/g food ingested, or a total of less than 1,000 cells (Swaminathan et al., 2007).

In nonpregnant, immunocompromised adult, listeriosis primarily presents with septicemia, meningitis, and meningeocephalitis. Less common manifestations include endocarditis (in patients with preexisting cardiac lesions), and other types of focal infections including endoophthalmitis, septic arthritis, osteomyelitis, pleural infection, and peritonitis (Slutsker and Schuchat, 1999). Mortality in this disease population is between 20 and 30% (Swaminathan et al., 2007).

In pregnant women infected with *Listeria*, although disease features are often mild and mainly febrile in nature, *L. monocytogenes* is teratogenic and readily crosses the materno-fetal (i.e. placental) barrier, resulting in feto-maternal infection. Most cases of fetal infection result in spontaneous abortion or stillbirth. Surviving neonates of feto-maternal listeriosis less than 7 days old present with pneumonia and sepsis, while those over 7 days old manifest meningitis and sepsis (Slutsker and Schuchat, 1999).

Compelling evidence from two investigated outbreaks support the idea that gastrointestinal listeriosis can also occur in individuals having no predisposing condition, with febrile gastroenteritis being the most consistent symptoms (Aureli et al., 2000; Dalton et al., 1997; Slutsker and Schuchat, 1999). The outbreak investigated by Aureil et al. (2000) exclusively involved adolescent school children.

Pathogenesis of *Listeria monocytogenes*

The unique pathogenesis of *L. monocytogenes* is such that it is able to cross three host barriers; the intestinal barrier, the blood-brain-barrier (BBB), and the materno-fetal (placental) barrier. *Listeria monocytogenes* is an intracellular, neurotropic pathogen that can cause a variety peripheral local infections, including infections of CNS (Drevets et al., 2008; Vazquez-Boland et al., 2001). There are many gaps in our understanding of the precise mechanisms of *L. monocytogenes* pathogenesis (Swaminathan et al., 2007), but much of what is known comes from cell kinetic and animal model studies.

Internalin A (InlA) and InIB are the two main *L. monocytogenes* invasion proteins, with the transmembrane protein E-cadherin being the host-cell receptor site for InlA (Cossart and Toledo-Arana, 2008). InlB interacts with several host-cell receptors including gCiqR/p33, Met/HGFR (hepatocyte growth factor receptor), and GAGs (glycosaminoglycans). gC1qR/p32 is a peripheral membrane-bound protein known to interact with many viral proteins (Cossart and

Toledo-Arana, 2008). Met, a tyrosine kinase, is the receptor for HGF (hepatocyte growth factor), and likely the most important receptor for InlB (Cossart and Toledo-Arana, 2008). Both InlA and InlB functionally mimic the host signaling apparatus to exploit receptor-mediated endocytosis and gain cell entry.

Once within the host cell, *L. monocytogenes* neutralizes host cell vacuolar hydrogen peroxide through the production of superoxide dismutase. Vacuole escape may be through the co-opting of phagosomal machinery (Archambaud et al., 2006).

After penetrating the gastrointestinal epithelium, *L. monocytogenes* forces its path from cell to adjoining cell within the host using an actin-based propulsion system (Ramaswamy et al., 2007). Once monocytes, macrophages, or polymorphic leukocytes are invaded, the host becomes septicemic. Because *L. monocytogenes* is able to penetrate monocytes, it can be transported across the blood-brain-barrier (BBB) (Drevets et al., 2008; Ramaswamy et al., 2007). Specifically in mice, *L. monocytogenes* crosses the BBB through transport within the Ly-6C^{high} monocyte subpopulation (Drevets et al., 2008).

Listeriosis is not an exclusively foodborne disease. Although uncommon, nosocomial outbreaks of neonatal cross-infection, both through equipment cross-contamination and by cutaneous-route infection associated with the use of contaminated mineral oil, have been documented (Pejaver et al., 1993; Schuchat et al., 1991). Focal cutaneous infections by *L. monocytogenes* have also been observed in persons whom occupationally handle live-stock, such as farmers and veterinary workers (Swaminathan et al., 2007).

Epidemiology of Listeriosis

Pregnant women and prenatal infants are probably the most at risk subpopulations for listeriosis (Jackson et al., 2010). The recent emergence of *Listeria monocytogenes* as a major pathogen of public health concern is likely due in part to the convergence of many medical,

industrial, and social factors that have resulted in larger high-risk populations (Swaminathan et al., 2007). Medical factors include increased life expectancy from medical and public health improvements, the AIDS epidemic, and increased use of immunosuppressive therapies. Industrial and social factors include the centralization and consolidation in food production and processing, the global expansion of food distribution, an increase in the use of refrigeration, changes in handling practices, an expanding market for frozen convenience foods (or RTE; read-to-eat) and fresh produce (Swaminathan et al., 2007). Improved diagnostic methods and active public health surveillance may also contribute to the increase in reported incidences of listeriosis (Swaminathan et al., 2007).

Since the first confirmed foodborne outbreak of listeriosis in 1981, investigation of over 30 outbreaks have repeatedly concluded that consumption of contaminated food is the primary mode of listeriosis transmission (Swaminathan et al., 2007).

While outbreaks get the most publicity, many cases of human listeriosis are sporadic, although some cases thought to be sporadic may share an unrecognized common source (Swaminathan et al., 2007). Adding to the difficulty inherent to outbreak investigations, non-enteric listeriosis can manifest after a very long incubation time (up to 5 weeks), which can make obtaining accurate food histories especially difficult (Swaminathan et al., 2007). This practical difficulty, along with the inability to examine incriminated foodstuffs after such an extended period, often makes *L. monocytogenes* traceback an impossible task. For this reason, although the majority of cases of listeriosis are thought to be foodborne, it is often impossible to positively identify a source (Swaminathan et al., 2007).

Listeriosis Outbreaks Associated with Fresh Produce

In 1979, an outbreak of *L. monocytogenes* associated with raw produce—specifically raw celery and lettuce served as garish with three foods—was suspected, although the food source

was not positively confirmed (Ho et al., 1986). In the 1981 Nova Scotia outbreak—which afflicted 34 pregnant women and 7 nonpregnant adults—coleslaw was the suspected vehicle of transmission. The epidemic strain was later isolated from an unopened package of this locally prepared product, made with cabbage that had been fertilized with the manure of diseased sheep being the likely source (Schlech et al., 1983). In the above case, harvested cabbage had been stored in an unheated shed over winter and spring; a possible environmental advantage for the survival of this psychrophile.

In 2010, an outbreak of listeriosis occurred in Texas involving 10 cases and 5 deaths over an 8-month period. Sangar Fresh Cut Produce Company was ordered by the Texas Department of State Health Services (DSHS) to recall all products shipped since January 2010, after their investigation discovered chopped celery positive for *L. monocytogenes*. Genetic subtyping of isolates from the implicated celery linked it to 6 of the 10 cases of listeriosis. At the Sangar processing plant, inspectors cited several sanitation problems including condensation above food products, soil on a preparation table, and hand-washing lapses (FDA, 2010).

Reservoirs of *Listeria monocytogenes*

Listeria monocytogenes has been isolated from fecal matter of healthy birds, deer, ruminates, and many other animals (Swaminathan et al., 2007; Weis and Seeliger, 1975). *Listeria monocytogenes* has been found in grasses and silage—associated with transmission of animal listeriosis—as well as on various other naturally decaying vegetation (Husu et al., 1990; Weis and Seeliger, 1975; Welshimer, 1968). In the Netherlands, *Listeria* spp. have been found in samples from canal surface water, in lakes, in polder ditch water, and in California sewage and freshwater bay tributaries (Colburn et al., 1990; Dijkstra, 1982; Geuenich et al., 1985). In 2010, a survey conducted at Finger Lakes National Forest in New York confirmed the presence of four *Listeriae* isolates with phylogenetic relatedness close to, but clearly distinct from *L.*

monocytogenes in the soil, as well as in stagnate, and flowing waters (Graves et al., 2010). *Listeria* spp. have also been found on alfalfa and other crops grown in soil enriched with sewage sludge (Alghazali and Alazawi, 1990). In one study involving radishes growing in soil inoculated with *L. monocytogenes*, 50% of recovered samples were confirmed positive after 3 months (Vanrenterghem et al., 1991). The apparent abundance of *L. monocytogenes* in soil is like due to contamination through detritus and fecal matter, encouraged by the cool, moist soil ecosystem with nutrients being supplied from the decaying organic materials (Fenlon, 1999).

Human Carriage of *Listeria monocytogenes*

Although listeriosis does not generally afflict healthy individuals, the healthy human population are possible asymptomatic carriers of *L. monocytogenes*, and may aid in its environmental distribution through fecal shedding (Swaminathan et al., 2007). *Listeria monocytogenes* has been isolated from up to 6% of fecal samples from health individuals (Slutsker and Schuchat, 1999), and 21.6% of symptomatic patients (Jensen, 1993). Fecal specimens from patients with listeriosis were much higher than asymptomatic carriers, with 21% carrying $\geq 10^4$ CFU/g fecal matter. Supporting the notion that *L. monocytogenes* is subject to gastrointestinal carriage and spread between people, one study involving six sporadic cases found that 21% of symptomatic patients and 18% of asymptomatic household contacts defecate the same serotype and multilocus enzyme type of *L. monocytogenes* as the patient (Schuchat et al., 1993). However, it should be noted that in some of these cases, an undetermined shared food vector between symptomatic and asymptomatic contacts may have been responsible for the intestinal carriage of both. Also of note, the prevalence of *L. monocytogenes* in human stool is low, and the duration of fecal shedding is short, which argues against the appropriateness of routine screening of workers whom handle high-risk foods as a prevention tool for the fecal

spread of this pathogen, and possible transmission to food, or to at-risk worker contacts (Grif et al., 2003; Sauders et al., 2005).

Listeria monocytogenes in Food

Considering the high degree of environmental adaptability and ubiquity of *L. monocytogenes*, there are many potential routes of transmission between ecosystems; animal fecal transport and water carriage being particularly suspect (Swaminathan et al., 2007). By whatever means distributed in nature, the transmission of *L. monocytogenes* to food processing plants and into food products can be attributed to worker contamination, transport equipment, from animal feces or hides, from raw plant or animal tissue, and in produce from contaminated food crops themselves, pre-harvest (Swaminathan et al., 2007).

Since the growth of *L. monocytogenes* is favored by the presence of water (though requirements are small) and nutrients, its detection in moist areas of food processing plants such as water drains, residues, and in processing equipment, is little surprise (Cox et al., 1989). The bacteria can strongly attach to many kind of surfaces including stainless steel, glass, and rubber, and is known to form single-, as well as multi-species biofilms on food contact surfaces at 10 and 21ºC (Jeong and Frank, 1994a; Jeong and Frank, 1994b), making it particularly difficult to eliminate from processing facilities (Gravani, 1999). Contaminated contact surfaces and dicing machinery can repeatedly contaminate RTE foods (Lunden et al., 2002). *Listeriae* have also been observed to remain on human hands after washing, and can be aerosolized (Swaminathan et al., 2007). The presence of *L. monocytogenes* inside food processing plants is so common that they could be considered a reservoir, as contaminated effluents from these plants can noticeably increase environmental spread of this species (Swaminathan et al., 2007).

Epidemiological investigations of outbreaks, along with active surveillance of sporadic cases have demonstrated that some ready-to-eat (RTE) foods are high-risk vehicles for the

transmission of listeriosis within susceptible populations (Swaminathan et al., 2007). These foods are typically preserved through refrigeration and supply *L. monocytogenes* with an appropriate environment for growth during manufacture, transportation, and storage. Such foods include fresh-cut produce, soft unfermented cheeses, minimally reheated frankfurters, certain delicatessen meats, foods mixed with mayonnaise, and some seafoods (Farber, 2000; Swaminathan et al., 2007). Multiple risk assessments for *L. monocytogenes* in RTE foods have been performed (Crepet et. al., 2007; Erickson, 2010; Farber, 2000; FDA, 2004; Harris et al., 2003; McLauchlin et al., 2004; WHO, 2004). Canadian regulatory policy mandates inspection and compliance action on RTE foods that are known to support the growth of *L. monocytogenes*, with the highest priority for high-risk foods that have a shelf life greater than 10 days (Farber, 2000). In 2003, the FDA in collaboration with the USDA Food Safety and Inspection Service (FSIS) and the CDC, published a table documenting the results of a risk assessment for RTE foods, with each food-type ranked in order of risk. According to the table, delicatessen meats were the greatest risk of all foods tested, and was predicted to be the vector for over 1,598 cases of listeriosis, annually (Swaminathan et al., 2007). Also according to this study, fruits and vegetables were considered low-risk vectors for the transmission of human listeriosis, with a median of less than one case per year. However, it should be noted that this does not account for the further handling, processing, packaging, and shipping that is involved with the commercial distribution of fresh-cut vegetables.

To model this risk assessment, five factors were determined to affect consumer exposure to *L. monocytogenes* during food consumption; 1) the amount and frequency of consumption of each food thought to pose a potential risk, 2) the frequency and level of the bacteria in each type of RTE food, 3) the growth potential of *L. monocytogenes* in a food during refrigeration, 4)

storage temperature, and 5) duration of storage prior to consumption (Swaminathan et al., 2007). By changing the parameters of the risk assessment model, the researchers were able to estimate the impact of control strategies. For example, according to the model, the number of estimated cases of listeriosis could be reduced by 69% if all home refrigerators consistently operated at or below 7.2°C. It was also estimated that the median number of case of listeriosis among the elderly could be reduced by 13.6% if the maximum storage time of deli meat was reduced from 28 to 14 days (Swaminathan et al., 2007).

Listeria monocytogenes in Celery and Other Fresh Produce

Once contaminated, fresh vegetables carrying *L. monocytogenes* are often eaten without cooking, irradiation, or chemical treatment, making listeriosis a serious problem, particularly with regard to high-risk populations. The relatively small number of outbreaks for which *L. monocytogenes* is responsible is likely due to the low number of high-risk persons.

Produce, and especially fresh-cut RTE produce items have been increasingly linked to foodborne outbreaks (Sivapalasingam et al., 2004). Crop contamination occurs both pre- and post-harvest, with cross-contamination documented to occur through both water and harvest equipment, though the most frequent contamination vector is the incoming product (Erickson, 2010). Pathogen survival during storage is dependent on storage conditions and product type. Chemical intervention can effectively reduce the potential for cross-contamination from harvest and processing equipment or through water, but its ability of remove pathogens from the product is quite limited (Erickson, 2010).

In 2007, a Bayesian approach meta-analysis of 165 studies designed to estimate the prevalence and concentration of *L. monocytogenes* on fresh vegetables (including celery) contaminated with more than 1, 2, and 3 log/CFU were observed in 1.44, 0.63, and 0.17% of all samples, respectively (Crepet et al., 2007). In North America, Europe, and Asia, *L.*

monocytogenes has been isolated from many vegetables, including celery, bean sprouts cabbage, cucumbers, leafy greens, potatoes, prepackaged salads, radishes, and tomatoes (Audurier et al., 1980; Crepet et al., 2007).

In many cases of foodborne human listeriosis, food contamination does not occur on the production and processing end of the supply chain, but through persistent *L. monocytogenes* strains in retail environments (Vazquez-Boland et al., 2001). With the myriad of possible cross-contamination points, it is often impossible to ascertain the precise contamination point in any given case or outbreak. In one survey on the microbial safety of fresh-cut RTE celery and other vegetables, *L. monocytogenes* was detected on 8 of 120 (6.7%) fresh-cut celery samples stored at 10°C, with 0 of 175 samples positive at 4°C (Odumeru et al., 1997). One Chilean survey detected *L. monocytogenes* on fresh-cut celery in supermarket-prepared salads in 2 of 13 (15.4%) samples taken (Cordano and Jacquet, 2009).

Listeria monocytogenes, Industrial Control Points

Much discussion of the problem of *L. monocytogenes* in various food-processing environments, as well as possible control methods were provided by Gravani (Gravani, 1999). In 2002, researchers proposed a six-step *Listeria* control program for processing plants (Tompkin, 2002). The steps included, 1) prevention of establishment of *Listeria* spp. in niches and other sites where the bacteria may take hold, 2) implementation of sampling programs, 3) rapid and effective response to positive sampling, 4) follow-up verification, 5) short-term assessment of the most recent samplings to facilitate early detection and determine trends or growth patterns, and 6) long-term assessment to identify scattered contamination events and measure progress. The environmental sampling design and response to positive findings determines the effectiveness of the *Listeria* control program in food-processing facilities (Tompkin, 2002).

Modern methods of food preservation including the use of chemical preservatives, vacuum-packaging, and modified atmosphere packaging (MAP) may not be sufficient hurdles to the foodborne transmission of listeriosis (Swaminathan et al., 2007). Bacteria-derived antibiotic agents such as pediocins, bacteriocins, and lactic acid can inhibit or destroy *L. monocytogenes* cells to varying degrees. However, caution is warranted in the use of bacteriocins as anti-listerial agents since it is known that *L. monocytogenes* can become antibiotic resistant to bavaricin A and nisin (Davies and Adams, 1994; Davies et al., 1996; Larsen and Norrung, 1993). Certain essential oils such as carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde, and cinnamic acid have demonstrated anti-listerial activity by partitioning lipid membranes and thereby increasing cell permeability and osmolytic potential (Burt, 2004).

Low dose irradiation and heating to ≥50ºC effectively destroys *L. monocytogenes* on fresh produce (Prakash et al., 2000; Swaminathan et al., 2007). As well, heat-stress can reduce the virulence of *L. monocytogenes* (Swaminathan et al., 2007).

Food Regulations for *Listeria monocytogenes*

Due to its frequent occurrence of in foods and processing environments, and the impracticality of maintaining zero tolerance, regulatory agencies in some countries—including Canada and France—have legislated policies which direct inspection and compliance to set tolerance levels for the *L. monocytogenes* in high-risk foods (Swaminathan et al., 2007). In contrast, the U.S. and U.K. both currently maintain a "zero tolerance" (0.04 CFU/g tolerance in the U.S.), despite their acknowledgement of the widespread distribution of *L. monocytogenes* and difficulty in the production of RTE foods free from contamination (Shank et al., 1996). This zero tolerance position is argued for based on the idea that setting "acceptable" levels for *L. monocytogenes* would require more data than is currently available on the number of *Listeriae* which do not cause human disease (Shank et al., 1996).

Salmonella

Biology of *Salmonella*

Salmonella is widely distributed throughout nature and has been isolated from soil, water, foods, and animal gastrointestinal tracts (Anderson and Ziprin, 2001; Nisbet and Ziprin, 2001) Coupled with this widespread distribution, intensive husbandry practices used in the meat, poultry, fish, and shellfish industries along with the recycling of offals and inedible raw materials into animal feed has favored the persistence of *Salmonella* in the global food chain (D'Aoust, 1994; Doyle, 1989).

In 1885, Salmon and Smith were the first to isolate and identify *Salmonella* (S*almonella enterica* serovar Choleraesuis) from swine suffering from hog cholera (Le Minor, 1981). In the early 1900s, serological advances led to methods for detection of the somatic (O) and flagellar (H) antigens of *Salmonella* through exploitation of antigen-antibody specificity (Le Minor, 1981). Subsequently in 1941, White and Kauffmann devised an antigenic scheme for *Salmonella* classification—the Kauffmann-White scheme—now consisting of more than 2,500 serovars (Popoff et al., 2004).

Salmonellae grow optimally at 37°C, but have a growth range of 2 - 54°C (D'Aoust et al., 1975). They are gram-negative, rod-shaped facultative anaerobes of the family *Enterobacteriaceae.* Salmonellae are usually mobile via peritrichous flagella, with serovar Pullorum and Gallinarum the aflagullate exceptions, along with various mutant strains (D'Aoust and Maurer, 2007). Salmonellae are chemoorganotrophs with both respiratory and fermentative metabolic pathways. Often identified through presumptive biochemistry, salmonellae produce acid and gas byproducts from carbohydrate catabolism on Triple Sugar Iron growth media. Generally, during *Salmonella* metabolism of *D*-Glucose, thiosulfate is utilized as a terminal electron acceptor, being reduced to hydrogen sulfide gas (D'Aoust and Maurer, 2007). H_2S can

also react with ferrous sulfate to form ferrous sulfide, a black precipitate. Additionally, most salmonellae enzymatically decarboxylate lysine to cadaversine in a lysine iron media, are oxidase negative, catalase positive, can metabolize citrate as a sole carbon source, and do not hydrolyze urea (D'Aoust and Maurer, 2007).

Although the above describes a template for biochemical identification, *Salmonella* is far from a metabolically homogeneous genus, and most serovars cannot be specifically distinguished through biochemical testing. Distorting the diagnostic landscape is the increasing occurrence of biotypes that can hydrolyze urea, produce indole from tryptophan, are not inhibited by KCN (potassium cyanide), and do not decarboxylate lysine (D'Aoust and Maurer, 2007). Because *Salmonella* metabolism of lactose and sucrose is plasmid-mediated and therefore subject to genetic transfer and exchange, genetically atypical serovars and strains may escape detection by disaccharide nutrient-source plating (Doyle, 1989). For this reason, bismuth sulfide agar is the preferred media, which utilizes secondary metabolic production of H_2S gas as an indicator (Doyle, 1989). However, when serovar-specific identification is needed, targeted biomolecular analysis aimed at identifying stable genetic loci or protein products may be required.

Salmonellae demonstrate high inter- and intraspecific variability and are adaptable to extreme environments. Although considered mesophiles, some strains can grow at elevated temperatures (≥54°C) (Droffner and Yamamoto, 1991), while others exhibit psychrotrophic properties in their ability to grow on foods at refrigeration temperature (2 to 4°C) (D'Aoust et al., 1975), which raises concerns regarding the efficacy of cold-induced bacteriostasis. The adaptability of salmonellae has also been demonstrated in their ability to grow at pH values ranging from 4.5 to 9.5, with an optimum growth pH of 6.5 to 7.5 (D'Aoust and Maurer, 2007). In addition, the ecological adaptability inherent to a facultatively anaerobic nature contributes to

salmonellae adaptability. *Salmonella* has been shown to grow under modified atmospheric conditions containing low levels CO_2 (20 to 50% [vol/vol]), on raw beef and cooked crabs meat (Bergis et al., 1994; Ingham et al., 1990).

Salmonella Pathogenesis

To cause infection, *Salmonella* must survive and surpass an array of non-specific host defense hurtles including lactoperoxidase in saliva, gastric acidity, mucoid secretions from intestinal goblet cells (which containing IgA antibodies), intestinal peristalsis, sloughing off of luminal epithelial cells, and various nonspecific phagocytes, coupled with adaptive immunity associated with T and B lymphocytes (particularly those localized to Peyer's patches), and the immunologically innate complement system of pathogen inactivation (D'Aoust and Maurer, 2007). Invading *Salmonella* must also outcompete the normal intestinal microflora for resources and the space necessary for enterocyte penetration.

Diarrhea is a physiological response to *Salmonella* infection and is the result of enterocolitis coinciding with increased goblet cell mucus secretion, and extensive leukocyte translocation into the infected tissues, and release of leukocytic prostaglandins triggering mucosal inflammation as well as activating epithelial cell adenyl cyclase, and signaling increased fluid secretion into the intestinal lumen (Guttman and Finlay, 2008; Kaufmann et al., 2001; Polotsky et. al., 1994). Failure of host defense systems can result in septicemia and degenerate into chronic sequelae such as aseptic reactive arthritis, Reiter's syndrome, and ankylosing spondylitis (D'Aoust and Maurer, 2007).

Upon contact with luminal epithium surface, *Salmonella* are induced to synthesize type 1 fimbriae, and transient, external, membrane-bound proteinaceous invasion appendages (Collazo et al., 1995; Ginocchio et al., 1994). Once attached, signal transduction leads to the host-cell

pathogen pinocytation, followed by bacterial proliferation (Garcia-Del Portillo and Finlay, 1994; Ginocchio et al., 1994; Jones et al., 1994).

Confined to endocytotic vacuoles within the host cell, *Salmonella* replicate within hours following internalization (Garcia-Del Portillo and Finlay, 1994). Infected vacuoles migrate from apical to basal pole, where *Salmonella* is exocytosed into the lamina propria mucosae (Isberg and Tran Van Nhieu, 1994; Polotsky et al., 1994). Although the mechanism which allows *Salmonella* to migrate into deeper layers of tissue is poorly understood, it is likely that the proteolytic enzyme plasmin present on the bacterial cell surface facilitates further transcytosis (Sjobring et al., 1994).

The putative and highly conserved *Salmonella* virulence factor that causes diarrhea is diarrheagenic enterotoxin. Release of the toxin into host epithelial cell cytoplasm precipitates activation of membrane-bound adenyl cyclase and increased cytoplasmic concentrations of cyclic AMP in host cells. A concurrent fluid efflux into the lumen results in increased concentration of Cl^- ions in the mucosa and decreased Na^+ absorption, increasing net fluid exsorption (D'Aoust, 1991). In addition to enterotoxin, *Salmonella* serovars generally manifest an outer-membrane-bound heat-labile cytotoxin which functions pathogenically through the inhibition of host protein synthesis and lysis of host cells, promoting further salmonellae dissemination into host tissue (D'Aoust, 1991; Koo et al., 1984).

Systemic migration exposes invading bacteria to phagocytes, leukocytes, and prevailing antibacterial conditions in the cytoplasm (D'Aoust, 1991). Host genotype, as much as the virulence of the invading salmonellae, determines whether a systemic infection will take hold and whether development of enteric fever occurs (Schwan et al., 2000; Skamene et al., 1998).

Iron is an essential micronutrient found only in small concentrations within eukaryotic cells. Having invaded a host, the virulence of *Salmonella* is proportional to its ability to outcompete host cells and scavenge intracellular iron through an extracellular membrane bound siderophoric receptor (an enterochelin) (D'Aoust, 1991).

Environmental factors such as low pO_2 and high osmolarity also enhance bacterial virulence by altering the superhelicity of chromosomes, which influences the level of transcription of invasion-related genes such as *invA* (Falkow et al., 1992; Garcia-Del Portillo and Finlay, 1994). It is interesting to note that these conditions are consistent with those found within mammalian intestinal epithelial cells (Falkow et al., 1992).

Genetic Determinants of Virulence in *Salmonella* Spp.

Comparative genomic studies (between *E. coli* and *Salmonella* spp.) have revealed that many *Salmonella*-specific genes are clustered together in chromosomal loci known as pathogenicity islands. These islands generally map next to tRNA genes and are often bordered by phage gene remnants. Additionally, pathogenicity islands usually have a lower GC (guanine-cytosine) content than is found in metabolic and housekeeping gene sequences, suggesting horizontal gene transfer was involved in the evolution of virulence in *Salmonella* spp. (Schmidt and Hensel, 2006). In *Salmonella enterica*, these genomic islands encode for virulence factors essential to pathogenesis, and are the defining characteristics of this genus and species (Baumler et al., 1998). However, not all genes that enhance virulence are associated with pathogenicity islands. For example, genes of various fimbriae operons—which are believed to be important contributors to *Salmonella* host-cell colonization—and exogenous, gastrointestinal hydrogen sequestration and respiration—which is thought to provide invading *Salmonella* with a competitive advantage—are not localized within pathogenicity islands (Maier et al., 2004; van Der Velden et al., 1998). The *Salmonella* pathogenicity island SPI1 is highly conserved within

the species and between serovars, making it a useful gene target for PCR-based detection in foods (Chen et al., 1997).

There is a great deal of intraspecific, genetic variation in *S. enterica*, with 2 to 8% of the Typhimurium LT2 gene complement (ca. 4,500 genes) absent in one or more serovars (Chan et al., 2003; Porwollik et al., 2004). Much of this variability is attributed to various virulence genes found, mobile gene elements (i.e. phages, plasmids, and transposons) and pseudogenes (i.e. those derived from point mutation, insertion, or deletions that result in nonsense or frameshift mutations) and the distribution of these elements among serovars (Ahmer et al., 1999; Bacciu et al., 2004; Brussow et al., 2004; Edwards et al., 2002; Parkhill et al., 2001). *Salmonella* spp. have evolved such that acquisition of mobile gene elements and pseudogenetic polymorphisms can have a profound impact on the behavior specific serovars in vivo (D'Aoust and Maurer, 2007). Epigenetically, subtle changes in expression specific to a serovar (Monsieurs et al., 2005; Winfield and Groisman, 2004) may have an even more profound impact on host adaptation than mobile element variation (Encheva et al., 2005; McClelland et al., 2004; Monsieurs et al., 2005; Porwollik et al., 2004; Porwollik et al., 2005; Winfield and Groisman, 2004). Subtle mutations in promoter and regulatory sequences may also explain differences in behavior that occur within bacterial populations (Koutsolioutsou et al., 2001; Robbe-Saule et al., 2003).

Etiology and Clinical Features of Human Salmonellosis: Nontyphi Serotypes

Salmonellosis is the enteric or systemic disease caused by *Salmonella*. Manifestations of the disease can vary depending on serotype and host. Certain highly host-adapted serotypes cause disease almost exclusively in their specific hosts, while others exhibit a broad range of host specificity (Ziprin and Hume, 2001). In humans, the most highly host-adapted organism known to medicine is *Salmonella* Typhi, which causes typhoid fever (Anderson and Ziprin, 2001).

Exclusive to humans, *Salmonella* Typhi is contracted from water or food contaminated by another human source (Ziprin and Hume, 2001; Ohashi, 1988).

Salmonella Choleraesuis is a swine pathogen that also causes highly fatal systemic infections in humans (Ziprin, 1994). The high fatality rate of *Salmonella* Choleraesuis is due in part to the fact that the infection can cause mycotic aneurysms (infection and weakening of the arterial walls) in human hosts. Fortunately the incidence of *Salmonella* Choleraesuis is low, which may be attributable to the fact that most people are aware of the importance of properly cooking pork in foodborne disease prevention.

Salmonella Enteritidis and *Salmonella* Senftenberg are somewhat host-adapted to chickens and turkeys, respectively (Ziprin, 1994). Both are capable of causing severe human illness and death. *Salmonella* Enteritidis is considered to be one of the major problem organisms among human bacterial diseases (Ziprin and Hume, 2001), and of particular public health concern because chicken eggs are sometimes contaminated by this serovar, which can lead to infection if eggs are not properly cooked (Ziprin, 1994). *Salmonella* Dublin is another host-adapted serovar. *Salmonella* Dublin causes septicemia in cattle and can cause zoonotic infections in human beings through the consumption of unpasteurized milk.

Except as noted above, most *Salmonella* serovars that infect humans are not host-adapted, but are instead found in a wide range of animal products, fruits, vegetables, and processed foods (Anderson, 2001). Although these serovars tend to be less virulent, they are responsible for the majority of cases, primarily presenting as uncomplicated enterocolitis (Hunter, 1997; Underman, 1997; Ziprin, 1994).

All human cases of nontyphoid salmonellosis share many common clinical features. Onset is rapid (but ranges from hours to days depending on dose), and symptoms typically

include enterocolitis and diarrhea, although systemic infections can occur (Hui, 2001). Death is possible, especially with more virulent strains, and is more likely to occur in high-risk subpopulations (i.e. the very young, aged, and immunologically compromised). The etiological sources of non-Typhi *Salmonella* spp. include food, water, animals and animal products, and human feces (Hui, 2001). Although many cases of salmonellosis are self-resolving, antibiotics such as choloramphenicol and ampicillin are the mainstay treatments in cases where medical intervention is necessary (Hui, 2001). Possibly because of global overuse of antibiotic therapy, the prevalence of multidrug resistant (MDR) strains is ever-increasing (Hui, 2001).

Epidemiology of Salmonellosis Associated with Produce

Between 1990 and 2005, 18% of all cases of produce-associated foodborne outbreaks were cause by *Salmonella* (CSPI, 2009). In all cases from 1998 to 2002 where the causative agents were determined to be bacterial and were serologically identified, *Salmonella enterica* accounted for the greatest proportion (9%). In 2001, the CDC reported 46 confirmed outbreaks of *Salmonella* Enteritidis (Tucker, 2003). Those outbreaks resulted in 1,681 reported illnesses, 102 hospitalizations, and no deaths. In 24 (52%) of the 2001 cases where phage typing was performed, type 8 accounted for 28%, despite being sourced to different geographic locations and food items. The dispersed character of these outbreaks from a common strain suggests that contamination likely occurred at a critical point prior to distribution (i.e. during the production or processing stages). Of the 2001 outbreaks where a location of consumption was known, 61% occurred in commercial food establishments (e.g. restaurants, caterers, etc.), 2% in long-term care facilities, 4% in correctional facilities, and 30% in the general community (i.e. private homes, day care facilities, community centers, and religious meeting places). From 1998 to 2002, 306 (31%) of outbreaks of foodborne bacterial pathogens where etiology was sourced to a location of consumption occurred in the home (Lynch et al., 2006).

Proportional to the total volume consumed, the amount of contaminated fresh produce is relatively small (CAST, 2009). However, the total number of outbreaks involving fresh fruits and vegetables is significant and increasing. In the 1990s, at least 12% of foodborne outbreaks were associated with fresh produce (FDA, 2004). Between 1973 and 1997, 190 produce-associated outbreaks involving 16,058 illnesses and 8 deaths were reported to the CDC (Sivapalasingam et al., 2004). The mean number of these outbreaks increased from 4.4 per year between 1973 and 1987, to 9.75 per year between 1987 and 1997 (Tauxe, 1997). As a percentage of all reported outbreaks, those associated with produce increased from 0.7% in the 1970s to 6% in the 1990s (Sivapalasingam et al., 2004). Also during this time, the annual median number of reported incidences per outbreak related to fresh produce increased from 21 to 43. Continuing on this upward trend over the next 4 years from 1998 to 2002, the number of reported outbreaks increased to 279, with 10,533 illnesses and 7 deaths (Lynch et al., 2006). Therefore, despite significant effort and governmental resources dedicated to prevention (CAST, 2009), the frequency of produce-related foodborne illness outbreaks has increased, and outbreaks tend to involve a larger numbers of people. According to De Roever (1998), these increases are due to, 1) centralization and amplification of production; 2) increased regional and global distribution; 3) increased consumption of fresh or minimally processed products; 4) increased popularity of salad bars and restaurant dinning; and 5) increased consumer preference for organically cultivated produce, which may increase risk of using improperly composted manure. However, new technologies in biomolecular traceback and serotyping are epidemiological confounding factors, contributing to the increase in documented outbreaks but not necessarily reflecting an actual increase in disease frequency.

CHAPTER 3
MATERIALS AND METHODS

Strain Information

Antibiotic resistant bacterial stock strains of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella*, from the Danyluk laboratory culture collection were used in all experiments. These cultures were stored at -80°C in tryptic soy broth with 15% glycerol on 3 mm glass beads.

A five-strain cocktail of *E. coli* O157:H7, was used (Table 3-1). These strains included, H1730 (isolate from lettuce outbreak; human feces), F4546 (isolated from alfalfa sprout outbreak; human feces), 223 (isolated from the Odwalla apple juice outbreak), EC4042 (isolated from spinach outbreak), and F658 (isolated from cantaloupe outbreak).

Four strains of *L. monocytogenes* were used (Table 3-2), including LCDC 81-861 (isolated from raw cabbage outbreak), SCOTT A (isolated from perinatally transmitted human milk outbreak), 101M (isolated from beef outbreak), and strain V7 (isolated from cow milk outbreak).

Five serovars of *Salmonella* were used to create a cocktail (Table 3-3), which included: Michigan (LJH 521, isolate from cantaloupe outbreak; human feces), Montevideo (G4639, isolate from tomato outbreak; human feces), Enteritidis phage type 30 (ATCC BAA-1045, isolated from raw almond outbreak; human feces), Agona (LJH 517, isolated from alfalfa sprout outbreak; human feces), and Gaminara (F2712 isolated from orange juice outbreak; human feces).

All strains used were antibiotic resistant. *Escherichia coli* O157:H7 and *Salmonella* strains were resistant to 100 μg/ml rifampicin (Rif), while *L. monocytogenes* strains were resistant to 50 μg/ml nalidixic acid (NA). Antibiotic resistance was achieved through a stepwise

exposure to increasing concentrations of antibiotic, for the purpose of inhibiting the growth of background microflora (Parnell et al., 2005).

Preliminary Experiments

For each pathogen, small, trial experiments were performed before full-scale experiments in order to determine the necessary inoculum levels and to minimize excess plating during recovery. These experiments were performed at three temperatures (4, 12, and 22°C) and followed the same procedure as the statistically significant replications discussed below. For purposes of increasing the level of detection, some trials varying the volume of recovery buffer were performed to determine the lowest volume necessary to achieve adequate sample maceration.

To determine whether atmospheric levels of O_2 and CO_2 within sealed bags and containers were altered by sample respiration, six uncontaminated celery samples (three in bags and 3 in containers) were held at 4, 12, or 22°C (N=18), with atmospheric prob readings recorded once every 24 h. Data were recorded in $\%O_2$, and assumed $\%CO_2$ accumulation was calculated as the difference from initial O_2. At 22°C, data was collected over 3 days, and at 4 and 12°C, data was collected over 7 days.

Celery

Prior to each experiments, bags of fresh-cut celery advertised as "washed and ready-to-eat", were obtained from a local supermarket in Winter Haven, FL. Celery was purchased in the afternoon, within one day of store delivery and held at 4 ± 2°C for up to 24 h. Immediately prior to inoculation, celery sticks were further cut to 10 ± 1 g using a sterile, serrated knife and cutting board.

Antibiotic and Growth Medium Preparation

Stock solutions of NA were prepared by dissolving 0.5 g in 100 ml of deionized water, resulting in a 5,000 μg/ml solution, which was then filter-sterilized (0.20 μm pore size) and stored in the refrigerator (4 ± 2°C) until use. For *L. monocytogenes* experiments, 10 ml of stock NA was added to 1 L cooled (ca. 45°C) modified Oxford media (MOX), resulting in a final concentration of 50 μg/ml NA in the media (MOXN).

Stock solutions of Rif were prepared by dissolving 1.0 g in 20 ml methanol, resulting in a 50 μg/ml solution, which was filter-sterilized as described above, and stored in the refrigerator until use. For *E. coli* O157H7 and *Salmonella* experiments, 2 ml of stock Rif was added to 1 L of liquid media (ca. 45°C), resulting in a final concentration of 100 μg/ml. For *E. coli* O157:H7, the nonselective media used was tryptic soy agar (TSA), and sorbitol MacConkey agar (SMAC) the selective media. For *Salmonella*, TSA was also the nonselective media used, and bismuth sulfite sgar (BSA) as the selective media.

All growth media, were supplemented with one of the above-referenced antibiotics (TSAR, BSAR,SMACR, MOXN), with the exception of TSA destined for enumeration of background microflora.

Inoculum Preparation

Prior to each replication, frozen stock cultures of each strain were streaked onto TSAR or TSAN and incubated at 35 ± 2°C for 24 h. A single colony from each strain was then transferred to 10 ml tryptic soy broth (TSB) with 100 μg/ml Rif (TSBR) or 50 μg/ml NA (TSBN) two times at 24-h intervals, and incubated at 35 ± 2°C for 24 h, prior to their use as inocula. Each culture was centrifuged at 3,000 × g for 10 min. Cells were washed twice in 10 ml 0.1% peptone after pouring off the supernatant and resuspending. Washed cells were resuspended in half the original culture volume. Each strain was plated out on TSAR or TSAN to confirm cell

concentration. All cocktail strains were then combined in equal volumes (2 ml of each strain) to make final inoculum cocktail. The cocktail was then diluted in 0.1% peptone to arrive at a final inoculum concentration of ca. 10^7 CFU/ml. The cocktail was stored on ice for up to 1 h, prior to sample inoculation.

Inoculation and Storage

Celery was spot-inoculated with 10 µl (3-5 drops) of the cocktail on either the cut or the uncut surface, resulting in ca. 10^5 CFU/10 g sample, prior to drying at ambient temperature for 1 h in a biosafety cabinet. Post-drying concentrations were ca. 10^3 CFU/g.

After drying, each sample was transferred to either a 16.5 x 8.25 cm polyethylene plastic press-to-seal snack bag (Great Value®) or 414 ml polyethylene "double-seal lidded" container (Gladware® Side Dish) and stored at one of three temperatures: 4 ± 2°C, 12 ± 2°C, or 22 ± 2°C.

Enumeration

For samples held at 4 or 12 ± 2°C, populations were enumerated after 0, 1, 3, 5, and 7 days, and for samples held at 22 ± 2°C, populations were enumerated after 0, 0.33, 0.71, 1, and 2 days. At each sampling point, samples were transferred into 1.6 L whirl-pak bags, combined with 40 ml 0.1% peptone solution, and placed in a stomacher at high speed for 1 minute. Serial dilutions where made in 0.1% peptone and surface plated (0.1 ml) in duplicate onto selective and nonselective media containing Rif or NA. To increase the limit of detection to 0.6 CFU/ml, 1 ml of the lowest dilution was distributed over four plates (0.25 ml/plate) of nonselective and selective media. Control samples were plated onto nonselective and selective media supplemented with the antibiotic, as well as onto TSA to determine background microflora. Plates were incubated at 35±2 ˚C, nonselective for 24 hours and selective media for 48 hours. After incubation, colonies were counted by hand and population levels were calculated in log CFU/g celery.

Statistics

Data were calculated in mean log CFU/g. Values were the average of duplicate plate counts, and the average again of six replicates. One experiment was performed at each of the three temperatures for each pathogen. Data were statistically evaluated via multiple Tukey's-adjusted ANOVAs using SAS 9.2 for Windows. For each pathogen, all experimental factors were compared simultaneously. Comparisons of mean log CFU/g were made between bagged and container-stored samples, cut and uncut inoculation surfaces, media types, and between as well as within time intervals. Possible combined effects between storage container type and site of surface inoculation were also examined. Comparisons were also made between temperatures, but not between pathogens. Differences between mean values were considered significant at $P \leq 0.05$.

Table 3-1. *Escherichia coli* O157:H7 serotypes used, confirmed original source of isolate, original isolate designation, and laboratory identification code.

Serotype	Source	Designation	Lab code
E. coli O157:H7	Odwalla outbreak	223	MDD 20
E. coli O157:H7	Clinical cantaloupe	F658	MDD 326
E. coli O157:H7	Clinical lettuce	H1730	MDD 18
E. coli O157:H7	Clinical alfalfa sprout	F4546	MDD 19
E. coli O157:H7	Clinical spinach	EC4042	MDD 321

Table 3-2. *Listeria monocytogenes* strain used, confirmed original source of isolate, original isolate designation, and laboratory identification code.

Species	Source	Strain/Designation	Lab code
L. monocytogenes	Raw cabbage	LCDC 81-861	MDD 328
L. monocytogenes	Milk outbreak, human	SCOTT A	MDD 329
L. monocytogenes	Beef outbreak	101M	MDD 330
L. monocytogenes	Milk outbreak, bovine	V7	MDD 331

Table 3-3. *Salmonella* serovar used, confirmed original source of isolate, original isolate designation, and laboratory identification code.

Serovar	Source	Designation	Lab code
Enteritidis PT30	Raw almonds	ATCC BAA-1045	MDD 2
Agona	Alfalfa sprouts	LJH 517	MDD 17
Gaminara	Orange juice	F2712	MDD 21
Michigan	Cantaloupe outbreak	LJH 521	MDD 24
Montevideo	Tomato outbreak	G4639	MDD 22

CHAPTER 4
RESULTS

Background Microflora

Throughout all experiments, control samples were enumerated for background microflora on TSA. Background microflora ranged from ca. 6 to 10 log CFU/g of celery and were highly variable between samples (data not shown).

Escherichia coli O157:H7

Escherichia coli O157:H7 populations held at 4 ± 2ºC decreased significantly over 7 days ($P \leq 0.05$) by ca. 1-2.0 log, with the greatest decrease observed in samples inoculated on uncut surfaces and stored in polyethylene containers (Table 4-1). No significant differences existed between populations on days 0 and 1 ($P \geq 0.05$) at 4 ± 2ºC. Between days 1 and 3, mean population size decreased significantly at 4 ± 2ºC under all conditions ($P \leq 0.05$). Decreases in mean population size from day 3 to 5 at 4 ± 2ºC were insignificant ($P \geq 0.05$), while significance was observed in population decreases between days 5 and 7 ($P \leq 0.05$) at 4 ± 2ºC. Under all conditions, there was no significant difference observed between nonselective (TSAR) and selective (SMACR) media types ($P \geq 0.05$).

Initial (0 d) mean population size was significantly different on cut versus uncut inoculation surfaces ($P \leq 0.05$). Significant differences were observed between populations on celery stored in bags and containers on days 3, 5, and 7 at 4 ± 2ºC, with bagged samples having larger mean population sizes than those in containers (all $P \leq 0.05$).

At 12 ± 2ºC, although under all conditions there was a significant increase from day 0 to day 1 ($P \leq 0.05$) and a significant decrease between days 1 and 3 or 5 ($P \leq 0.05$); *E. coli* O157:H7 populations remained relatively stable over 7 days (Table 4-2). Initial (0 d) population

size was smaller on uncut versus cut surfaces at 12 ± 2ºC. No significant differences in mean population sizes were observed between bags and containers (all $P \geq 0.05$).

At 22 ± 2ºC, mean *E. coli* O157:H7 populations did not change dramatically, although from day 0 to day 1, under all conditions there was a slight but significantly increase in samples stored in bags ($P \leq 0.05$) (Table 4-3). Between 0 and 0.33 days, overall population size did not change significantly ($P \geq 0.05$). However, from 0.33 to 0.71 days, there was a significant increase in population size ($P \leq 0.05$). From day 0.71 through day 2, there were no significant differences in population sizes (all $P \geq 0.05$). Throughout the 22 ± 2ºC experiment, samples inoculated on cut surfaces had larger mean populations than those inoculated on uncut surfaces ($P \leq 0.05$). There were no significant differences in mean population size observed between bags and containers at any sample point ($P \geq 0.05$).

Listeria monocytogenes

At 4 ± 2ºC over the course of the experiment, mean *L. monocytogenes* populations decreased significantly under all conditions ($P \leq 0.05$), by ca. 1 log CFU/g (Table 4-4); the only significant decrease between consecutive sample points was between days 0 and 1 ($P \leq 0.05$). Relatively large standard deviations (SD) were seen for all the *L. monocytogenes* experiments. No significant differences were observed between container type or inoculation location ($P \geq 0.05$). Samples inoculated on cut surfaces and stored in bags always had larger mean populations than those inoculated on uncut surfaces and stored in containers ($P \leq 0.05$). Samples inoculated on uncut surfaces and stored in bag had larger mean populations than those inoculated on uncut surfaces and stored in container ($P \leq 0.05$).

The behavior of *L. monocytogenes* populations held at 12 ± 2ºC was variable, with little significant change over 7 days (Table 4-5). The only significant changes in mean population size was a slight but significant increase between days 3 and 5 ($P \leq 0.05$), which occurred under all

condition other than samples inoculated on cut surfaces and stored in bags ($P \geq 0.05$). Day 7 samples showed significantly larger mean populations than days 0, 1, or 3 ($P \leq 0.05$), and day 5 had significantly larger mean populations than days 0 or 1 ($P \leq 0.05$). Initial (0 d) population size was significantly smaller on uncut versus cut surfaces ($P \leq 0.05$). No significant differences were observed between inoculation sites ($P \geq 0.05$), other than on day 0 and day 3 when populations inoculated on cut surfaces were larger than those inoculated on uncut surfaces ($P \leq 0.05$). No significant differences were observed between bags and containers at any sampling point ($P \geq 0.05$).

At 22 ± 2ºC, a ca. 0.3 log CFU/g increase was observed in mean *L. monocytogenes* populations over 2 days, under all conditions (Table 4-6). This small increase was determined to be significant (P=0.04), with mean populations on day 0 smaller than those on day 2 ($P \leq 0.05$). Changes in population sizes were not linear. Mean population of samples inoculated on uncut surfaces decreased significantly ($P \leq 0.05$) by ca. 1 log CFU/g over the first 0.33 days, then increasing significantly to near initial size over 0.33 to 0.71 days ($P \leq 0.05$). From 0.71 to 1 day, there was no significant change in mean population size (all $P \geq 0.05$). However, between day 0.71 and day 2, mean populations increased slightly, though significantly ($P = 0.01$). No significant differences in mean population size were observed between container type or inoculation sites at any sample point ($P \geq 0.05$).

Salmonella

Cut celery held at 4 ± 2ºC were inoculated with the *Salmonella* cocktail at ca. 3 log CFU/g. Populations decreased significantly under all conditions, by ca. 1 log CFU/g, at a rate of 0.1-0.2 log CFU/g/day ($P \leq 0.05$) (Table 4-7). There were no significant differences in survival between bags and containers ($P \geq 0.05$). Mean populations were significantly greater on cut verses uncut sample inoculation sites throughout the experiment ($P \leq 0.05$), with the exception of

day 0 ($P \geq 0.05$). At each sample point, the difference between selective and nonselective media was not significant ($P \geq 0.05$).

Salmonella populations remained relatively stable between 3.0 to 3.5 log CFU/g for 7 days at 12 ± 2ºC, under all conditions (Table 4-8). Although there was a significant decrease between day 0 and day 7 ($P \leq 0.05$), differences between consecutive sample days were insignificant ($P \geq 0.05$). The greatest, and most significant decrease was seen between days 1 and 5 in samples inoculated on uncut surfaces ($P \leq 0.05$). Initial (0 d) population size was significantly smaller on uncut versus cut surfaces ($P \leq 0.05$). There was no significant difference between samples stored in bags and those in containers ($P \geq 0.05$), or between nonselective and selective media at any sampling point ($P \geq 0.05$), and no significant combined effects were observed between experimental factors (all $P \geq 0.05$).

All *Salmonella* populations increased significantly by ca. 1.5-2 log CFU/g over 2 d at 22 ± 2ºC, under all conditions ($P \leq 0.05$) (Table 4-9). The greatest growth (2.1 log CFU/g over 2 days) was observed on the cut surface of samples stored in containers, which on day 7 were significantly greater than those stored in bags, regardless of inoculation site ($P \leq 0.05$). Under all conditions, growth appears to manifest as a sinuous curve, with the majority occurring at earlier sampling points and beginning to taper off after 0.71 days. After 0.71 days, changes in population size were statistically insignificant ($P \geq 0.05$). With the exception of initial inoculation on day 0, population size was significantly greater on cut versus uncut surfaces ($P \leq 0.05$). With the exception of day 7, there were no significant differences between bags and containers ($P \geq 0.05$). No significant difference was observed between growth media at any sampling point ($P \geq 0.05$).

Table 4-1. Recovery of *Escherichia coli* O157:H7 from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 4 ± 2ºC for up to 7 days. Values are the mean[a] (log CFU/g) of duplicate samples (n=6) enumerated on TSAR and SMACR.

	Bag				Container			
	Cut		Uncut		Cut		Uncut	
Days	TSAR	SMACR	TSAR	SMACR	TSAR	SMAR	TSAR	SMACR
0	$4.5\pm0.1_{A,a}$	4.3±0.1	$3.5\pm0.9_{A,b}$	2.4±0.6	$5\pm0.1_{A}$	4.3±0.1	$3.5\pm0.9_{A,b}$	2.4±0.6
1	$4.4\pm0.1_{A}$	3.6±0.7	$4.3\pm0.2_{A}$	3.4±0.8	$4\pm0.2_{A}$	3.8±0.7	$3.6\pm1.1_{A}$	3.3±1.2
3	$3.5\pm0.8_{B,1}$	2.8±0.8	$3.4\pm1.3_{B}$	2.2±1.3	$2\pm0.8_{B}$	2.4±0.5	$1.9\pm1.4_{B,2}$	1.2±0.9
5	$3.7\pm0.7_{B,3}$	2.6±0.2	$2.6\pm1.4_{B,}$	1.6±0.9	$5\pm0.2_{B,4}$	1.9±0.4	$2.5\pm1.3_{B}$	1.6±0.9
7	$3.0\pm1.0_{C,5}$	2.2±0.6	$2.5\pm1.1_{B,}$	1.7±0.8	$5\pm0.5_{B,a,6}$	1.8±0.6	$1.0\pm0.5_{C,b}$	0.8±0.3

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

Table 4-2. Recovery of *Escherichia coli* O157:H7 from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 12 ± 2ºC for up to 7 days. Values are the mean[a] (log CFU/g) of duplicate samples (n=6) enumerated on TSAR and SMACR.

	Bag				Container			
	Cut		Uncut		Cut		Uncut	
Days	TSAR	SMACR	TSAR	SMACR	TSAR	SMACR	TSAR	SMACR
0	$4.7\pm0.1_{A}$	4.6±0.1	$4.2\pm0.7_{A}$	3.3±0.9	$4.7\pm0.1_{A}$	4.6±0.1	$4.2\pm0.7_{A}$	3.3±0.9
1	$5.1\pm0.1_{B}$	5.1±0.1	$4.9\pm0.2_{B}$	4.9±0.3	$5.1\pm0.1_{B}$	5.0±0.1	$4.6\pm0.2_{A}$	4.5±0.2
3	$4.9\pm0.6_{B}$	4.8±0.5	$4.3\pm0.4_{A}$	4.2±0.4	$4.5\pm0.8_{A}$	4.4±0.7	$4.2\pm0.5_{A}$	4.3±0.3
5	$4.6\pm0.6_{A}$	4.3±0.7	$4.2\pm0.6_{A}$	4.1±0.6	$4.2\pm0.4_{A}$	4.4±0.3	$4.5\pm0.2_{A}$	4.4±0.3
7	$4.7\pm0.6_{A}$	4.5±0.5	$4.3\pm0.6_{A}$	4.3±0.6	$4.4\pm0.3_{A}$	4.2±0.2	$3.9\pm0.6_{A}$	4.0±0.2

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

Table 4-3. Recovery of *Escherichia coli* O157:H7 from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 22 ± 2ºC for up to 2 days. Values are the mean [a] (log CFU/g) of duplicate samples (n=6) enumerated on TSAR and SMACR.

	Bag				Container			
	Cut		Uncut		Cut		Uncut	
Days	TSAR	SMACR	TSAR	SMACR	TSAR	SMACR	TSAR	SMACR
0	$4.6\pm0.1_{A}$	4.6±0.1	$4.4\pm0.1_{A}$	3.5±0.8	$4.6\pm0.1_{A}$	4.6±0.1	$4.4\pm0.1_{A}$	3.5±0.8
0.33	$4.7\pm0.5_{A,a}$	4.8±0.6	$4.1\pm0.4_{A,b}$	4.0±0.4	$4.9\pm0.3_{A,a}$	4.7±0.3	$4.1\pm0.6_{A,b}$	4.0±0.6
0.71	$5.0\pm0.2_{B,c}$	4.8±0.2	$4.5\pm0.5_{A,d}$	4.4±0.5	$5.1\pm0.4_{A,c}$	4.8±0.3	$4.5\pm0.4_{A,d}$	4.4±0.4
1	$5.3\pm0.5_{B,e}$	5.1±0.4	$4.8\pm0.2_{B,f}$	4.6±0.1	$5.2\pm0.4_{A,e}$	4.9±0.3	$4.7\pm0.6_{A,f}$	4.3±0.6
2	$5.0\pm0.3_{B}$	4.8±0.3	$4.8\pm0.3_{B}$	4.5±0.6	$4.9\pm0.4_{A}$	4.7±0.4	$4.7\pm0.5_{A}$	4.4±0.5

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

Table 4-4. Recovery of *Listeria monocytogenes* from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 4 ± 2ºC for up to 7 days. Values are the mean[a] (log CFU/g) of duplicate samples (n=6) enumerated on MOXN.

	Bag		Container	
Days	Cut	Uncut	Cut	Uncut
0	$3.7\pm0.7_{A}$	$3.5\pm0.6_{A}$	$3.7\pm0.7_{A}$	$3.5\pm0.6_{A}$
1	$2.9\pm0.6_{B}$	$3.4\pm0.7_{A}$	$2.9\pm0.7_{B}$	$2.7\pm0.7_{B}$
3	$2.6\pm0.2_{B,1}$	$2.9\pm0.5_{A}$	$2.9\pm1.0_{B}$	$1.6\pm0.6_{C,2}$
5	$2.4\pm0.2_{B}$	$2.6\pm0.2_{B}$	$2.0\pm0.3_{B}$	$2.1\pm0.2_{B}$
7	$2.3\pm0.9_{B,3}$	$2.4\pm0.2_{B}$	$2.2\pm0.4_{B}$	$1.7\pm0.2_{C,4}$

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

Table 4-5. Recovery of *Listeria monocytogenes* from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 12 ± 2ºC for up to 7 days. Values are the mean[a] (log CFU/g) of duplicate samples (n=6) enumerated on MOXN.

	Bag		Container	
Days	Cut	Uncut	Cut	Uncut
0	$3.9\pm0.5_{A,a}$	$3.2\pm0.6_{A,b}$	$3.9\pm0.5_{A,a}$	$3.2\pm0.6_{A,b}$
1	$3.5\pm0.6_{A}$	$3.3\pm0.8_{A}$	$3.3\pm0.6_{A}$	$3.5\pm0.7_{A}$
3	$4.5\pm0.3_{B,c}$	$2.6\pm0.3_{B,d}$	$3.5\pm0.9_{A}$	$3.2\pm0.9_{A}$
5	$4.1\pm1.0_{C}$	$4.0\pm0.8_{C}$	$4.2\pm0.8_{B}$	$4.2\pm1.0_{B}$
7	$4.1\pm1.2_{B,C}$	$4.0\pm0.9_{C}$	$4.5\pm1.1_{B}$	$4.2\pm0.7_{B}$

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

Table 4-6. Recovery of *Listeria monocytogenes* from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 22 ± 2ºC for up to 2 days. Values are the mean[a] (log CFU/g) of duplicate samples (n=6) enumerated on MOXN.

	Bag		Container	
Days	Cut	Uncut	Cut	Uncut
0	$3.3\pm0.8_{A}$	$3.4\pm0.8_{A}$	$3.3\pm0.8_{A}$	$3.4\pm0.8_{A}$
0.33	$2.9\pm0.2_{A}$	$2.5\pm0.3_{B}$	$2.8\pm0.2_{A}$	$2.7\pm0.3_{B}$
.071	$3.4\pm0.3_{B}$	$3.2\pm0.5_{A}$	$3.6\pm0.8_{B}$	$3.0\pm0.3_{A}$
1	$3.8\pm0.9_{B}$	$3.2\pm0.4_{A}$	$3.2\pm0.7_{B}$	$3.5\pm0.3_{C}$
2	$3.8\pm0.5_{B}$	$3.8\pm0.3_{C}$	$4.1\pm1.0_{B}$	$3.8\pm0.5_{C}$

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

Table 4-7. Recovery of *Salmonella* from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 4 ± 2ºC for up to 7 days. Values are the mean[a] (log CFU/g) of duplicate samples (n=6) enumerated on TSAR and BSAR.

	Bag				Container			
	Cut		Uncut		Cut		Uncut	
Days	TSAR	BSAR	TSAR	BSAR	TSAR	BSAR	TSAR	BSAR
0	$3.5\pm0.1_{A,a}$	3.7±0.2	$3.3\pm0.2_{A,b}$	2.8±0.4	$3.5\pm0.1_{A,a}$	3.7±0.2	$3.3\pm0.2_{A,b}$	2.8±0.4
1	$3.5\pm0.2_{A,c}$	3.4±0.1	$2.7\pm0.1_{B,d}$	2.5±0.3	$3.5\pm0.3_{A,c}$	3.5±0.3	$2.9\pm0.4_{A,d}$	2.7±0.4
3	$3.0\pm0.2_{B}$	3.0±0.3	$2.7\pm0.5_{B}$	2.4±0.5	$3.0\pm0.5_{A}$	3.4±0.4	$2.4\pm0.5_{A}$	2.4±0.4
5	$3.2\pm0.2_{B,e}$	3.0±0.1	$2.5\pm0.7_{B,f}$	2.3±0.7	$2.9\pm0.6_{A,e}$	2.67±6	$1.8\pm0.6_{B,f}$	1.7±0.5
7	$2.5\pm0.3_{C,}$	2.5±0.2	$2.0\pm0.5_{B}$	1.9±0.6	$2.8\pm0.4_{B}$	2.8±0.5	$2.1\pm0.6_{B}$	2.0±0.6

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

Table 4-8. Recovery of *Salmonella* from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 12 ± 2ºC for up to 7 days. Values are the mean[a] (log CFU/g) of duplicate samples (n=6) enumerated on TSAR and BSAR.

	Bag				Container			
	Cut		Uncut		Cut		Uncut	
Days	TSAR	BSAR	TSAR	BSAR	TSAR	BSAR	TSAR	BSAR
0	$3.5\pm0.1_{A,a}$	3.6±0.2	$3.0\pm0.3_{A,b}$	3.0±0.3	$3.5\pm0.1_{A,a}$	3.6±0.2	$3.0\pm0.3_{A,b}$	3.0±0.3
1	$3.7\pm0.1_{A,c}$	3.7±0.2	$3.1\pm0.2_{A,d}$	2.9±0.3	$3.7\pm0.2_{A,c}$	3.8±0.1	$3.0\pm0.3_{A,d}$	2.9±0.4
3	$3.4\pm0.2_{A}$	3.6±0.2	$3.0\pm0.3_{A}$	3.0±0.4	$3.5\pm0.2_{A}$	3.6±0.1	$3.1\pm0.4_{A}$	3.3±0.2
5	$3.2\pm0.3_{B}$	3.3±0.3	$3.0\pm0.3_{A}$	2.8±0.5	$3.3\pm0.3_{B}$	3.3±0.3	$3.1\pm0.4_{A}$	3.2±0.2
7	$3.2\pm0.4_{B}$	3.1±0.4	$2.9\pm0.5_{A}$	3.1±0.5	$3.2\pm0.4_{B}$	3.2±0.4	$2.6\pm0.4_{A}$	2.6±0.4

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

Table 4-9. Recovery of *Salmonella* from celery inoculated at approximately 3 log CFU/g onto cut or uncut surface and stored in either a bag or container held at 22 ± 2ºC for up to 2 days. Values are the mean[a] (log CFU/g) of duplicate samples (n=6) enumerated on TSAR and BSAR.

	Bag				Container			
	Cut		Uncut		Cut		Uncut	
Days	TSAR	BSAR	TSAR	BSAR	TSAR	BSAR	TSAR	BSAR
0	$3.6\pm0.1_{A}$	3.8±0.1	$3.4\pm0.2_{A}$	3.4±0.1	$3.6\pm0.1_{A}$	3.8±0.1	$3.4\pm0.2_{A}$	3.4±0.1
0.33	$4.8\pm0.1_{B,a}$	4.6±0.4	$3.4\pm0.3_{A,b}$	3.5±0.3	$4.8\pm0.2_{B,a}$	4.8±0.2	$3.7\pm0.3_{A,b}$	3.7±0.8
0.71	$5.2\pm0.5_{C,c}$	5.3±0.4	$4.6\pm0.3_{B,d}$	4.6±0.5	$5.4\pm0.2_{C,c}$	5.4±0.2	$4.2\pm0.4_{B,d}$	4.5±0.0
1	$5.1\pm0.5_{C,e}$	5.0±0.4	$4.5\pm0.0_{B,f}$	4.5±0.0	$5.4\pm0.3_{C,e}$	5.3±0.2	$4.6\pm0.1_{C,f}$	4.6±0.1
2	$5.2\pm0.4_{C,g,1}$	5.1±0.5	$4.8\pm0.1_{B,h,}$	4.8±0.2	$5.7\pm0.1_{C,I,2}$	5.7±0.1	$4.9\pm0.1_{D,j}$	4.9±0.0

[a]Mean ± standard deviation of duplicate samples from each of six replications. Within columns, means with different capital letters are significantly different. Within rows, means with different lower case letters (cut vs. uncut) or numbers (bag vs. container) are significantly different ($P \leq 0.05$).

CHAPTER 5
DISCUSSION

The behavior of foodborne pathogens at refrigeration and ambient temperatures on a variety fresh and fresh-cut produce is well established (Aruscavage et al., 2006; Heaton and Jones, 2008; Strawn et al., 2011). However, little is known about the fate of human foodborne pathogens on celery. At 4 ± 2°C, populations of all three pathogens decreased by ca. 1 log CFU/g under all the conditions evaluated. This result was expected for *E. coli* O157:H7 and *Salmonella.* In a companion study of chopped romaine lettuce and shredded carrots (Zhao et al., 2010), populations of both *E. coli* O157:H7 and *Salmonella* behaved in a similar manner, decreaseing by ca. 0.5 log CFU/g over 7 days when held at 4°C. *Listeria monocytogenes* was not evaluated by Zhao et al. (2010). Comparable behavior of *Salmonella* and *E. coli* O157:H7 has also been observed on minimally processed peaches, diced celery, leafy greens and herbs, zucchini squash, rutabaga, soybean sprouts, and alfalfa sprouts (Alegre et al., 2010; Prakash et al., 2010; Duffy et al., 2005; Weissinger et al., 2000; Hsu et al., 2006; Castro-Rosa et al., 2010; Francis and O'Beirne, 2001; and Gandhi et al., 2001). On cut pineapple and papaya held at 4°C, *E. coli* O157:H7 and *Salmonella* populations declined slowly, but survived for 28 days (Strawn and Danyluk, 2010a; Strawn and Danyluk, 2010b). On mango held at 4°C, *Salmonella* populations increased by ca. 1 log CFU/g after 1 day, but *E. coli* O157:H7 populations remained stable over 28 days (Strawn and Danyluk, 2010b).

In contrast to the reduction we saw for *L. monocytogenes* on celery, *L. innocula* populations inoculated onto peaches held at 5°C increased by ca. 0.5 log CFU/g over 6 days (Alegre et al., 2010). However, *L. monocytogenes* did not grow at 4°C on rutabaga and shredded lettuce, and populations significantly decreased by 2 and 1.5 log CFU/g on dry coleslaw and

soybean sprout, respectively (Francis and O'Beirne, 2001). On both shredded carrots and lettuce held at 4°C, *L. monocytogenes* populations did not change (Kakiomenou et al., 1998)

Survival of a pathogenic microorganism on produce is dictated by its metabolic capabilities, which can be greatly influenced by intrinsic (e.g. surface moisture) and extrinsic environmental factors (e.g. atmospheric changes) (Beuchat, 2002). Modified atmospheres develop within sealed storage devices containing fresh produce due to plant cell respiration (Francis and O'Beirne, 2001). Atmospheric changes may affect the fate of some bacteria. Atmospheric conditions of up to 30% CO_2 do not inhibit the growth of *E. coli* O157:H7 on vegetables (Diaz and Hotchkiss, 1996; Francis and O'Beirne, 2001), but *L. monocytogenes* is known to be sensitive to certain environmental conditions (Belessi et al., 2011; Francis and O'Beirne, 2001). The concentrations of O_2 and CO_2 within packages can vary by food product (Francis and O'Beirne, 2001). In semipermeable (permeability to O_2 of 1200 ml/m^2/day/atm and to CO_2 of 4000 ml/m^2/day/atm) polypropylene packaging bags containing lettuce and rutabaga, O_2 fell and CO_2 increased to achieve 6%/6% (O_2/CO_2) and 4%/10%, respectively, over 7 days. With soybean sprouts and coleslaw mix, atmospheric changes were much greater, equilibrating at 0%/23% and 0%/25% (O_2/CO_2), respectively, over 7 days. The relatively modest atmospheric changes observed in packages containing lettuce and rutabaga were not found to affect the fate of *L. monocytogenes*. However, growth of *L. monocytogenes* was suppressed by the atmospheric changes observed in packages containing soybean sprouts and coleslaw mix (Francis and O'Beirne, 2001). The inhibitory effect of high CO_2 concentrations on *L. monocytogenes* is related to decreased pH and interference with various metabolic machinery (Chen et al., 2003; Dixon and Kell, 1989; Hudson et al., 1994). However, we observed very little passive

accumulation of CO_2 at any temperature (data not shown), and do not believe that the respiration of fresh-cut celery within sealed containers was sufficient to influenced pathogen behavior.

The diversity and abundance of background microflora can also affects the growth of *L. monocytogenes*, with lactic acid bacteria, enterobacteria, and pseudomonads being proven competitive inhibitors (Carlin et al., 1995; Francis and O'Beirne, 1998a; Francis and O'Beirne, 1998b; Vescovo et al., 1996). The indigenous aerobic microflora of 13 different vegetables obtain from Spanish farms and markets has been evaluated, and it was determined that celery ranked third highest in background cell concentration after beet leaves and artichoke, with average an background MPN of 2.2 $x10^{10}$ cells/100g celery (Garcia-Villanova et al., 1987). Similarly, we observed high populations (10^6 to 10^{10} log CFU/g), and what visually appeared to be diverse populations (noticeable differences in color and colony formation on TSA plates) of background microflora on control samples. This large background microflora inherent to celery may have contributed to the behavior of *L. monocytogenes*. An atmosphere modified by sample respiration, large endogenous populations of microflora, and the relatively limited nutrient availability of celery (FDA, 2009)—with likely differences in nutrient availability and water activity between cut and uncut surfaces (Beuchat and Scouten, 2002; Sapers et al., 2006)—are factors which may have contributed to the observed decrease in *L. monocytogenes* populations at 4°C. Headspace atmosphere, sample pH, and background microflora have also been hypothesized as factors attributing to the lack of *L. monocytogenes* growth on shredded carrots and lettuce (Kakiomenou et al., 1998). With a reported pH of 5.7 to 6.0 for fresh celery (FDA, 2007), we do not believe that pH had an inhibitory role on pathogen behavior in this study.

Less is known about the fate of foodborne pathogens on produce at abusive temperatures (i.e. above refrigeration but below normal room temperatures). For all three pathogens, we

observed little to no change in population size at 12 ± 2°C, with *L. monocytogenes* having the greatest increase. At 12°C, *Salmonella* populations inoculated at ca. 5 log CFU/g decreased on cut pineapple by ca. 2 to 3 log CFU/g over 7 days (Strawn and Danyluk, 2010a). *Salmonella* inoculated at ca. 3 log CFU/g increased by ca. 3.5 log CFU/g on cut mango and papaya after 3 and 5 days, respectively (Strawn and Danyluk, 2010b). However, at 12°C, *E. coli* O157:H7 populations inoculated at 5 log CFU/g on cut pineapple decreased by ca. 4 log CFU/g over 14 days, but increased on papaya by ca. 3 log CF/g over 3 days, and did not change on mango over 10 days (Strawn and Danyluk 2010a; Strawn and Danyluk 2010b). Populations of *L. innocua* on fresh cut peaches stored at 10°C increased by ca. 1.6 log CFU/g over 6 days (Alegre et al., 2010), while those of *L. monocytogenes* increased by ca. 1.5 log CFU/g on shredded lettuce and ca. 1 log CFU/g on rutabaga, did not change significantly on soybean sprouts, and decreased by 1.5 log CFU/g on packaged coleslaw over 5 days at 8°C (Francis and O'Beirne, 2001). *Escherichia coli* O157:H7 populations had a similar behavior to *L. monocytogenes* on shredded lettuce, rutabaga, soybean sprouts, and packaged coleslaw over 5 days at 8°C (Francis and O'Beirne, 2001).

On fresh-cut celery at 22 ± 2°C, populations of all three pathogens increased. *Escherichia coli* O157:H7, *Salmonella*, and *L. monocytogenes* populations increased by ca. 1.0, 0.5, and 2.0 log CFU/g, respectively, with the majority of growth occurring over the first 0.71 days in all cases. *Salmonella* and *E. coli* O157:H7 populations on both romaine lettuce and freshly shredded carrots held at 23°C increased by 2.5 log CFU/g within 25 h of inoculation, similar to the results we see on celery (Zhao et al., 2010). *Salmonella* Montevideo populations inoculated onto raw tomatoes and stored at 30°C increased from ca. 0.5 to 5 log CFU/g over 1 day before populations stabilized (Zhuang et al., 1995). In this study, similar to Zhao et al

(2010) and Zhuang et al. (1995), maximum populations of *Salmonella* at 5-6 log CFU/g were observed, indicating a potential carrying capacity of *Salmonella* spp., as well as *E. coli* O157:H7 on some fresh produce items stored at or above ambient temperatures. However, this pattern is certainly not a rule. Strawn and Danyluk (2010b) observed that *Salmonella* and populations on papaya inoculated at three different inoculum levels (1, 3, and 5 log CFU) held at 23°C reached carrying capacity at ca. 7.2 log CFU/g after 1 day. Under the same conditions, *E. coli* O157:H7 populations inoculated on papaya at two inoculum levels (3 and 5 CFU/g) reached a stable carrying capacity at 6.5 CFU/g after 3 days (Strawn and Danyluk, 2010b). On minimally processed peaches held at 25°C, *E. coli* O157:H7 and *Salmonella* populations reached 8 log CFU/g within one day (Alegre et al., 2010a). However, starting concentrations of 10^5 CFU/ml and storage in controlled atmosphere containers (semi-oxygen-permeable film) may have influenced the results on peaches. The large numbers of competitive background microflora found on celery may also be a determining factor in the observed carrying capacity for both *E. coli* O157:H7 and *Salmonella* spp. on celery.

At 25°C, *Salmonella* populations inoculated onto fresh Italian parsley increased from ca. 2.5 to 4.1 log CFU/g over 7 days (Duffy et al., 2005). These findings are similar those we observed on fresh-cut celery. However, when held at 25°C, *Salmonella* populations inoculated onto whole zucchini squash and zucchini slices decreased from ca. 7 to 1.5 log CFU/sample over 7 days, and increased from ca. 2.5 to 8 log CFU/sample over 2 days, respectively (Castro-Rosas et al., 2010). In the same study, a single strain of *E. coli* O157:H7 inoculated onto whole zucchini and zucchini slices decreased from ca. 7 to 5 log CFU/g, and increased from ca. 2.2 to 7 log CFU/sample, respectively, over 7 and 2 days, respectively (Castro-Rosas et al., 2010). Compared with *Salmonella* spp., *E. coli* O157:H7 fared better on the skin of whole zucchini

(Castro-Rosas et al., 2010). On sliced, but not whole zucchini, *E. coli* O157:H7 and *Salmonella* behavior was similar to that observed on fresh-cut celery. In our experiments, both *E. coli* O157:H7 and *Salmonella* populations were greater on cut versus uncut celery surfaces. Microniches exist at epidermal cell junctions where cuticular waxes are less dense, water accumulates, and nutrients are more available than in other surface locations (Sapers et al., 2006). It has been hypothesized that microbial resource competition is particularly intense at these junctions (Castro-Rosas et al., 2010).

Salmonella populations inoculated onto alfalfa sprouts decrease over 1 day storage at 25°C by ca. 1.4 CFU/g (Gandhi et al., 2001). This observation is difficult to explain, but the researchers suggested that the waxy outer material of the sprout repelled the inoculum mixture, thereby repelling the bacteria. Another study on alfalfa sprouts found that at both 25 and 37°C, pathogen population decreases were greater than that observed at 5°C (Taormina and Beuchat, 1999). These observations are not consistent with those observed on fresh-cut celery, and it is apparent that pathogen behavior is often unique to the produce item in question.

Similar to the Castro-Rosas et al. (2010) study comparing whole and sliced zucchini held at 25°C, we frequently observed that samples inoculated with either *E. coli* O157:H7 or *Salmonella* populations on cut surfaces grew more than those inoculated onto uncut surfaces. However, as a modified leaf bundle, celery petioles are not analogous to the epidermis of whole zucchini fruit, and some human pathogens may have the capability to invade through stomatal openings (Kroupitski et al., 2011). In an inoculation study of mechanically damaged lettuce, *E. coli* O157:H7 populations where shown to survive longer on damaged than on undamaged samples (Aruscavage et al., 2008). These findings are also consistent with our observations, since cut may be considered analogous to a damaged surface.

Storage container type had little effect on pathogen fate. For celery inoculated with *Salmonella* held at 22 ± 2°C, *L. monocytogenes* held at 4 ± 2°C, and *E. coli* O157:H7 held at 4 ± 2°C, significant, combined effects were observed, where populations inoculated on cut celery surfaces and stored in bags were larger than those inoculated on uncut surfaces and stored in containers. This suggests that storage type may have a small effect on pathogen behavior, and rigid containers may be preferable to bags in this respect.

CHAPTER 6
CONCLUSIONS AND FUTURE WORK

At 4°C, populations of all three pathogens decreased by ca. 0.5 to 1.0 log CFU/g over 2 days, under all conditions. Behavior at 4°C was as expected for the mesophilic *E. coli* O157:H7 and *Salmonella* spp., but not for the psychrophilic *L. monocytogenes*. The unexpected decline in populations of *L. monocytogenes* may have been due to a combination of environmental and ecological factors including passive MAP, interspecific competition with normal microflora, and low nutrient accessibility.

At 12°C, population sizes of *E. coli* O157:H7 and *Salmonella* spp. did not change over 7 days, under all conditions, while populations of *L. monocytogenes* increased by ca. 0.5 log CFU/g. Behavior at 12°C was as expected, although more growth for *L. monocytogenes* populations was anticipated.

At 22°C, as expected, populations of *E. coli* O157:H7 increased by ca. 1 log CFU/g, *L. monocytogenes* increased by ca. 0.3 to 0.5 log CFU/g, and *Salmonella* increased by ca. 1.5 to 2 log CFU/g, with the majority of growth for all three pathogens occurring during the first 0.71 days. Significant differences in populations were frequently observed between inoculation location, with samples inoculated on cut surfaces being larger than those inoculated on uncut surfaces. This result may have been due to differences in water activity and nutrient availability at the surface locations. Significant differences between container type did occur, but were infrequent. This result suggests only a minimal influence of the container types used on pathogen behavior.

This work demonstrates that *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. can survive and grow on improperly stored fresh-cut celery.. We have also shown that strict maintenance of refrigeration temperatures can inhibit the growth of several pathogens on fresh-

cut celery. Therefore, we suggest that maintaining the cold-chain during transportation, distribution, storage, handling, in markets, and by consumers is essential to prevent disease proliferation. That pathogen populations did not fall below the limits of detection over 7 days, even at 4°C, underscores the importance of implementation and strict adherence to preventative food safety programs at all stages of production and processing to minimize the risk of contamination.

Pathogen behavior has been shown to vary based on initial inoculum concentrations (Strawn and Danyluk, 2010b). At 22°C, pathogen populations recovered from tomato samples after 1 h of drying was significantly large than those obtained after 24 h drying time (Lang et al., 2004). That we observed some differences in the effect of drying time between samples inoculated on cut versus uncut surfaces is methodologically significant. During future experiments involving different locations of inoculation, we suggest varying drying times on the basis of site of inoculation to achieve a more precise initial inoculum load. Alternatively, different initial inoculum concentrations could be applied to reach a uniform level of contamination between samples. Different strains of *E. coli* O157:H7, *L. monocytogenes, Pseudomonas,* and *Salmonella* attach to different regions of cut lettuce, suggesting different species-specific (and possibly strain-specific) attachment mechanisms (Takeuchi et al., 2000). This may also be true for celery, and future research on this topic is recommended to further develop our understanding of the behavior of various pathogens on celery. A greater understanding of the biomechanics of pathogen-produce attachment and invasion could lead to more effective intervention and decontamination strategies, if these mechanisms can be chemically or biophysically disrupted (Teplitski et al., 2009).

Spoilage and physiological changes in fresh-cut celery occur over time (Robbs et al., 1996). The predominant spoilage bacteria was identified as *Pseudomonas fluorescens* and *P. marginalis*, which caused water soaking, soft rot, and discoloration in celery tissues stored at 5 or 25°C. *Leuconostoc mesenteroides* was also isolated and hypothesized to have been responsible for slime production (Robbs et al., 1996). Because these changes alter the microenvironment and likely affect water activity and nutrient availability, future studies on the effects of spoilage and natural microflora on pathogen behavior is suggested.

Because of the growth potential of pathogens on fresh-cut celery, further investigations into the factors influencing this behavior are warranted. Such studies could include an analysis of the abundance and behavior of natural microflora on fresh-cut celery, as well as the respiration of cut celery in consumer storage containers and atmospheric conditions within the packaged product. Further examination of the effect of storage container type is also warranted, as the level of gas exchange may vary based on material permeability and seal integrity.

Studies on the potential for cross-contamination in the consumer kitchen, as well as the efficacy of various prevention strategies (e.g. washing, chemical treatments, etc.) are suggested. While the fresh-cut industry is generally known to practice strict food safety programs, once a consumer purchases a product, disease prevention becomes a matter of proper consumer handling, and should be based on public education disseminated from an informed government.

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BIOGRAPHICAL SKETCH

Joshua Peter Vandamm was born in Silver Spring, Maryland to Michael and Deborah Vandamm. Joshua attended Towson State University, where he graduated in 2008, with a B.S. degree in biology with an emphasis in zoology. Joshua began his M.S. degree in 2009, at the University of Florida under the instruction of Dr. Michelle Danyluk. At the University of Florida, Joshua studied food science, with an emphasis on food microbiology and safety. Future plans include perusing a career in food safety and/or public health in industry or government.