GROWTH PROBABILITY OF *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* IN PACKAGED FRESH-CUT ROMAINE MIX AT FLUCTUATING TEMPERATURES DURING SIMULATED TRANSPORT, RETAIL STORAGE AND DISPLAY

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ABSTRACT

GROWTH PROBABILITY OF *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* IN PACKAGED FRESH-CUT ROMAINE MIX AT FLUCTUATING TEMPERATURES DURING SIMULATED TRANSPORT, RETAIL STORAGE AND DISPLAY

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Temperature abuse during commercial transport and retail sale of leafy greens negatively impacts both microbial safety and product quality. The effect of fluctuating temperatures on *Escherichia coli* O157:H7 and *Listeria monocytogenes* growth in commercially bagged salad greens was simulated during transport, retail storage, and display. Time/temperature profiles for bagged salads were obtained from 16 transportation routes (432 profiles) and during retail storage (4867 profiles) and display (3799 profiles) at 9 supermarkets. Five different time/temperature profiles collected during transport, retail storage, and display were then duplicated in a programmable incubator to assess pathogen growth in bags of romaine mix. Microbial growth predictions using the Baranyi/Ratkowsky and McKellar models were validated by comparing the root mean square error (RMSEs) and biases between the growth data and model predictions. Both models yielded acceptable RMSEs and biases. Monte Carlo simulations were then performed to calculate the probability distribution of microbial growth from 8,122,127,472 scenarios. *E. coli* O157:H7 and *L. monocytogenes* populations increased a maximum of 3.1 and 3.0 log CFU/g at retail storage. Based on the simulation results, both pathogens generally increased < 2 log CFU/g. However, retail storage duration can significantly impact the extent of *E. coli* O157:H7 and *L. monocytogenes* growth. This first large-scale US study should be useful in filling some of the data gaps in current risk assessments for leafy greens.

To my mom Changbin Sun, my dad Jinxiang Zeng, and my husband Yuanteng Pei
I dedicate this thesis
谨以此献给我的母亲, 父亲和我的爱人

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CHAPTER 1

INTRODUCTION

Due to the abundant health benefits and year-round availability of fresh produce, consumption of fresh and fresh-cut leafy greens in the United States has increased over the past few decades (Calvin and others 2003; ERS 2003; Meng and Doyle 2002; Nicola and others 2006), with per capita consumption of romaine lettuce increasing from 1.5 to 5.1 kg from 1982 to 2003 (ERS 2003). Salad greens, which are consumed raw, are highly prone to microbial contamination in the field. Although relatively small quantities of leafy greens are presumed to be contaminated with bacterial pathogens, outbreaks of illness continue to be reported.

Since 1995, at least 19 outbreaks of foodborne illness were caused by *Escherichia coli* O157:H7 linked to fresh or fresh-cut lettuce as the vehicle of infection (Falkensten, 2010). These outbreaks can result from low levels of contamination since as few as 10 cells of *E. coli* O157:H7 are able to cause human illness (FDA 1998). Although traceability to growers was not completed in all outbreak investigations, eight of the outbreaks associated with lettuce and spinach were traced back to Salinas, California.

In addition to *E. coli* O157:H7, *Listeria monocytogenes* can also grow on fresh produce when present (De Simon and others 1992; Francis and others 1999; Szabo and others 2000). *L. monocytogenes* has thus far been linked to 16 produce-related outbreaks internationally, with a recent large-scale cantaloupe outbreak involving 139 cases, including 29 deaths and 1 miscarriage (CDC 2011b). Several recent recalls also have been issued for *Listeria*-contaminated produce, including bagged salads, baby spinach, Napa kimchi, chopped or shredded romaine, and diced red onions in the U. S. and Canada.

These outbreaks have greatly shaken consumer confidence concerning the consumption of ready-to-eat fresh produce. As a result, the produce industry is being driven to make major changes in how leafy greens are grown, harvested, and processed. The industry is reviewing current operation guidelines for minimizing microbial food safety hazards as well as other available information regarding pathogen reduction strategies for fresh produce. This thesis focuses on the effect of temperature abuse during postharvest transportation and distribution, with this research expected to provide practical solutions that can be used to enhance safety and keeping quality of leafy greens during transportation, retail storage, and display.

Produce can be easily contaminated at any point in the farm-to-fork continuum. The high moisture and nutrient content, together with the large surface area of fresh-cut produce, make these products highly susceptible to microbial contamination and subsequent growth. The many benefits of low temperature storage on product quality and microbial growth during transport and distribution have been well documented (Jacxsens and others 2002; Koseki and Isobe 2005a; Luo and others 2010). Therefore, the U. S. along with many other countries has developed temperature guidelines for most perishable foods during transport and retail sale. The U. S. Food and Drug Administration 2009 Food Code requires that ready-to-eat fruits and vegetables be refrigerated and received at or below 5°C (41°F) to limit growth of organisms of public health concern (FDA 2009). The Canadian Food Inspection Agency code of practice for minimally processed ready-to-eat vegetables requires that such products be maintained at 4°C during transportation and storage (Canadian Food Inspection Agency 2009). The EU food hygiene legislation (2006) requires foods which are likely to support the growth of pathogenic micro-organisms to be held at or below 8°C. Nunes and others (2008) summarized the recommended storage temperatures of 1 to 3°C for various fruits, vegetables, and bagged salads. While these

temperature ranges are considered ideal, temperatures of 5 or 8°C are more realistic in the distribution chain.

Temperature monitoring during distribution, retail storage, and display provides a major advantage in the ability to manage temperature sensitive products in the cold chain with most food and pharmaceutical suppliers of perishable goods already tracking time/temperature histories. Many large-scale retailers have now mandated the use of temperature sensors and data loggers for their suppliers, with others using various temperature monitoring systems to track specific cases and pallets of product in the supply chain. In this study, temperature sensors were used to monitor temperature fluctuations and collect time/temperature profiles during transport, at retail storage room, and retail display cases.

However, maintaining a consistently low temperature throughout the distribution chain is challenging. Opening of the truck doors during loading and unloading, outside temperature extremes, and retail storage/display conditions can all lead to temperature fluctuations. Thus far, only three studies from Canada, Japan, and Belgium have assessed the growth of *E. coli* O157:H7 and *L. monocytogenes* at real-time temperatures recorded during preharvest, transportation, and retail sale of fresh-cut bagged salad greens (McKellar and others 2012; Koseki and Isobe 2005a; Rediers and others 2008). Moreover, the time/temperature profiles from these three studies may not sufficiently reflect that segment of the U. S. cold chain during transport and distribution.

Consequently, the objectives of this study were to:

1. Assess the impact of various real-time time/temperature histories on the growth of *E. coli* O157:H7 and *L. monocytogenes* as well as psychrotrophic and mesophilic bacteria in commercial romaine mix during transport, retail cold storage, and display;

2. Validate the Baranyi/Ratlowsky and McKellar models using laboratory data;

3. Determine the probability distributions for *E. coli* O157:H7 and *L. monocytogenes* growth during transport, retail storage, and display.

LITERATURE REVIEW

1.1 Foodborne pathogens

Foodborne pathogens cause widespread and growing public health problems over the world. The global incidence of foodborne disease leads to approximately 1.8 million deaths annually, with most cases from less developed countries (WHO 2007). In developed countries foodborne pathogens are responsible for millions of cases of gastrointestinal diseases each year, resulting in significant medical cost as well as productivity loss. Emerging foodborne pathogens and foodborne diseases are also likely to occur in recent years, driven by factors such as genetic evolution, changes in agricultural and manufacturing practices, and the diversity of host status. There are also growing concerns for terrorism reasons to contaminate food and water supplies in attempts to incapacitate people and disrupt economic growth. Fuelled by these concerns, 25 major microbial pathogens are monitored by various surveillance programs in the U. S., such as the Foodborne Diseases Active Surveillance network (FoodNet), the National Notifiable Diseases Surveillance System (NNDSS), the Cholera and other *Vibrio* Illness Surveillance (COVIS) system, the National Tuberculosis Surveillance System (NTSS), and the Foodborne Disease Outbreak Surveillance System (FDOSS).

1.1.1 *Escherichia coli*

Escherichia coli is a facultative anaerobic, Gram-negative, non-sporulating bacterium predominantly found in the colonic flora of warm-blooded animals (Todar 2008a). Although most strains are harmless, some pathogenic serotypes such as *E. coli* O157:H7 are responsible for serious food poisoning and product recalls. Serotyping of pathogenic *E. coli* is based the presence of different O (lipopolysaccharide, LPS), H (flagellar), and K (capsular) antigens

(Nataro and Kaper 1998). Among the estimated 50,000 to 80,000 serotypes, six categories of pathogenic *E. coli* are recognized based on serological characteristics and virulence properties - enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative (EAEC), and diffuse-adhering *E. coli* (DAEC) (Guerrant 2005; Nguyen 2006; Scaletsky 2002). *E. coli* O157:H7 belongs to EHEC, causing human diseases including non-bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS), acute kidney failure, and thrombotic thrombocytopenic purpura.

1.1.2 *Listeria monocytogenes*

The genus *Listeria* comprises a group of facultative anaerobic, Gram-positive, non-spore forming, short rod-shaped bacterium and contains the following species: *L. monocytogenes, L. innocua, L. invanovii, L. seeligeri, L. welshimeri, L. grayi, L. marthi and L. rocoyrtiae. Listeria monocytogenes* is of greatest concern as a foodborne pathogen of the aforementioned species due to its ability to grow at refrigeration temperatures (Rocourt 1999). Its resistance to acid, salt, and heat makes this pathogen a major concern to food manufacturers. Thus, while typically found in the natural environment, including soil and water, as well as wild and domesticated animals, *L. monocytogenes* can be commonly found in many food-processing environments and typically enters food as a post-processing contaminant.

L. monocytogenes is the causative agent of listeriosis, which has a fatality rate of about 20% (Scallan and others 2011). Thirteen serotypes are recognized, with serotypes 1/2a, 1/2b, and 4b responsible for most foodborne illnesses (Farber and others 1991). Listeriosis usually lasts 7-10 days and occurs predominantly in newborns, the elderly, and immunocompromised individuals (Hof 1996). Symptoms of listeriosis include fever, muscle aches, vomiting, headache, nausea and diarrhea. After crossing the blood-brain barrier, *L. monocytogenes* can

cause meningitis, an infection of the covering of the brain and spinal cord, characterized by headache, stiff neck, confusion, loss of balance, and convulsions (Todar 2008b).

1.2 Foodborne illness

Foodborne illnesses have become a considerable public health burden in the United States. An estimated 48 million foodborne illnesses, 128,000 hospitalizations, and 3,000 associated deaths occur annually, with 55% of outbreaks traced to bacterial pathogens with known etiology (CDC 2006b; Scallan and others 2011). A total of 63,153 annual cases of shiga toxin-producing *E. coli* O157 (STEC O157), 112,752 cases of non-O157 shiga toxin-producing *E. coli* (non-STEC O157), and 1,591 cases of *L. monocytogenes* infection were estimated by Scallan and others (2011). The hospitalization rate for laboratory-confirmed cases was highest for *L. monocytogenes* (94%), followed by *Vibrio vulnificus* (91%) and *Clostridium botulinum* (83%), whereas the death rate was highest for *C. botulinum* (83%), followed by *V. vulnificus* (35%) and *L. monocytogenes* (16%) (Scallan and others 2011). The estimated annual cost of medical care and premature death for the major bacterial pathogens exceeds $6.9 billion (ERS 2010). The cost of *E. coli* O157 alone is at least $478 million (ERS 2010).

Numerous foodborne outbreaks involving *E. coli* O157:H7 have shaken consumer confidence. An estimated 63,153 illnesses are caused by *E. coli* O157:H7 in the United States annually, with 2,138 hospitalizations and 20 deaths (Scallan and others 2011). *E. coli* O157:H7 was first identified as a foodborne pathogen in 1982 during an investigation of hemorrhagic colitis (Riley and others 1983). It has remained a threat to the food industry since 1993 when a large multistate outbreak was linked to ground beef. Ground beef, produce, and dairy products are the most common vehicles associated with *E. coli* O157:H7 outbreaks.

During 1999-2008, contaminated produce accounted for 21% of all foodborne outbreaks and 31% of all foodborne illnesses in the United States, with leafy greens frequently implicated (DeWaal and others 2012). The term leafy greens includes iceberg lettuce, romaine lettuce, leaf lettuce, butter lettuce, baby leafy lettuce, escarole, endive, spring mix, spinach, cabbage, kale, arugula and chard. Since 1993, at least 800 cases of illness and 8 deaths have been traced to leafy greens (FDA 2005a), particularly California-grown lettuce (iceberg, romaine, red leaf, and mesclun) and spinach (Table 1.1). In one 2005 outbreak, 250,000 bags of pre-washed and pre-cut salad greens were recalled. In late summer of 2006, *E. coli* O157:H7 contaminated California-grown baby flat-leaf spinach was responsible for 199 cases of illness in 28 states, including 31 cases of hemolytic uremic syndrome, 141 hospitalizations, and 3 deaths (FDA, 2006). In December 2006, commercially-shredded lettuce used by Taco Bell sickened over 71 people with 53 hospitalizations and 8 cases of kidney failure traced to *E. coli* O157:H7 (CDC 2006c). Produce outbreaks usually peak in summer and fall. A total of 34% of produce outbreaks were linked to lettuce, 18% apple cider or apple juice, 16% to salad, 11% to coleslaw, 11% to melons, 8% to sprouts and 3% to grapes (Rangel and others 2005). With increasing frequency in recent years, the median number of cases in produce-associated outbreaks was significantly larger than that of ground beef outbreaks.

Other serogroups of shiga toxin-producing *E. coli* are also becoming a major concern incuding serotypes O26, O45, O103, O111, O121 and O145 – now known as ‘the big six’. In June 2011, *E. coli* O104:H4 contaminated sprouts were responsible for 2229 reported infections, 852 cases of hemolytic uremic syndrome and 32 deaths in Germany as well as 6 confirmed cases and 1 death in the US (CDC 2011a). In May 2010, a multistate outbreak of human *E. coli* O145

Table 1.1 Lettuce- and leafy green-associated outbreaks (Anonymous, 2011; Falkensten).

Date	Causative Agent	Illnesses Reported	Source	Location
May 2011	*Salmonella* Typhimirum	15	Salad?	IL
May 2010	*E. coli* O145	23	Lettuce (Romaine)	MI, OH, NY, TN
Spring 2010	*Salmonella* spp	?	Lettuce	Upper Midwest
Jul.-Aug. 2009	*Salmonella* spp	> 100	Lettuce	5 States
Aug. 2009	*Salmonella* spp	124	Lettuce?	?
Feb. 2009	*Salmonella* spp	5	Lettuce?	MS
Nov. 2008	*E. coli* O157:H7	130	Lettuce	Ontario, Canada
Oct. 2008	*E. coli* O157:H7	59	Lettuce	Multistate, US; Ontario, Can
Sep. 2008	*E. coli* O157:H7	34	Lettuce	MI
Aug. 2008	*E. coli* O157:H7	5	Spinach	OR
Jun. 2008	*E. coli* O157:H7	9	Lettuce	WA
May 2008	*E. coli* O157:H7	9	Lettuce	CA
Jul. 2007	*Shigella sonnei*	72	Salad	CA
Jul. 2007	*E. coli* O157:H7	26	Lettuce	AL
Feb. 2007	Norovirus	8	Lettuce	TN
Jan. 2007	Norovirus	9	Salad	ID
Nov. 2006	*E. coli* O157:H7	77	Lettuce	Multistate, U. S.
Oct. 2006	*E. coli* O157:H7	199 (3 deaths)	Spinach	Multistate, U. S.
Sep. 2006	Norovirus	9	Salad	VA
Sep. 2006	*E. coli* O157:H7	30	Lettuce	Ontario, Canada
Sep. 2005	*E. coli* O157:H7	11	Lettuce (Romaine)	?
Nov. 2004	*E. coli* O157:H7	6	Lettuce	NJ
Nov. 2003	*E. coli* O157:H7	16	Spinach	CA
Sep. 2003	*E. coli* O157:H7	51	Lettuce (Mixed salad)	CA
Dec. 2002	*E. coli* O157:H7	3	Lettuce	MN
Nov. 2002	*E. coli* O157:H7	13	Lettuce	IL
Jul. 2002	*E. coli* O157:H7	55	Lettuce (Romaine)	WA
Nov. 2001	*E. coli* O157:H7	20	Lettuce	TX
Oct. 2000	*E. coli* O157:H7	6	Salad	IN
May 2000	*Campylobacter jejuni*	13	Salad	CT
May 2000	Norovirus	3	Salad	OH
Feb. 2000	Norovirus	7	Salad	OH
Oct. 1999	*E. coli* O157:H7	40	Lettuce	PA
Oct. 1999	*E. coli* O157:H7	47	Lettuce	OH
Oct. 1999	*E. coli* O157:H7	5	Salad	OR
Oct. 1999	Norovirus	16	Salad	WA
Sep. 1999	*E. coli* O157:H11	6	Lettuce	WA
Sep. 1999	Norovirus	115	Lettuce	WA
Sep. 1999	*E. coli* O111:H8	58	Salad	TX
Aug. 1999	Norovirus	25	Salad	MN

Table 1.1 (cont'd)

Date	Causative Agent	Illnesses Reported	Source	Location
May 1999	Norovirus	28	Salad	FL
Feb. 1999	*E. coli* O157:H9	65	Lettuce (Iceberg)	NE
May 1998	*E. coli* O157:H7	2	Salad	?
Jun. 1996	*E. coli* O153:H49	7	Lettuce	NY
May 1996	*E. coli* O157:H10	61	Lettuce (Mesclun)	?
Oct. 1995	*E. coli* O153:H46	11	Lettuce (Iceberg)	OH
Sep. 1995	*E. coli* O153:H47	30	Lettuce (Iceberg)	ME
Sep. 1995	*E. coli* O157:H7	20	Salad (Romaine)	ID
Jul. 1995	*E. coli* O153:H48	74	Lettuce (Romaine)	MT

was linked to shredded romaine lettuce, with 26 confirmed and 7 probable cases in Michigan, New York, Ohio, Pennsylvania, and Tennessee (CDC, 2010).

In addition to *E. coli* O157:H7, *L. monocytogenes* can also grow on fresh produce when present (De Simon and others 1992; Francis and others 1999; Szabo and others 2000). *L. monocytogenes* was first recognized as a foodborne pathogen in 1981 when contaminated coleslaw sickened at least 41 people and caused 18 deaths (Schelech and others 1983). *L. monocytogenes* has this far been linked to 16 produce-related outbreaks (Table 1.2). In September of 2011, cantaloupes grown in Colorado were implicated in a multistate outbreak that included 139 cases of illness, 29 deaths and 1 miscarriage (CDC 2011b). From January to October 2010, *L. monocytogenes*- contaminated celery from Sangar Fresh Cut Produce in Texas was responsible for 10 listeriosis cases and 5 deaths (FDA 2010a). Several recent recalls have also been issued for Listeria-contaminated produce. These include the 2010 recall of Tuv Taam salads brand Nova Lox packaged salad (FDA 2010b), the 2011 recalls of various bagged salad products from River Ranch Fresh Foods, chopped or shredded romaine from True Leaf Farms, and organic baby spinach and cut Napa Kimchi, and the 2012 recall of romaine lettuce, bagged salads and diced red onions in the U. S. and Canada.

Table 1.2 Outbreaks of listeriosis associated with produce (Hoelzer and others 2012).

Year	Source	Illnesses Reported	Location
1979	Tuna fish and chicken salads with celery, lettuce, and tomatoes; cheese	20 (5 death)	U. S.
1981	Coleslaw	41 (18 death)	Canada
1986	Vegetables or unpasteurized milk	28 (5 death)	Australia
1988	Vegetable rennet	1 miscarriage	United Kingdom
1989	Salad containing lettuce (hypothesized)	1	United Kingdom
1989	Salted mushrooms	1	Finland
--	Frozen broccoli, cauliflower	7	U. S.
1993	Rice salad (cheese, pickled vegetables, frozen vegetables, hard-boiled eggs)	18	Italy
1997	Corn and tuna salad	1566	Italy
1998-1999	Fruit salad	6 (5 death)	Australia
--	Melon and watermelon	--	U. S.
2001	Potato salad	56	U. S.
2006	Taco/nacho salad	2	U. S.
2010	Alfalfa sprouts	20	U. S.
2010	Celery	10 (5 death)	U. S.
2011	Cantaloupe melon	146 (30 death)	U. S.

Produce outbreaks have been most commonly associated with fresh-cut bagged products. The increasing number of outbreaks traced to fresh and fresh-cut produce points to the need to develop farm-to-table strategies to better unsure their safety.

1.3 Contamination

1.3.1 Pre-harvest contamination

The potential routes for the spread of *E. coli* O157:H7 and *L. monocytogenes* in the natural environment are numerous and include the following: irrigation water, airborne dust, cattle feed-lot run-off, wild animals (e.g., feral pigs, deer, birds), manure particulates, improperly treated compost, field processing (e.g., cores/trimmings, farm equipment), and employee hygiene (Diez-Gonzalez and Mukherjee 2009). Once present in the soil, survival of *E. coli* O157:H7 and *L. monocytogenes* is further influenced by variations in soil type, background microflora and climate (e.g., UV light, temperature, relative humidity). In addition, cattle, feral pigs, and other animals may also become asymptomatic carriers of *E. coli* O157:H7 and *L. monocytogenes* (Mandrell 2009). When leafy greens become contaminated, pathogen survival is based on relative humidity, temperature, overall health, nutrients, and maturity of the plant. The high moisture and nutrient content, together with the large surface area, make fresh produce highly susceptible to microbial growth.

Increased outbreaks of *E. coli* O157:H7 and *L. monocytogenes* in recent years have prompted significant efforts towards the development and implementation of improved Good Agricultural Practices (GAPs) to minimize pathogen contamination in the field. One such outcome was issuance of the California Leafy Green Products Handler Marketing Agreement in 2007, together with modified Good Agricultural Practices specifically for the production and

harvest of leafy greens. While adherence to GAPs will aide in pathogen reduction, current leafy green harvesting, post-harvest processing, and distribution practices cannot guarantee that these products will be free of *E. coli* O157:H7, *L. monocytogenes*, *Salmonella*, norovirus or other foodborne pathogens of public health concern.

1.3.2 Postharvest contamination

Leafy greens are prone to contamination during washing and sorting in the packinghouse, distribution in retail stores, food-service facilities, and at the home (Fig. 1.1). Contamination can result from contact with wash water, cooling ice, manure and compost, equipment in the field, transportation vehicles, cross-contamination from other foods, and improper storage, packaging, and preparation (FDA, USDA, and CDC 1998). Leafy greens destined for the fresh-cut market are typically vacuum-cooled, shredded, washed in disinfectant water, dewatered by shaking and centrifugation, and packaged in a modified atmosphere. Key contamination points observed by Kaneko and others (1999) at two fresh-cut produce facilities included the trimming knives, wash water, the blades located in the interior surfaces of a mechanical slicer, and the interior of a dewatering centrifuge, with most samples taken during production yielding aerobic plate counts above 5 log CFU/cm^2.

Washing is an essential step for removing soil and debris, decreasing the microbial load, and increasing product shelf life. While washing has abundant benefits, the wash water may become a vehicle for pathogen cross-contamination since the water is recirculated in the wash system (Buchholz and others 2009; FDA 2008). Therefore, addition of sanitizers to the water during leafy green processing is a widely used in the industry to reduce microbial populations and cross contamination. Chlorine-based sanitizers, which are most widely used in industry,

function by oxidizing intracellular enzymes (Cho and others 2010) and typically reduce microbial populations by 1 - 2.5 log CFU/g (Beuchat and others 2004).

In some cases, these sanitizers may be no more effective than water, particularly when the organic load increases in the wash water during processing, with the ongoing recalls reinforcing the unreliability of commercial sanitizers (Parish 2003; Beuchat and others 2004). Furthermore, those cells that enter the stomata of leafy greens or those that reside in hydrophobic regions, cavities, and on rough surfaces are likely to escape sanitizer exposure (Takeuchi and Frank 2000; Zhou and others 2009). Biofilm production by *E. coli* O157:H7 and *L. monocytogenes*, which has been observed on stainless steel surfaces in the presence of lettuce juice (Ryu and others 2004), can also protect pathogens from sanitizers and increase the extent of cross contamination. Moreover, internalization of pathogens from any processing step that cools, tears, cuts, or shreds leafy greens can withstand the exposure to sanitizers.

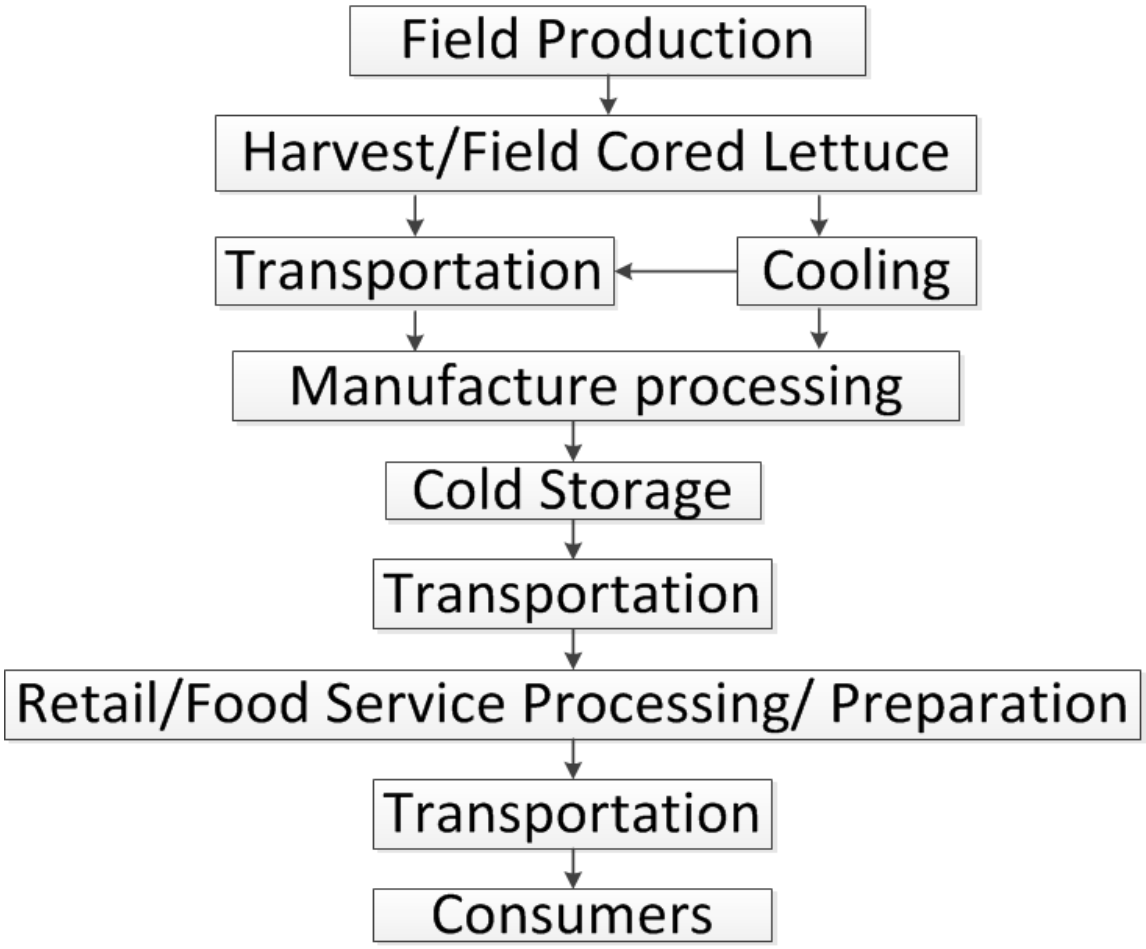


Figure 1.1 Production and distribution chain for leafy greens (Carrasco and others 2010).

After processing, bagged salads are shipped through the cold chain transport networks with no effective strategies availble to reduce microbial contamination after processing. Therefore, the safety of fresh-cut leafy greens is primarily based on temperature control. Temperature abuse leading to growth/survival of *E. coli* O157:H7 and *L. monocytogenes* during storage, transport, retail sale, and home/restaurant refrigeration is one of the major concerns in the current risk assessments for leafy greens.

1.4 Temperature

1.4.1 Growth of *E. coli* O157:H7 and *L. monocytogenes* at constant temperatures

E. coli O157:H7 will grow in a temperature range from 8 to 48°C, with the optimal growth at 37°C (Buchanan and Klawitter 1992). A comprehensive study on the growth kinetics of *E. coli* O157:H7 as impacted by temperature, pH, and salt concentration was conducted by Buchanan and Klawitter (1992), then calculated the lag phase durations, generation times, and maximum population densities. When grown in a laboratory medium at 37°C (pH 6.5) containing 5 g/L NaCl, *E. coli* O157:H7 exhibited a fastest generation time of 0.3 h, lag phase duration of 1.4 h, maximum population density of 9.6 log CFU/ml, and exponential growth rate of 1.0 log CFU/ml/h.

Growth and survival of *E. coli* O157:H7 in fresh produce, meat products, and juices under various temperatures have been extensively investigated under laboratory conditions (Abdul-Raouf and others 1993a; Abdul-Raouf and others 1993b; Del-Rosario and Beuchat 1995). Studies monitoring the growth of *E. coli* O157:H7 in shredded lettuce, sliced cucumber, cantaloupe, watermelon, and ground beef under various storage temperatures demonstrated no growth or even slightly reductions in population at 5°C, and rapid growth at 10°C or higher

(Abdul-Raouf and others 1993a; Abdul-Raouf and others 1993b; Del-Rosario and Beuchat 1995; Kauppi 1996). Significant growth of *E. coli* O157:H7 can occur on lettuce at temperatures of 12^{o}C or above (Koseki and Isobe 2005a; Luo and others 2010; McEvoy and others 2009; O'Beirne 2007). McKellar and others (2012) and Jones and others (2005) showed slight die-off of *E. coli* O157:H7 when incubated for 4 - 12 days at temperatures below 6°C. *E. coli* cells will elongate without dividing, with all cells regardless of length losing viability at similar rates (Visvalingam and others 2012).

The impact of temperature fluctuations on safety of commercially packaged produce remains poorly understood. Most growth studies have used laboratory rather than commercially prepared leafy greens, with the levels of background spoilage microorganisms likely differing from commercially packaged products. Additionally, Luo and others (2009) and Lee and Baek (2008) demonstrated that commercially packaged baby spinach leaves inoculated with *E. coli* O157:H7 stored at 7°C or above would support pathogen growth. However, these baby spinach leaves were packaged using micro perforated films. Hence, more studies on commercially bagged salads fresh-cut lettuce under modified atmosphere are needed.

Unlike *E. coli* O157:H7, *L. monocytogenes* is psychrotrophic and will grow under refrigeration conditions. Hoelzer and others (2012) synthesized and compared the available information regarding the growth of *L. monocytogenes* on produce with the listeriosis outbreak data. They found that growth rates and maximum population densities for *L. monocytogenes* differed markedly among produce commodities at 10°C. Post-harvest processing also had a major impact on the growth dynamics for certain types of fresh produce. In general, the minimum growth temperatures were 1.0°C for *L. monocytogenes* serovars 1/2a, 1/2b, 1/2c and 1.3°C for serovar 4b (Junttila and others 1988). The maximum growth temperature was 48°C.

Populations of *L. monocytogenes* on lettuce increased after eight days at 5°C (Beuchat and Brackett 1990). Growth of *L. monocytogenes* on lettuce at 5°C, 10°C, 15°C, 20°C, and 25°C was also demonstrated by Koseki and Isobe (2005b), with the fastest growth rate and shortest lag phase seen at 20°C.

1.4.2 U. S. and international guidelines on temperature control

The U. S. Food and Drug Administration 2009 Food Code requires that ready-to-eat fruits and vegetables be refrigerated and received at or below 5°C (41°F) to limit growth of organisms of public health concern (FDA 2009). The Canadian Food Inspection Agency code of practice for minimally processed ready-to-eat vegetables requires that such products be maintained at 4°C during transportation and storage (Canadian Food Inspection Agency 2009). The EU food hygiene legislation (2006) requires foods which are likely to support the growth of pathogenic micro-organisms to be held at or below 8°C. In Ireland, the core food temperature must be maintained at 5°C or below during transport (Food Safety Authority of Ireland 2006). The Food and Agriculture Organization (FAO) recommends optimum refrigeration temperatures of 2 to 5°C for fruits and vegetables (FAO 2003). Nunes and others (2008) summarized the recommended storage temperatures of 1 to 3°C for various fruits, vegetables, and bagged salads. While these temperature ranges are considered ideal, temperatures of 5 or 8°C are more realistic in the distribution chain.

1.4.3 Temperature fluctuations

Storage, processing, and distribution temperatures have a major impact on microbial quality and shelf-life of fresh-cut produce. The beneficial effects of low temperature storage (1 to 3°C) on product quality and microbial growth during transportation have been well documented (Jacxsens and others 2002; Koseki and Isobe 2005a; Luo and others 2010). Low temperature is

not only able to decrease the respiration rate and enzyme activity of fresh-cut leafy greens, but also reduces microbial growth and survival. Several studies have demonstrated the survival of *E. coli* and *L. monocytogenes* in salad vegetables at 4 to 5.5º C (Abdul-Raouf and others 1993a; Kauppi 1996; Koseki and Isobe 2005a,b). This temperature range is assumed to be frequently encountered during commercial transport of leafy greens in the U. S. Hence, under such ideal conditions, survival rather than growth of *E. coli* O157:H7 and limited growth of *L. monocytogenes* would be expected during cold chain distribution and retail handling of leafy greens.

Maintaining a consistent low temperature during transport from the manufacturer to the retail store is challenging due to both opening of the truck during loading and unloading of products as well as the extreme outside temperatures. The effect of these temperature fluctuations on growth and survival of pathogens is not yet fully understood. Thus far, only three published studies – one each from Canada, Japan and Belgium, have monitored the growth of *E. coli* O157:H7 or *L. monocytogenes* in fresh-cut bagged salad greens during actual fluctuating temperatures that were recorded during commercial harvest, transport, and retail sale (McKellar and others 2012; Koseki and Isobe 2005a; Rediers and others 2008).

Koseki and Isobe (2005a) monitored the growth of *E. coli* O157:H7 and *L. monocytogenes* for two time/temperature scenarios that were documented in Japan during commercial harvest, transport and retail sale of iceberg lettuce. The lettuce was harvested at a temperature of 16 to 17°C and then pre-cooled to below 5°C in the field. The temperature ranged from 4 to 8°C, 2 to 15°C, and 8 to 12°C, during storage, transport and display, respectively (Fig. 1.2). Overall, from the farm to retail sale, populations of *E. coli* O157:H7 and *L. monocytogenes* increased 1.3 and 1 log CFU/g, respectively. *E. coli* O157:H7 was sensitive to temperature variations on inoculated

Iceberg lettuce, with increased growth seen immediately after temperature abuse. Longer periods of temperature abuse at higher temperatures further increased the growth of *E. coli* O157:H7. In contrast, *L. monocytogenes* was less sensitive to temperature abuse with the growth rate being more consistent over time.

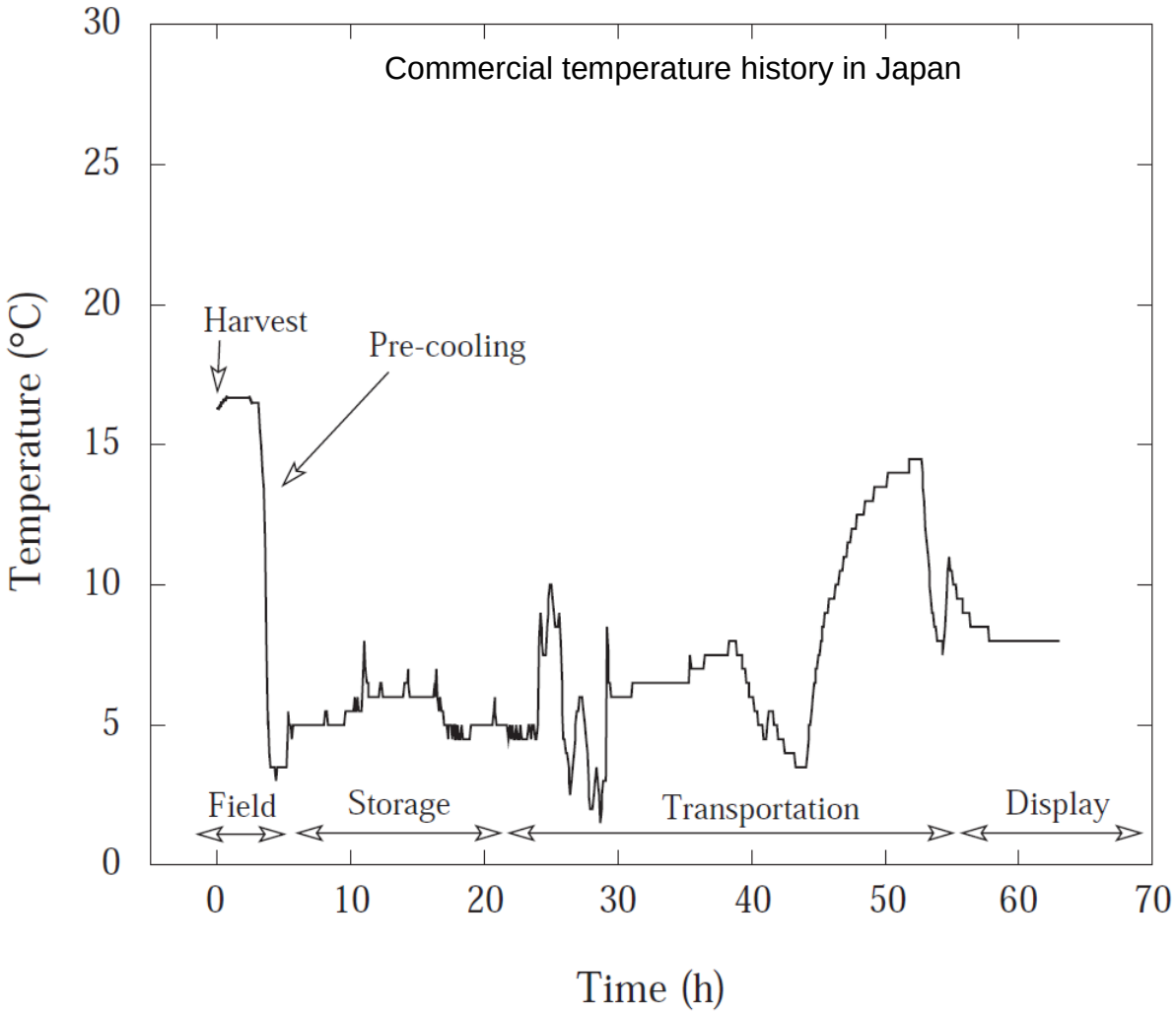


Figure 1.2 Temperature history for lettuce from the field to retail display in Japan (Koseki and Isobe 2005a).

Working in Canada, McKellar and others (2012) obtained 27 cold chain temperature profiles for fresh-cut lettuce during storage at the processing facility, commercial transport, storage at the distribution center, delivery, and retail storage. The distribution times and temperatures significantly varied for the different stores, routes, and the segments of the cold chain. In the worst case scenario, the temperature fluctuated from 2 to 4°C, 2 to 6.8°C, 1.6 to 6°C, and 1.6 to 5.4°C, and 3.5 to 6.3°C over 12.5 days during storage at the processor, transport, storage at the distribution center, delivery, and retail storage, respectively. These temperatures remained below the minimal growth temperature for *E. coli* O157:H7. Therefore, the mean estmated populations of *E. coli* O157:H7 declined 0.98 log CFU/g overall, indicating slight cell die-off. However, since this study was only conducted in winter, microbial growth could be higher during warmer months.

Rediers and others (2009) obtained three temperature profiles for endive throughout the supply chain from producer, via processor and distributor to a restaurant in Belgium. Similar to the above study, the time/temperature profiles indicated that the cold chain was generally properly maintained with only small temperature fluctuations observed. Total coliforms and Enterobacteriaceae populations were significantly higher in endive samples subjected to temperature fluctuations in the supply chain, compared to samples stored under optimal laboratory conditions. However, none of endive samples were inoculated with pathogens.

Nunes and others (2009) evaluated produce temperatures during retail storage, retail display, and household storage. The results showed that heat-sensitive fruits and vegetables were transported at higher temperatures than those recommended (Fig. 1.3). Upon arrival at three retail stores, the bagged salad temperatures were 6.8, 8.1, and 7.7°C, all of which were higher

than the recommended 1 to 3°C during cold chain transport. These recorded temperatures were higher than the above studies where sensors were placed in the refrigerated trucks. However, this study was conducted under warmer ambient conditions. At retail display, the temperature profiles for bagged salads varied widely from 1.5 to 16°C, with significant temperature differences seen in top, middle, and bottom layers of the display cases (Fig. 1.3).

Time/temperature profiles for chilled ready-to eat foods including salads from preparation and packaging in a centralized kitchen to refrigerated storage in a school canteen showed ample opportunity for growth of *L. monocytogenes*, especially during extended storage over the weekend (Rosset and others 2004). Time/temperature profiles for ready-to-eat foods stored in U. S. household refrigerators also indicate many opportunities for temperature abuse (Pouillot and others 2010). However, limited support was found for a correlation between home storage time and temperature.

Other studies monitoring temperatures during transport and retail display highlight the need to adopt temperature-monitoring technologies for perishable foods during transport. When Whyte and others (2006) evaluated meat temperatures during transport and retail display in New Zealand, more than 8% of the samples exceeded the 7ºC industry standard during summer. The retail storage temperature averaged 2ºC higher than the transport temperature, leading to potential quality and safety concerns. Another study monitoring transport of bulk beef showed broad fluctuations in product temperature during summer storage and transport (Gill and others 1997), again suggesting ample opportunity for growth of both pathogenic and spoilage organisms. A similar study in Australia monitoring chilled foods for temperature abuse during transport found that 60% of the products were above the maximum acceptable storage temperature of 5ºC (Estrada-Flores and others 2006), which resulted in abundant growth of

Pseudomonas. These findings reinforce the importance of strict temperature control during transport and retail display.

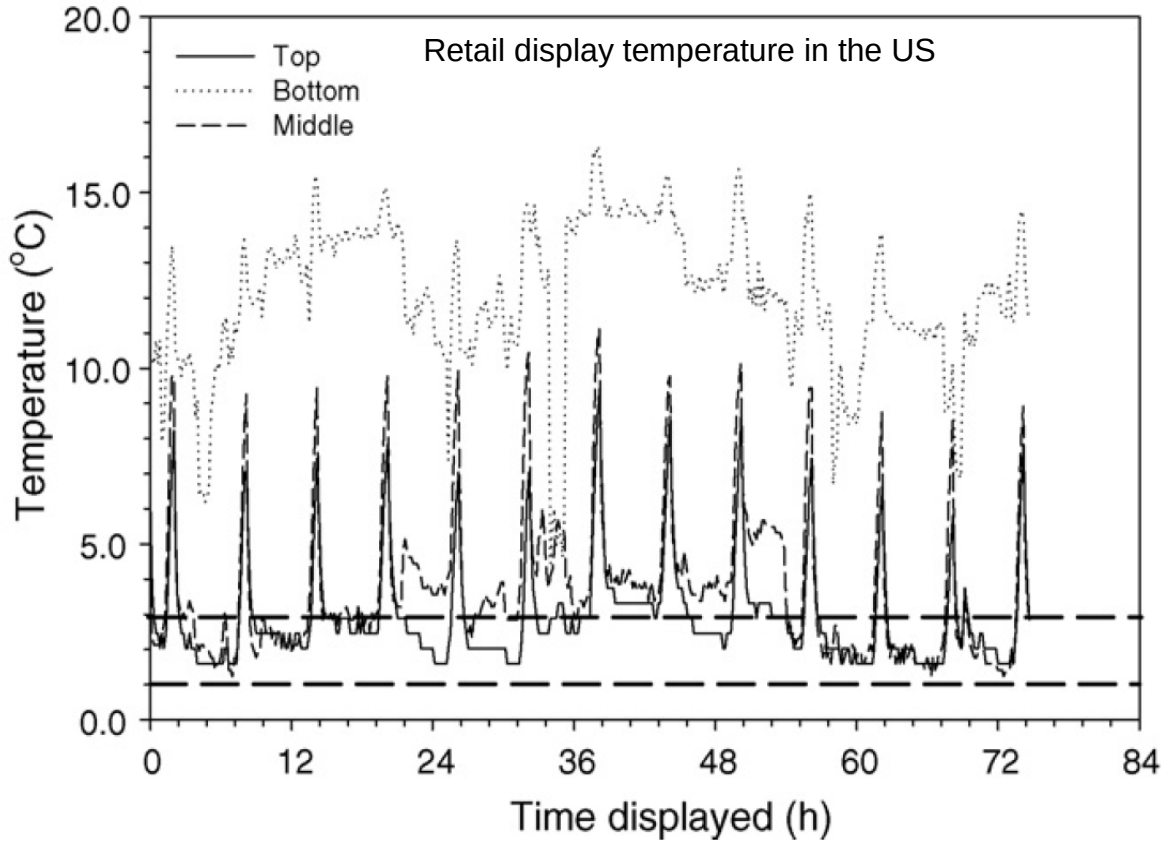


Figure 1.3 Temperature profiles for different locations (top, middle and bottom) of salad bags during retail display. Dashed horizontal lines correspond to the recommended temperature range for fresh-cut vegetable salads (Nunes and others 2009).

The effect of sudden temperature change on the intermediate microbial lag phase has been studied (Swinnen and others 2005). The intermediate lag phase is the phenomenon that a microbial population needs some time to adapt to changes in the environment when subjected to dynamic conditions, such as temperature fluctuations in the cold chain. Temperature fluctuations within the temperature range from 20 to 37°C do not result in a lag phase for *E. coli*, while temperature changes below this range lead to a period of adaptation. Therefore, at low temperatures during transport and retail sale, rapid and slight temperature changes caused by normal refrigeration cycles may have little effect on the growth of *E. coli* O157:H7.

Similarly, listeriosis outbreaks traced to fresh produce have been most often associated with fruits or vegetables that have been contaminated during processing and/or subjected to temperature abuse during storage (Hoelzer and others 2012). However, data are currently insufficient to reliably estimate growth behavior for many commodities.

In summary, the temperatures during large-scale commercial cold chain transport are generally properly maintained with only small temperature fluctuations. Temperature abuse is most likely to occur at retail storage and display. However, few temperature profiles with relative low temperatures are presently available, which may be not representative. Consequently, the impact of temperature fluctuations on both the microbial safety and quality of fresh produce in the U. S. is not yet fully understood.

1.4.4 Temperature monitoring in industry

Application of accurate temperature monitoring systems during perishable food transport and distribution can help maintain food quality and improve food safety, particularly during warm months. This information is also needed for HACCP validation and supplier compliance

records. One study used a Safety Monitoring and Assurance System to track temperatures during shipment of hams, and these time-temperature histories then were used to simulate and identify critical control points in the supply chain (Koutsoumanis and others 2005). The results showed that the percentage of unacceptable hams decreased from 16 to 4%. Surprisingly, over 12.5% of ham samples were beyond acceptable quality at the time of consumption, with the potential growth of *L. monocytogenes* and *Lactobacillus sakei* up to 10^7 CFU/g using growth models. These results reaffirm the need for monitoring transport temperatures to accurately assess current transport conditions.

Currently, various sensors with cold chain logistics are available for temperature monitoring. One of the latest innovations in temperature monitoring, TimestripPlus®, is a new temperature indicator used for temperature sensitive goods (Hafen 2012). The major advantage of this indicator is that it can be modified for each application to reflect different temperature requirements (freeze, refrigerated, and control room temperature) or different lengths of abuse time. The alert can also be color-scaled for different levels (high/moderate/low) of temperature abuse. This user-friendly design has the potential to significantly help truck drivers, retail personnel and FDA inspectors.

Implementation of Active Radio Frequency Identification (RFID) wireless sensors for temperature monitoring of perishable foods from harvest to retail sale has allowed both food processors and retailers to improve backroom operations for quality control (Songini 2006). Some large-scale retailers have even mandated the use of RFID sensors by their more than 1000 suppliers (Songini 2006). Using these RFID sensors resulted in a significant improvement in replenishment rate and stock-out reduction, and an estimated cost savings of over US $1.7 billion (Hardgrave and others 2005). Some grocery chains have also integrated RFID tracking for cases

and pallets of produce throughout its supply chain, improving customer demand chain management (Gonsalves 2004). Recent use of advanced sensor-based RFID systems has helped to minimize the growth of *E. coli* in meat (Gessner and others 2007). These sensor-based technologies are able to notify retailers of potential temperature abuse that could lead to unacceptable microbial growth at the unit level (Wessel 2006).

1.5 Modeling bacterial growth

Since the 1980s, various models have been developed to predict the growth of foodborne pathogens in foods (Zwietering and others 1990). When using isothermal growth models, curves are fitted to the experimental growth data using one of the sigmoid functions. Several sigmoid functions have been developed with part of the resulting curve defined as the lag phase (Gill 1984; Fu and others 1991). The lag phase is then modeled, together with the maximum specific growth rate, as a function of environmental factors such as temperature, pH, and water activity. In this approach, lag phase was defined empirically without mechanistic principles. Other models including logistic, vitalistic, Gompertz, Richards, Schnute, and Stannard can also be used to predict microbial growth (Cole and others 1990; Membre and others 1997; Sorrells and others 1989; Zwietering and others 1990).

Additional models have been developed to predict bacterial growth during non-isothermal temperature abuse. The U. S. Department of Agriculture – Agricultural Research Service (USDA – ARS) Pathogen Modeling Program 7.0 (PMP) has been widely applied in the food industry to estimate the growth, survival, and inactivation of *Listeria* and other pathogens based on pH, storage temperature, and salt concentration (Buchanan and others 1990; Houstsma and others 1996; Le Marc and others 2002). Under non-isothermal temperature conditions, pathogen growth can be predicted by dividing the time-temperature history into multiple small time/temperature

intervals and then applying the USDA PMP to predict the pathogen growth in each interval. The overall estimated pathogen growth is the sum of the predicted growth for each interval. However, this model was developed from pure-culture and broth-based studies. Because pure-culture systems contain high levels of nutrients without competitive microflora, the PMP model is generally assumed to provide conservative estimates of pathogen growth. Koseki and Isobe (2005a) also indicated that the PMP model tended to overestimate the pathogen growth. Therefore, USDA regulators have not generally accepted this approach as the sole means of scientific validation (USDA FSIS 2005).

Baranyi and Roberts (1994; 1995) developed a state-of-the-art approach to predict microbial growth based on the physiological state of the cell. This dynamic model has been successfully implemented for continuously changing growth conditions such as temperature, pH and water activity (Baranyi and others 1994; 1995). Combined with Baranyi's primary model and Ratkowsky's square-root model, the growth of *Brochothrix thermosphacta* was accurately predicted under gradually changing temperatures and sudden changes in temperature between 5 and 25°C. Accuracy of the model prediction depends on optimizing an appropriate value for model parameter α_0, the actual specific growth rate to the potential specific growth rate at the time of inoculation. However, the extent of growth was overestimated when the temperature profile contained sudden changes between 2.8 and 25°C (Baranyi and others 1995). This might be due to changes in the physiological state of the microorganism as a result of sudden cold shock.

Koseki and Isobe (2005a) applied the Baranyi model to predict the growth of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* on lettuce at fluctuating temperatures encountered from farm to retail store. The growth kinetic parameters (lag time, maximum growth rate, and

maximum population density) were obtained from pathogen growth studies on lettuce at isothermal temperatures (5 to 25°C). These model parameters were then used to predict pathogen growth under fluctuating temperatures in the Baranyi primary microbial growth model along with the Ratkowsky secondary model. Their results demonstrated that the Baranyi growth model was able to accurately predict pathogen growth under fluctuating temperatures in most cases when fresh produce was properly cooled after harvest. Most predictions agreed with the observed viable counts based on the small root mean square error (RMSEs) and biases. In comparison, the PMP model, the Gamma concept, and the model of Farber and others (1996) overestimated the growth rate with larger RMSEs (Sant'Ana and others 2012; Carrasco and others 2008; Farber and others 1996; Koseki and Isobe 2005a).

However, Baranyi's model has limitations. Overestimation of *E. coli* O157:H7 and *Salmonella* growth using Baranyi model occurred when the temperature history started high (25°C) for 5 hours and decreased later (Koseki and Isobe 2005a), while the prediction of *L. monocytogenes* under the same condition fitted the observed growth data. Danyluk and Schaffner (2011), McKellar and Delaquis (2012) also showed that Baranyi's model overestimated the growth rate of *E. coli* O157:H7. However, most data are from constant temperature studies (Chang and Fang 2007; Delaquis and others 2002; Lee and Baek 2008; McEvoy and Luo 2009; Theofel and Harris 2009). One explanation was that the estimated growth kinetics calculated from the isothermal environments were inadequately used for determining coefficients of differential equations for predicting growth in dynamic environments. In addition, the growth kinetic parameters used by Koseki and Isobe (2005a) were obtained under ambient conditions. The optimizing kinetic parameters for predicting microbial growth in commercial fresh-cut bagged salad greens under Modified Atmosphere Packaging (MAP) remain unknown which also

increases uncertainty. When Carrasco and others (2007) used the Baranyi model to predict the growth of *L. monocytogenes* on ready-to-eat MAP-packaged iceberg lettuce, *Listeria* growth was significantly inhibited.

Juneja and others (2008) recently modified the Baranyi model by adding a memory effect so that the kinetic parameter predictions depended on prior cell history. This modification allowed for a less restrictive assumption and might lead to models that provide more precise estimates of growth. Although limited to lag and exponential growth phase, this model can characterize the growth kinetic parameters relative to prior history in dynamic environments. Future studies are needed to validate this modified Baranyi model for predicting the growth of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* in bagged fresh-cut salads.

To improve the accuracy of model prediction, McKellar and others (2009) developed a dynamic growth-death model for *E. coli* O157:H7 in minimally processed leafy greens. McKellar's model contains a primary three-phase linear model for growth, a secondary square root model for growth, and a tertiary log-linear model for death. Therefore, McKellar's model has two advantages. Firstly, bacterial cell death and survival over time have been considered. Secondly, the model can accurately predict microbial growth at low temperatures. McKellar and others (2009) successfully used this model to predict pathogen behavior under both isothermal and non-isothermal conditions when fitted to the data from 13 published studies, with better predictions compared to Baranyi's model.

McKellar and others (2012) applied this dynamic growth-death model to simulate the behavior of *E. coli* O157:H7 in fresh-cut lettuce under the dynamic temperatures encountered during distribution from processing to retail. Since this study was conducted in Canada during winter, the 27 temperature profiles reported in this study showed only slight temperature abuse.

At these low temperatures, the model predicted an overall mean decline in cell numbers and slight growth for a few cases when the temperature rose above 5°C. However, no experimental growth data were available to validate the model predictions, making accuracy of the model prediction difficult to determine. In addition, since this model includes a linear death model, it has the potential to underestimate microbial growth if the parameters are not optimized. Therefore, model validation during periods of more severe temperature such as during summer is necessary since underestimation of microbial growth is usually not acceptable for model predictions.

1.6 Risk assessment

Quantitative microbial risk assessment (QMRA) has been an increasingly used approach to integrate and evaluate information from diverse sources concerning the origin and fate of pathogens in the food chain and to determine the magnitude of public health risks. The Sanitary and Phytosanitary Agreement (SPS Agreement) of the World Trade Organization (WTO) recognized the necessity of scientific verification and validation when determining food safety risks. The principles and guidelines for food safety risk analysis were defined by the Codex Committee on Food Hygiene (FDAP/WHO 1995). Risk is defined as the probability and the consequence of a hazard to occur. A traditional QMRA consists of four components: hazard identification, dose-response assessment, exposure assessment, and risk characterization, which when considered together provide an expression of public health risk.

Risk assessment has its roots in the concerns for toxic chemicals in food. While these assessments are mostly based on toxicology and carcinogenity studies, their application to microbial foodborne pathogens poses some significant challenges. One difficulty relates to the fact that unlike chemical, toxicological, or environmental contaminants, populations of bacterial

pathogens can change dynamically since conditions vary widely from farm to consumption. Fortunately, researchers are making progress in developing the predictive models, simulations, and other tools that will meet the technical requirements for quantifying estimates of risk. In addition to this technical challenge, there are many data gaps that limit the precision necessary for quantitative risk assessments. For instance, little information is available to precisely estimate the relationship between the quantity of a biological agent and the frequency and magnitude of adverse human health effects, particularly as this might relate to susceptible sub-populations. There is also limited information on exposure assessment - the amounts of foods consumed by populations and their probable contamination.

QMRA usually involves various mathematical models, probability distributions, and Monte Carlo simulation. Mathematical models are used to describe the introduction of pathogens into food, multiplication of microbes in food over time, microbial survival following various treatments, consumption of microbes in food, and subsequent illness. The inherent variability and uncertainty in the estimation of microbial risk can be described by probability distributions. A probability distribution is a mathematical representation of the relative likelihood of a random variable taking on a specific value. Monte Carlo simulation can be used to estimate the level of human illness and uncertainty associated with the model. Control strategies can be calculated in the same way and a cost/benefit can be conducted for prevention of illness.

The preliminary QMRA framework for the risks associated with leafy greens from farm to table began a few years ago. Up to now, three research groups have assessed the risk of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in leafy green vegetables from farm to consumption in the U. S., Spain, and the Netherlands (Carrasco and others 2010; Franz and others 2010; Danyluk and Schaffner 2011). Danyluk and Schaffner (2011) presented a

preliminary framework that identified currently available data and provided initial risk estimates for *E. coli* O157:H7 in leafy greens in the U. S. The risk model predicted a starting level of -1 log CFU/g and a prevalence of 0.1% which could have resulted in an outbreak approximately the size of the 2006 *E. coli* O157:H7 spinach outbreak. Temperature abuse will support growth of *E. coli* O157:H7, with populations increasing as much as 1 log CFU/day. Carrasco and others (2010) assessed the risk of *L. monocytogenes* in ready-to-eat lettuce salads in Spain. The estimated number of listeriosis cases was 10^2 and 10^5 for low- and high-risk subpopulations, with similar numbers of listeriosis cases reported in Spain. They also found that MAP was the most effective means to reduce the number of cases. Franz and others (2009) estimated the average number of cases per year associated with the consumption of leafy greens at salad bars in the Netherlands at 166, 187, and 0.3 for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, respectively. The temperatures in that study were maintained at < 5°C, with considerably greater growth of *L. monocytogenes* (194%) and minimal growth of *E. coli* O157:H7 (19%). The mathematical models and data used in these studies provide a preliminary database for future leafy green risk assessments. However, many data gaps still remain to be filled, including transport/distribution/retail sale/home storage temperature fluctuations, the correlation between storage time and temperature, the importance of lag time in microbial growth models, and validation of the importance of cross-contamination in wash water during processing.

Quantitative microbial risk assessment has become the framework for the scientifically-based decisions used in formulating sound risk management strategies. The work presented in this thesis on temperature abuse of leafy greens in the cold chain will help fill one of the important data gaps in the current risk assessment.

CHAPTER 2

MODELING THE GROWTH OF *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* IN PACKAGED FRESH-CUT ROMAINE MIX AT FLUCTUATING TEMPERATURES DURING SIMULATED TRANSPORT, RETAIL STORAGE AND DISPLAY

2.1 Abstract

Temperature abuse during commercial transport and retail sale of leafy greens negatively impacts both microbial safety and product quality. Consequently, the effect of fluctuating temperatures on *Escherichia coli* O157:H7 and *Listeria monocytogenes* growth in commercially bagged salad greens was assessed during transport, retail storage, and display. Over a 16-month period, a series of time/temperature profiles for bagged salad greens were obtained from 16 transportation routes covering four geographic regions (432 profiles), as well as during retail storage (4867 profiles) and display (3799 profiles) at nine supermarkets. Five different time/temperature profiles collected during 2 to 3 days of transport, 1 and 3 days of cold room storage and 3 days of retail display (including the best and worst scenarios) were then duplicated in a programmable incubator to assess *E. coli* O157:H7 and *L. monocytogenes* growth in commercial bags of romaine lettuce mix. Microbial growth predictions using the Baranyi/Ratkowsky and McKellar models were validated by comparing the root mean square error (RMSE) and bias between the laboratory growth data and model predictions. Monte Carlo simulations (~100,000 iterations) were then performed to calculate the probability distribution of microbial growth from a total of 8,122,127,472 scenarios during transport, cold room storage, and retail display. Using inoculated bags of retail salad, *E. coli* O157:H7 and *L. monocytogenes* populations increased a maximum of 3.1 and 3.0 log CFU/g at retail storage. Both models

yielded < 1 log CFU/g RMSEs and biases with the Baranyi model fitting slightly better to the laboratory data. Based on the simulation results, both pathogens generally increased < 2 log CFU/g. However, retail storage duration can significantly impact the extent of *E. coli* O157:H7 and *L. monocytogenes* growth. Greatest growth of *E. coli* O157:H7 would be expected in product located at the bottom of the pallet during summer transport. This large-scale U. S. study – the first using commercial time/temperature profiles to assess the microbial risk of leafy greens during transport, retail storage, and display, should be useful in filling some of the data gaps in current risk assessments for leafy greens.

2.2 Introduction

Consumption of fresh and fresh-cut leafy greens in the United States has increased over the past few decades due to the abundant health benefits and year-round availability (Calvin and others 2003; Meng and Doyle, 2002; Nicola and others, 2006). Leafy green vegetables consumed raw are highly susceptible to microbial contamination. Since 1993, numerous foodborne outbreaks involving *Escherichia coli* O157:H7 have shaken consumer confidence with at least 800 cases of illness and 8 deaths having been traced to leafy greens (FDA 2005 a), particularly California-grown lettuce (Iceberg, Romaine, red leaf, and mesclun) and spinach. In late summer of 2006, consumption of contaminated California-grown baby flat-leaf spinach was responsible for 205 cases of *E. coli* O157:H7 infection in 28 states, including 31 cases of hemolytic uremic syndrome and 3 fatalities (FDA 2006). In December 2006, commercially-shredded lettuce used in tacos that sickened over 150 people was again traced to *E. coli* O157:H7 (CDC 2006a).

In addition to *E. coli* O157:H7, *Listeria monocytogenes* is also of particular concern because of its wide distribution in the environment and its ability to grow during refrigeration. In

2011, 139 cases of listeriosis, including 29 deaths and 1 miscarriage, were traced to consumption of cantaloupe (CDC 2011b) with a smaller outbreak linked to celery one year earlier (FDA 2010a). While cases of listeriosis involving lettuce are few (FDA 2012), 8 recalls have been issued since 2010 for *L. monocytogenes*-contaminated leafy greens, thus legitimizing concern for the pathogen in lettuce.

Produce can become contaminated at any point in the farm-to-fork continuum. The high moisture and nutrient content, together with the large surface area of fresh-cut produce, make these highly susceptible microbial contamination and subsequent growth. Although washing and sanitizing can decrease microbial numbers, bacterial cells internalized in stomata or cut surfaces and those found in hydrophobic regions, cavities, and rough surfaces of leafy greens are likely to survive sanitizer exposure (Takeuchi and Frank 2000; Zhou and others 2009).

Temperature monitoring during distribution provides a major advantage in the ability to manage temperature sensitive products in the cold chain with most food and pharmaceutical suppliers of perishable goods already tracking time/temperature histories. Many large-scale retailers have now mandated the use temperature sensors and data loggers for their suppliers with others using various temperature monitoring systems to track specific cases and pallets of product in the supply chain. In this study, temperature sensors were placed in trucks during transport, at retail storage room, and retail display cases in order to monitor temperature fluctuations and collect time/temperature profiles.

The many benefits of low temperature storage on product quality and microbial growth during transport and distribution have been well documented (Jacxsens and others 2002; Koseki and Isobe 2005a; Luo and others 2010). The U. S. Food and Drug Administration Food Code requires that ready-to-eat fruits and vegetables be refrigerated at $\leq$ 5°C (41°F) to minimize

growth of foodborne pathogens (FDA 2009). However, maintaining a consistently low temperature throughout the distribution chain is challenging. Opening of the truck doors during loading and unloading, outside temperature extremes and retail storage/display conditions can all lead to temperature fluctuations. Thus far, only studies from Canada, Japan and Belgium have assessed the growth of *E. coli* O157:H7 and *L. monocytogenes* at real-time temperatures collected during pre-harvest, transportation and retail sale of fresh-cut bagged salad greens (McKellar and others 2012; Koseki and Isobe 2005a; Rediers and others 2008). However, limited time/temperature profiles in the above studies may not represent the U. S. cold chain transport and distribution.

Several mathematical models have been developed over the last 25 years to predict the likelihood for growth of *E. coli* O157:H7, *L. monocytogenes* and other pathogens in foods, primarily under constant temperature conditions (Buchanan and others 1990; Cole and others 1990; Membre and others 1997; Sorrells and others 1989; Zwietering and others 1990). The Baranyi/Ratlowsky model has been successfully used to predict microbial growth under continuously changing conditions (Baranyi and others 1994; 1995). Koseki and Isobe (2005a) applied this same model to predict the growth of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* on lettuce at fluctuating temperatures. While some studies showed a small root mean square error between observed bacterial growth and the model prediction (Koseki and Isobe 2005a; Carrasco and others, 2008), others found that the Baranyi/Ratlowsky model tended to overestimate *E. coli* O157:H7 growth (Danyluk and Schaffner 2011; McKellar and Delaquis 2011). A dynamic growth – death model was recently developed by McKellar and Delaquis (2011) to quantitatively predict the growth/survival of *E. coli* O157:H7 at variable temperatures during transport and retail sales.

A recent quantitative microbial risk assessment of leafy greens from farm to fork by Danyluk and Schaffner (2011) supported the growth of *E. coli* O157:H7 during periods of temperature abuse. However, the retail temperature profiles used were obtained from meat, cheese and yogurt rather than leafy greens, with temperature abuse during commercial transport from the manufacturer to retail stores also lacking.

Consequently, the objectives of this study were to: 1) assess the impact of various real-time time/temperature histories on the growth of *E. coli* O157:H7 and *L. monocytogenes* as well as psychrotrophic and mesophilic bacteria in commercial romaine mix during transport, retail cold storage and display, 2) validate the Baranyi/Ratkowsky and McKellar models using laboratory data, and 3) determine the probability distributions for *E. coli* O157:H7 and *L. monocytogenes* growth during transport, retail storage and display. To our knowledge, this is the first large-scale study to report the impact of fluctuating temperatures on microbial growth for fresh-cut leafy greens distributed in the United States.

2.3 Materials and methods

2.3.1 Temperature monitoring during commercial transport and retail

Temperature fluctuations were continuously recorded in typical refrigerated truck trailers using TempTale$^{@}$4 sensors (Sensitec Inc., Beverly, MA). During cross-country transport of bagged salads, five different commercial routes were assessed during four seasons – California to North Carolina (5 trials), Arizona to North Carolina (1 trial), California to Texas (3 trials), California to Ohio (5 trials) and California to Arizona (2 trials). Within each refrigerated cargo trailer, 20-30 sensors were placed in three key zones - front, midway and rear, midway up the sidewall of the truck and at three pallet levels - top, middle, and bottom (unpublished data).

Time/temperature readings were recorded at 5-minute intervals during 2 to 3 days of transport until delivery with a total of 432 time/temperature profiles generated.

Temperature fluctuations during retail storage and display were monitored at 9 supermarkets. Depending on the size of the store, 2 to 6 and 6 to 24 sensors were respectively placed in the storage room and display case for 1 to 2 months with two replications. The bagged salads were typically stored for 1 to 3 days and displayed for a maximum of 3 days. Therefore, each original temperature profile during storage and was generated at 1- and 3-day intervals. In total, 4867 retail storage and 3863 retail display temperature profiles were obtained.

Five temperature profiles, including the best and worst case scenarios as well as three in-between temperatures, were selected from those collected during transport, retail storage, and retail display for the microbial growth studies. Criteria for selection were based on the average, maximum and minimum temperatures, standard deviations, and model predictions (see model validation).

2.3.2 Culture preparation

The four avirulent, GFP-labeled strains of *Escherichia coli* O157:H7 chosen for study were ATCC 43888 (pGFPuv, *stx1*$^-$, *stx2*$^-$, ampicillin resistant), CV 2b7 (pGFPuv, *stx1*$^-$, *stx2*$^-$, ampicillin resistant), 6980-2 (pGFP, *stx1*$^-$, *stx2*$^-$, ampicillin resistant), and 6982-2 (pGFPuv, *stx1*$^-$, *stx2*$^-$, ampicillin resistant), all of which were obtained from Dr. Michael Doyle (University of Georgia, Griffin, GA). The four unlabeled strains of *Listeria monocytogenes* were N1-227 (serotype 4b, food outbreak), C1-056 (serotype 1/2a, human sporadic case), J1-108 (serotype 4b, human outbreak), and N3-031 (serotype 1/2a, hot dog sporadic case). All stock cultures were maintained at –80°C in Trypticase Soy Broth containing 0.6% (w/v) Yeast Extract (TSB-YE)

(Becton, Dickinson & Co., Franklin Lakes, NJ)] and 10% glycerol (Mallinckrodt Baker, Inc., Phillipsburg, NJ).

Prior to inoculation, the four strains of *E. coli* O157:H7 and *L. monocytogenes* were respectively streaked to plates of Trypticase Soy Agar containing 0.6% (w/v) Yeast Extract (TSA-YE) and 100 ppm ampicillin or TSA-YE which were incubated overnight at 37°C. Single colonies of each *E. coli* O157:H7 and *L. monocytogenes* strain were then subjected to two consecutive 24 h/37°C transfers in TSB-YE with or without 100 ppm ampicillin, respectively. The cultures were then combined in equal volumes to obtain two separate 4-strain cocktails containing ~10^8 CFU/ml as determined by optical absorbance and then diluted in sterile phosphate buffer solution (PBS) to a level of 2.6×10^6 CFU/ml.

2.3.3 Inoculation

Retail bags of pre-washed classic romaine mix (255 g/bag) were obtained from a local supermarket (Meijer, Okemos, MI) at least 15 days before the "best sell by" date and immediately placed in a walk-in cold room at 4°C with a maximum 2-day storage time before use. Each bag was surface-sterilized using 70% ethanol prior to inoculation. Working in the cold-room, each bag of romaine mix was inoculated with 2 ml of the *E. coli* O157:H7 or *L. monocytogenes* cocktail using a BD PrecisionGlide™ general use syringe (Becton, Dickinson & Co.) so as to contain ~10^3 CFU/g, immediately sealed with tape to maintain the same package atmosphere and then vigorously shaken for 1 min to evenly distribute the inoculum. The package atmosphere was measured using Gas Chromatography at the Department of Packaging, Michigan State University. The average oxygen and carbon dioxide concentration based on three bags of salads were 4.3% and 3.2%, respectively. Three bagged salads were similarly syringe-inoculated with Glow Germ (0.5 g in 2 ml of distilled water) and placed under UV light. Glow Germ

fluorescence under UV light indicated that the inoculum was evenly distributed inside the bags after the 1-min shake. In addition, three 50 g sub-samples from three bags of salads inoculated with *E. coli* O157:H7 were separatedly examined for *E. coli* O157:H7. Statistical results also showed no significant difference for *E. coli* O157:H7 populations in each sub-samples of salad bags to support the conclusion that the inoculum was evenly distribution in salad bags.

2.3.4 Incubation

The inoculated bags were immediately placed in a Thermo Forma Environmental Chamber (Model 3851, Thermo Fischer Scientific, Inc., Waltham, MA) that was programmed to duplicate the same time/temperature profiles recorded by the TempTale® 4 sensors during commercial transport and retail storage/display. All of the programmed time/temperature profiles were monitored and recorded at 5-min intervals using a HOBO data logger (Onset®, Bourne, MA). These profiles were then validated based on the root mean squared errors (RMSEs) and biases between the transport/storage/display and environmental chamber time/temperature data. For each individual temperature selected:

$$\mathbf{RMSE} = \sqrt{\frac{\sum (y_{sensor} - y_{lab})^2}{\mathbf{n}}}$$

and

$$\mathbf{Bias} = \frac{\sum \left(y_{sensor} - y_{lab} \right)}{\mathbf{n}}$$

Each experiment was repeated three times with two replicates per trial.

2.3.5 Microbial analyses

Bags of romaine mix were collected at pre-determined times (average of three to four times per day for transport and retail storage, and once per day for retail display) and quantitatively

analyzed for *E. coli* O157:H7 and *L. monocytogenes*. The salad bags were weighed and aseptically opened after surface decontamination with 70% ethanol. Duplicate 50-g lettuce samples from each bag were transferred to sterile Whirl-Pak® filter bags (1.7 L, Nasco, Fort Atkinson, WI) and macerated in 200 ml of sterile PBS using a Stomacher® 400 Circulator (Seward, London, U.K.) at 260 rpm for 1 min. After appropriate serial dilution in PBS, 100 µl aliquots were plated on TSA-YE containing 100 ppm ampicillin or Modified Oxford Agar (Neogen, Lansing, MI) to respectively quantify *E. coli* O157:H7 and *L. monocytogenes* after 48 h of incubation at 37°C and 35°C. Similarly diluted samples were surface-plated in duplicate on TSA-YE with these plates then incubated at 37°C for 48 h or 4°C for 10 days to quantify mesophilic and psychrotrophic bacteria, respectively.

2.3.6 Model validation

The Baranyi primary model, a square-root Ratlowsky secondary model for maximum growth rate, and a linear model for maximum population density (MPD) were applied to the time/temperature profiles, with the dynamic solution based on the following coupled differential equations (Baranyi et al., 1995):

$$\frac{dq}{dt} = \mu_{max} q \quad \text{and} \quad \frac{dx}{dt} = \mu_{max} \frac{q}{1+q}\left(1 - \frac{x}{x_{max}}\right)x$$

where x is the natural log of the bacterial concentration, q is the physiological state of the population, μ_{max} is the maximum specific growth rate, and x_{max} is MPD. The initial conditions for the above equations are fixed as $q(0) = q_0$ and $x(0) = x_0$. The secondary models are:

$$\sqrt{\mu_{max}} = b(T - T_{min})$$

where the model parameters used were those initially reported by Koseki and Isobe (2005a) for growth of *E. coli* O157:H7 and *L. monocytogenes* on iceberg lettuce (Table 2.1).

MATLAB® (R2010b, MathWorks, Natick, MA) codes were developed for the Baranyi/ Ratlowsky model so that microbial growth could be predicted by inputting the time/temperature profiles. Initial predictions were used to select the time/temperature profiles used in the previously described laboratory experiments.

Table 2.1 Baranyi and Ratlowsky model parameters for growth of *E. coli* O157:H7 and *L. monocytogenes* on Iceberg lettuce (Koseki and Isobe 2005a).

Pathogen	**α_0**	**b**	**T_{min} (°C)**
E. coli O157:H7	0.056	0.033	4.54
L. monocytogenes	0.072	0.016	-4.26

A dynamic growth-death model developed by McKellar and Delaquis (2011) was also applied to simulate the growth of *E. coli* O157:H7:

$$\frac{dN}{dt} = R_{ate \times N};$$

$$Rate = if\ (T > 5, Growth, Death);$$

$$Growth = (b * [(T - T_{min}))]^2;$$

$$Death = -k;$$

$$LogN_{t+\Delta t} = LogN_t + \left(Rate \times \frac{\Delta t}{2}.303\right);$$

$$b = 0.023\ (95\%\ prediction\ limits\ 0.01 - 0.035);$$

$$T_{min} = 1.335 - 5.766 \times b;$$

$$k = 0.013\ (\pm 0.01)$$

The validity of the above two growth models was quantified based on the root mean squared error (RMSEs) and biases between the predicted and observed experimental growth data. RMSEs and biases were evaluated as follows for each individual time/temperature profile selected:

$$RMSE = \sqrt{\frac{\sum(y_{observation} - y_{predition})^2}{n}}$$ and

$$Bias = \frac{\sum(y_{observation} - y_{predition})}{n}$$

The model with the smaller RMSE and bias was then used to predict the increase in growth rate for *E. coli* O157:H7 and *L. monocytogenes* at every temperature profile collected during transport, retail storage and display.

2.3.7 Monte Carlo simulation

Monte Carlo simulations (~100,000 iterations) were performed in MATLAB to calculate the probability distribution of microbial growth for 432 (during transport) × 4867 (24 h at retail storage) × 3863 (72 h at retail display) in a total of 8,122,127,472 time/temperature scenarios. Seasonal effect during transport, location of the bag (top, middle, bottom layer) within the pallets, retail storage time (24 h vs 72 h), and location in the retail display (front vs back of the display case) were examined to further simulate the probability distributions for microbial growth.

2.3.8 Statistical analyses

Data were log transformed to satisfy the assumption of homogeneity of variance. The standard errors for bacterial populations over time were calculated by ANOVA using the PROC MIXED procedure in the SAS package (v.9.2, SAS Institute, Cary, NC). Fisher's Least Significant Difference was used to compare the difference between the initial and maximum populations of mesophiles and psychrotrophs ($P < 0.05$).

2.4 Results

2.4.1 Temperature selection for lab validation

Over a 16-month period, a series of time/temperature profiles were obtained for bagged salad greens from 16 transport routes covering four geographic regions (432 profiles), as well as during retail storage (4867 profiles) and display (3863 profiles) at nine supermarkets. Overall,

the average temperature profiles ranged from -0.3 to 7.7°C, 0.6 to 15.4°C, and -1.1 to 9.7°C during transport (Fig. 2.1A), retail storage (Fig. 2.1B), and retail display (Fig. 2.1C),

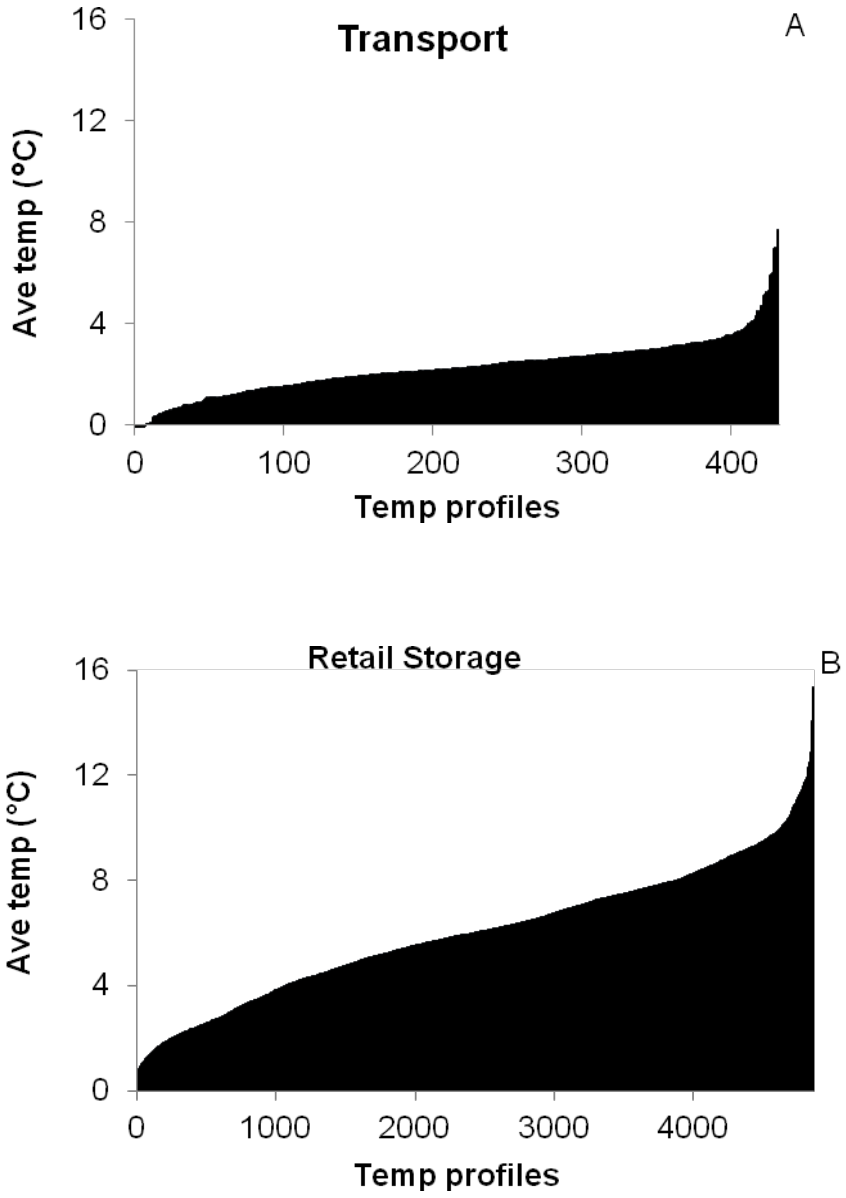
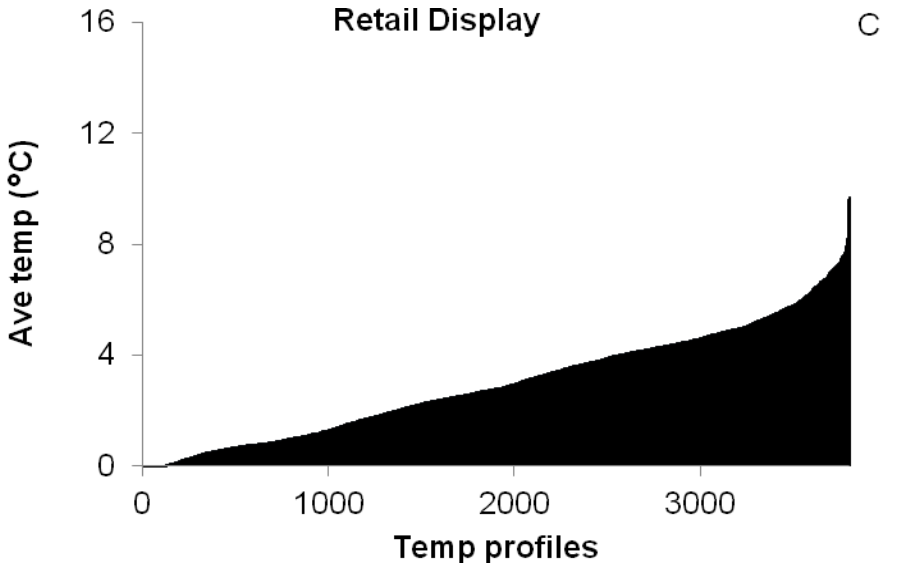
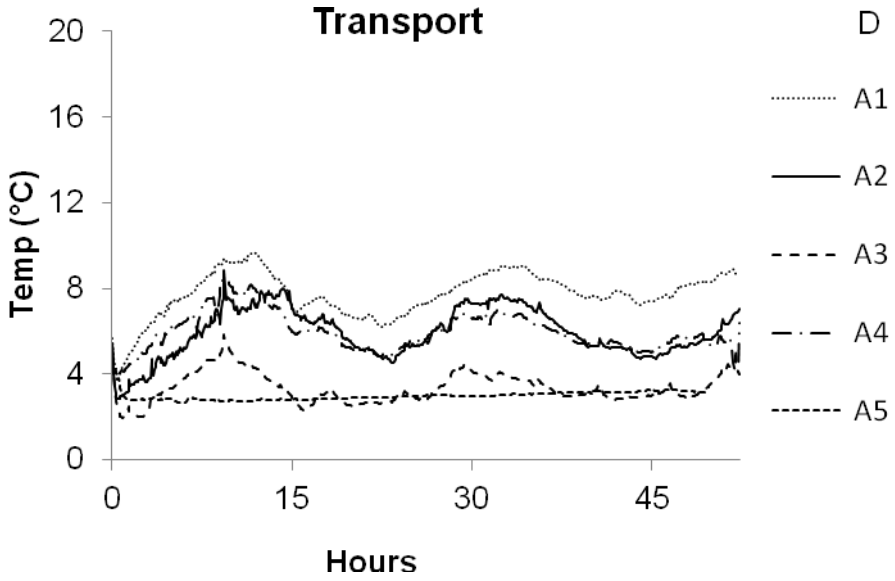


Figure 2.1 Average temperatures for all profiles collected during transport, retail storage, and display with the 5 selected temperatures indicated (A transport, B retail storage, C retail display) and the time/temperature profiles for each of the temperatures selected (D transport, E retail storage, F retail display).

Figure 2.1 (cont'd)





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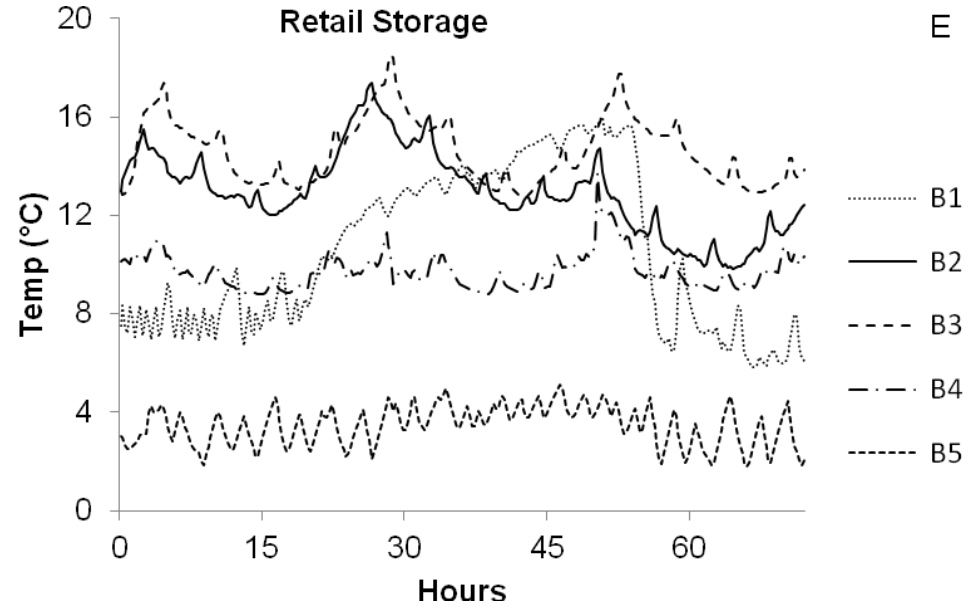
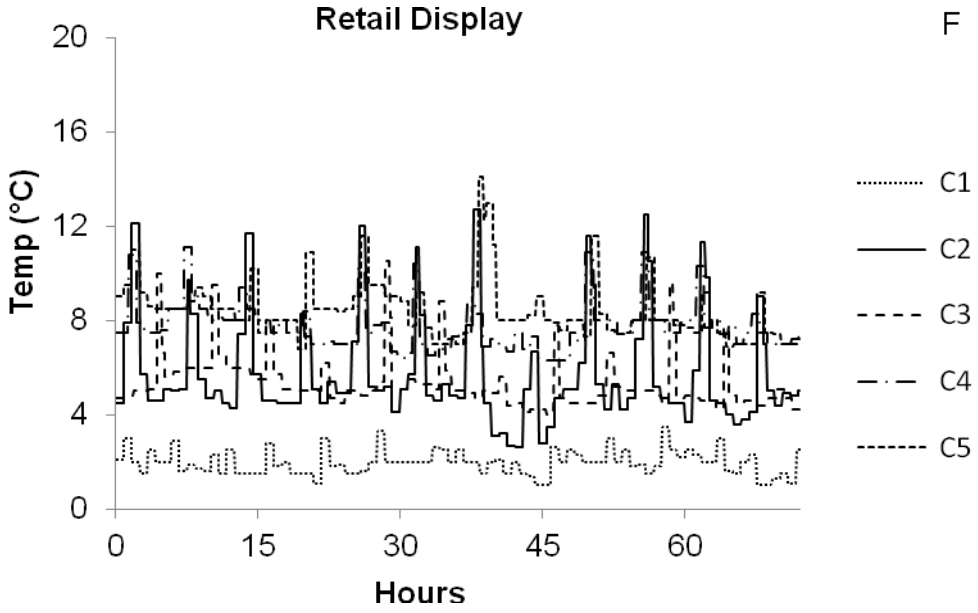




respectively. Five transport (Fig. 2.1D), retail storage (Fig. 2.1E) and retail display (Fig. 2.1F) time/temperature profiles having minimum and maximum temperature peaks ranging from 2.1 to 9.7°C, 1.8 to 18.2°C, and 1.0 to 14.1°C, respectively, were selected based on average, minimum and maximam temperatures, and preliminary model predictions for the microbial studies.

Root mean square errors (RMSEs) and biases between sensor and laboratory-based temperatures duplicated in the programmable incubator were calculated for the five transport, storage and display time/temperature profiles (Table 2.2). All biases were $\leq$ 1°C and all RMSEs except those for C1 and C2 were $\leq$ 1°C.

2.4.2 Microbial growth

In bags of inoculated romaine mix, *E. coli* O157:H7 populations remained at ~ 3 log CFU/g during 48 h to 52 h of incubation at simulated transport temperatures (Fig. 2.2 A1 – A5). Simulated retail storage led to *E. coli* O157:H7 population increases of 0.1 to 3.1 log CFU/g (Fig. 2.2 B1 – B5) with no significant growth seen during 72 h at retail display (Fig. 2.2 C1 – C5). *L. monocytogenes* populations increased $\leq$ 0.6 log CFU/g at simulated transport temperatures (Fig. 2.2 A1 – A5). Greater growth of *Listeria* was seen during 72 h at retail storage and display temperatures with populations increasing up to 3.0 (Fig 2.2 B1 – B5) and 1.1 log CFU/g (Fig 2.2 C1 – C5), respectively. Using the same time/temperature profiles as above, mesophilic and psychrotrophic populations increased significantly ($P < 0.05$) in all samples except A3 (Table 2.3). Similar to *E. coli* O157:H7 and *L. monocytogenes*, mesophiles and psychrotrophs exhibited greater growth – up to 3.1 and 3.3 log

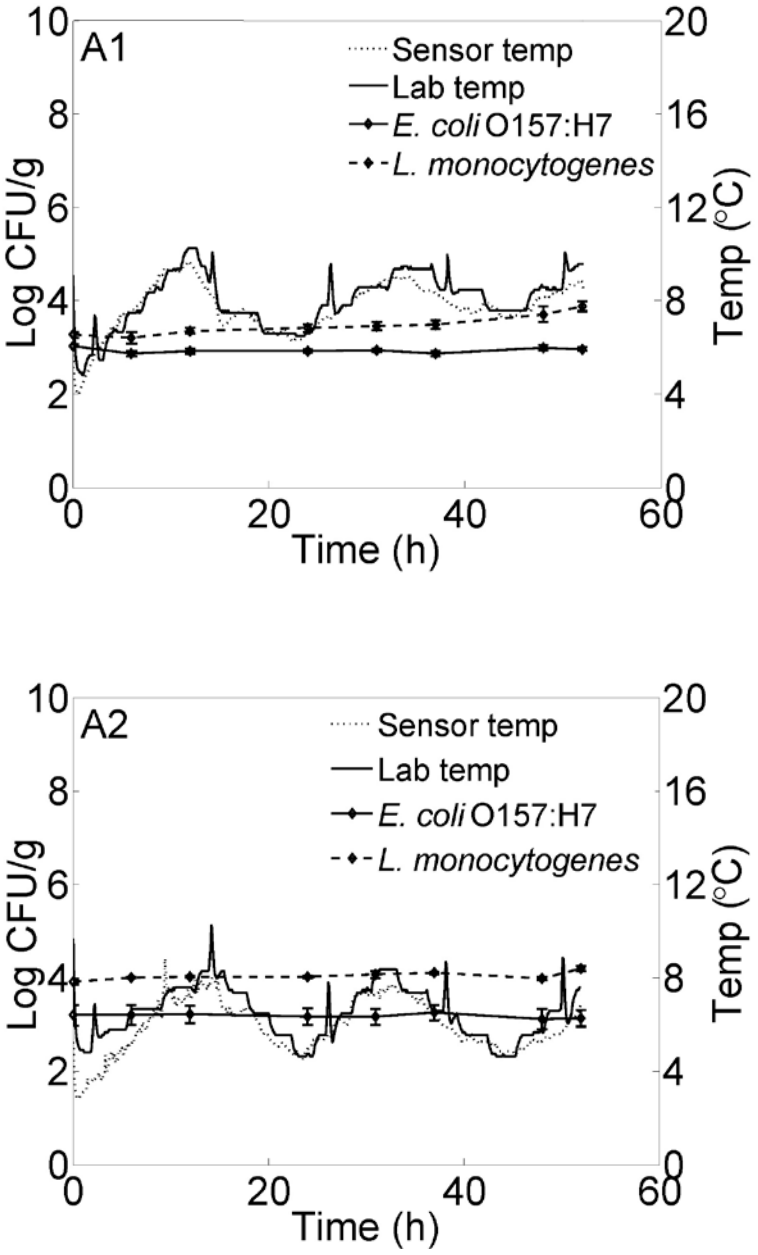
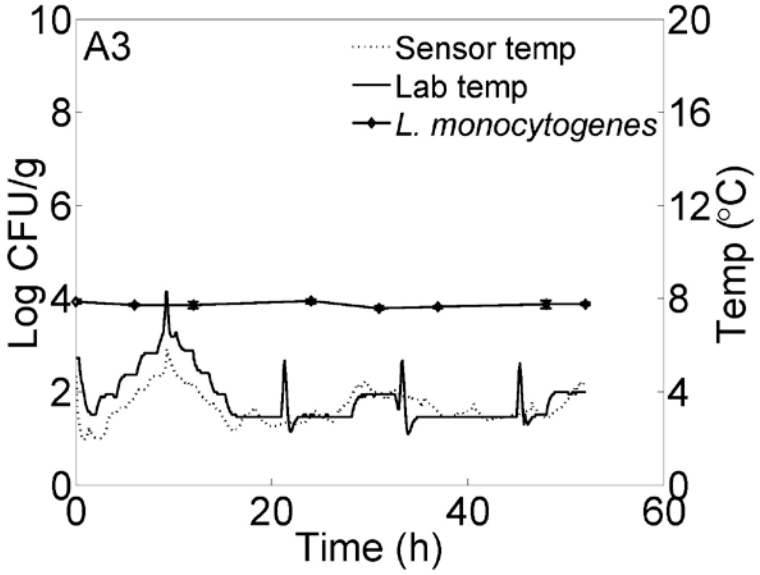
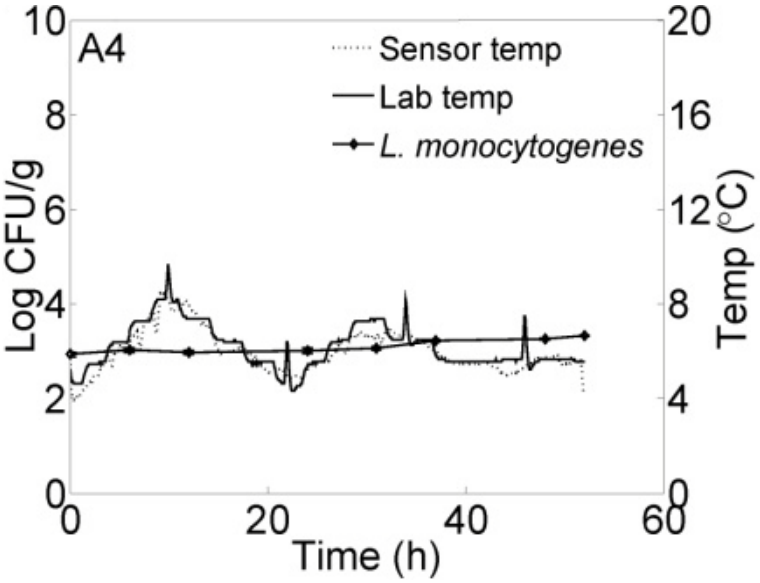


Figure 2.2 Growth of *E. coli* O157:H7 and *L. monocytogenes* under selected temperatures during transport (A1 – A5), retail storage (B1 - B5) and retail display (C1 – C5).

Figure 2.2 (cont'd)





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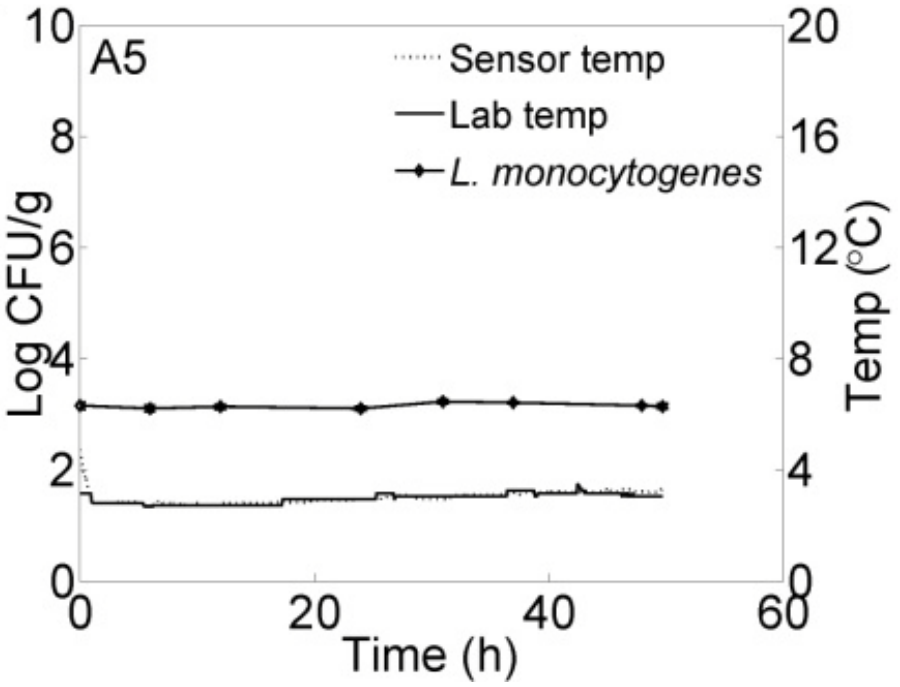

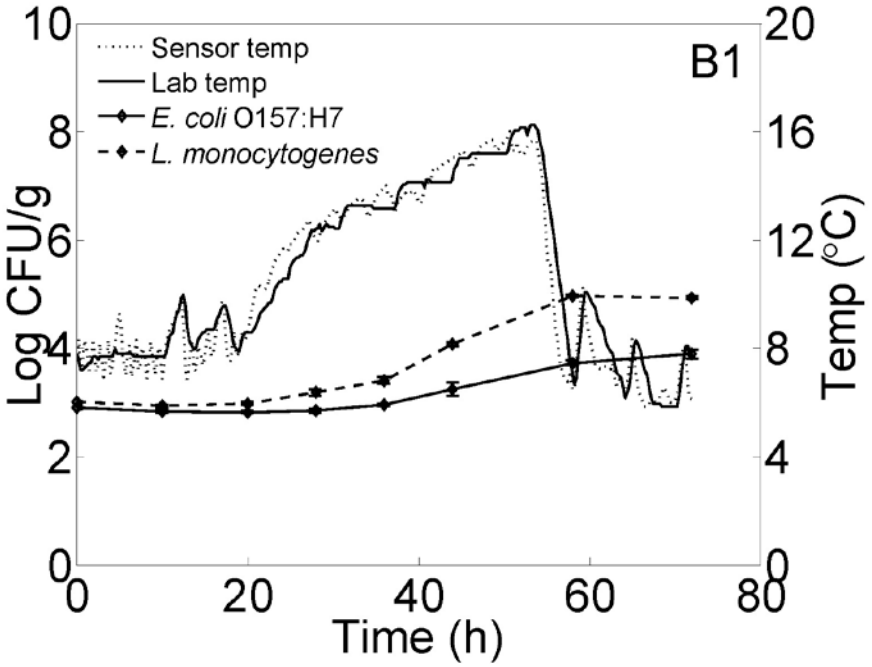

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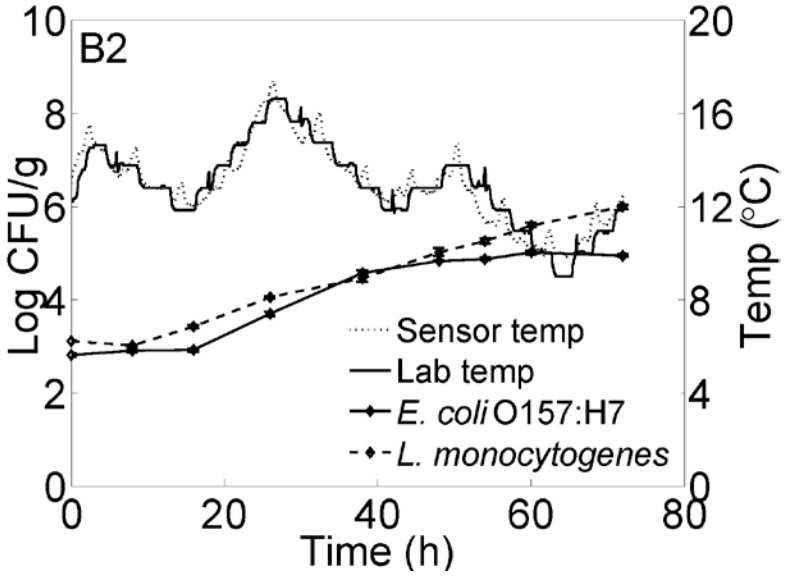

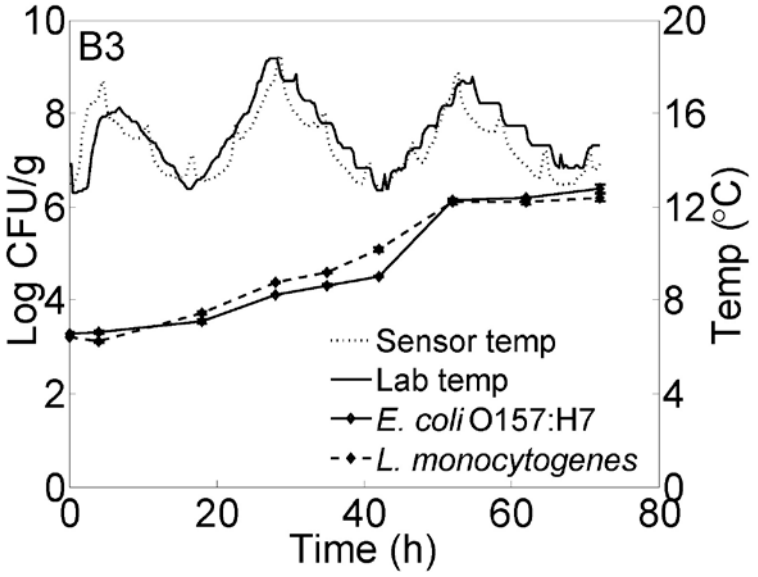

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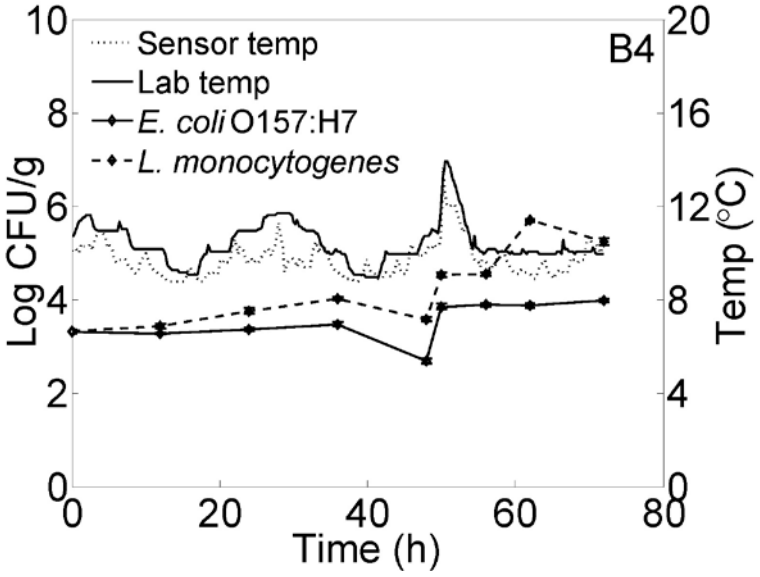

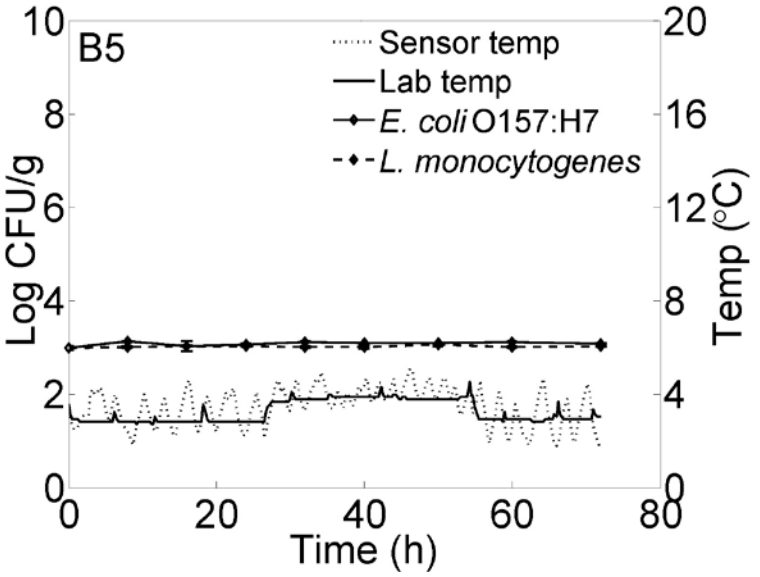

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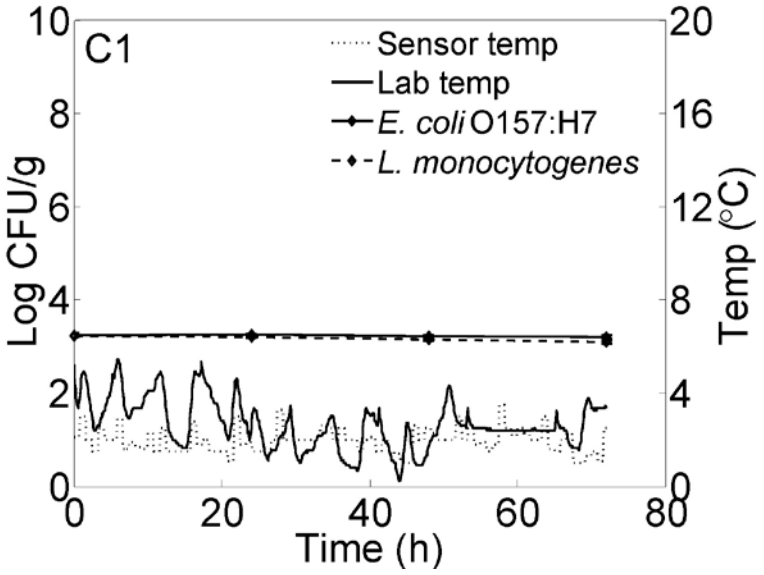

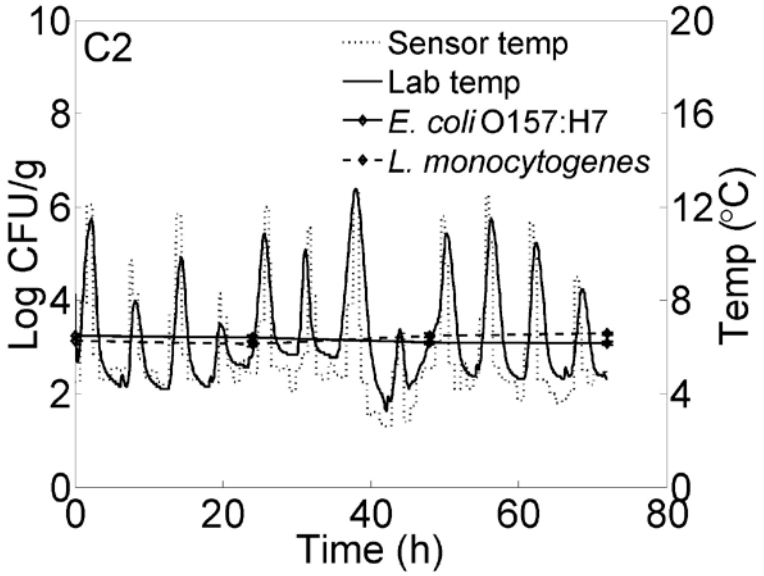

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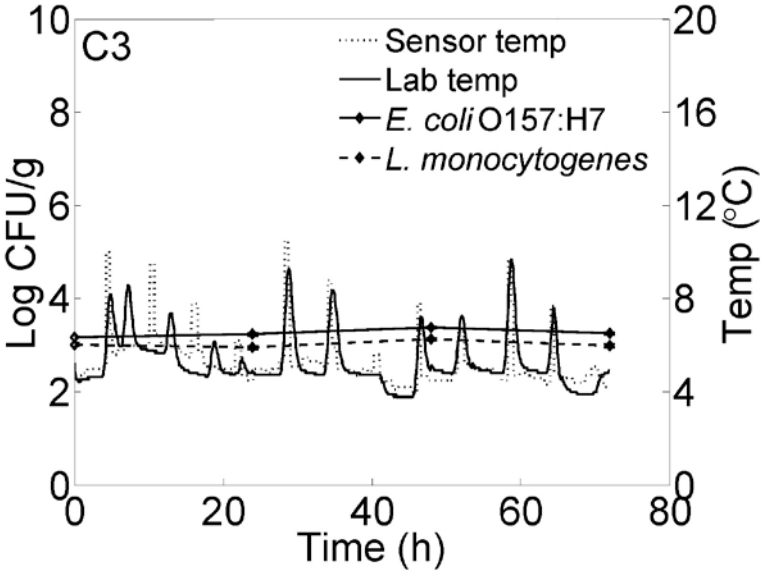

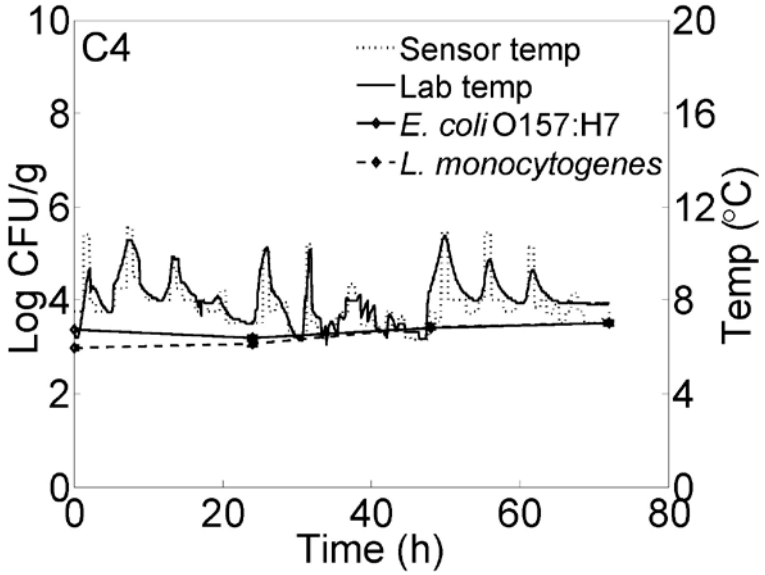

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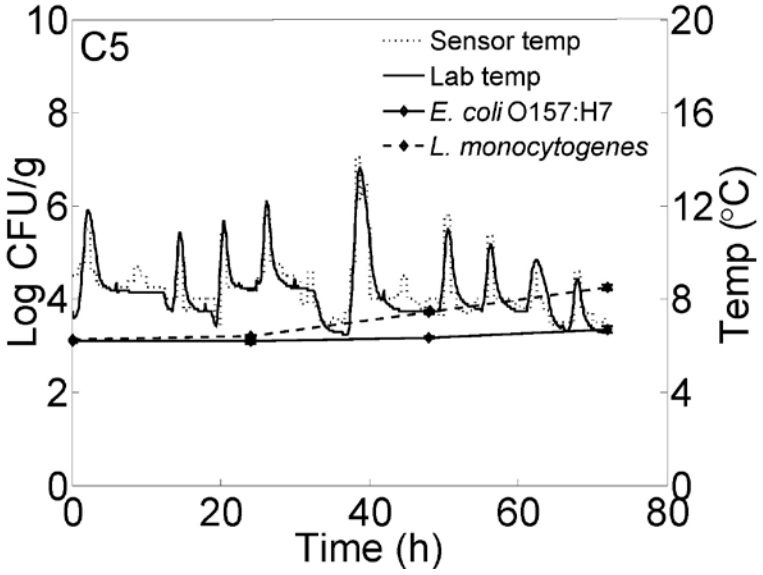


Table 2.2 Root Mean Square Errors and biases between selected sensor and duplicated lab temperatures during transport, retail storage and display.

Transport	RMSE (°C)	Bias (°C)	Retail Storage	RMSE (°C)	Bias (°C)	Retail Display	RMSE (°C)	Bias (°C)
A1	0.57	0.30	B1	0.88	0.11	C1	1.32	0.71
A2	0.88	0.57	B2	0.62	-0.06	C2	2.11	0.98
A3	0.88	0.57	B3	0.93	0.45	C3	0.81	0.21
A4	0.47	0.21	B4	0.89	0.69	C4	0.78	0.28
A5	0.14	-0.02	B5	0.69	-0.19	C5	1.00	-0.08

Table 2.3 Growth of mesophiles and psychrotrophs in bagged romaine mix during transport, retail storage, and display

	Mesophiles			Psychrotrophs		
Transport	**Initial (log CFU/g)**	**Final (log CFU/g)**		**Initial (log CFU/g)**	**Final (log CFU/g)**	
A1	5.19	6.62	*[a]	5.79	7.06	*
A2	6.28	6.83	*	6.61	7.54	*
A3	5.86	5.91		6.10	6.71	*
A4	5.06	6.29	*	5.54	6.70	*
A5	6.61	6.82	*	6.90	7.06	*
Retail Storage						
B1	5.50	7.36	*	5.48	8.37	*
B2	4.79	7.87	*	6.08	7.78	*
B3	4.52	6.30	*	4.83	6.69	*
B4	5.10	8.02	*	5.35	8.65	*
B5	5.78	6.86	*	5.89	7.21	*
Retail Display						
C1	5.99	6.16	*	5.94	7.14	*
C2	4.96	7.31	*	5.13	7.72	*
C3	6.03	6.73	*	6.35	7.39	*
C4	6.20	7.12	*	6.01	7.45	*
C5	5.26	6.65	*	6.06	7.19	*

[a]*-Significant growth during incubation ($P < 0.05$).

CFU/g, respectively, during retail storage where the average temperatures were higher as compared to transport and retail display. During transport, retail storage, and display, psychrotrophic populations were generally higher than the mesophiles.

2.4.3 Model validation

All RMSEs and biases between the observed microbial growth and the model predictions were < 1 log CFU/g (Table 2.4). These small RMSEs and biases indicated that both the Baranyi model and McKellar models were acceptable. Based on our growth data, Baranyi's model yielded smaller RMSEs and biases for most of the transport retail storage and display scenarios. Only one RMSE (Table 2.4 B4) and two biases (Table 2.4 B3 and C4) were slightly higher using Baranyi's in comparison to McKellar's model. Therefore, Baranyi's model was used for the Monte Carlo simulations.

2.4.4 Monte Carlo simulation

In most cases, the simulated populations of *E. coli* O157:H7 and *L. monocytogenes* increased < 1 and < 2 log CFU/g, respectively (Fig. 2.3, 2.4, 2.5, and 2.6). Retail storage duration had the greatest impact on *E. coli* O157:H7 and *L. monocytogenes* growth (Fig. 2.3). Storing the bagged salads for 24 and 72 h at retail storage yielded overall growth (> 1 log CFU/g) probabilities of 2 and 34%, respectively, for *Listeria*. Similarly, 24 and 72 h of storage produced overall *E. coli* O157:H7 growth (> 1 log CFU/g) probabilities of 0.5% and 3.6%.

Season, bag location in the pallet (top, middle, or bottom), and location in the retail display case (front or back) did not significantly impact ($P > 0.05$) microbial growth (Fig. 2.4, 2.5, and 2.6). However, the probability of *E. coli* O157:H7 exceeding 3 log CFU/g was 29.6 times greater during transport in summer as compared to fall, winter, and spring. The probability of *E. coli*

O157:H7 exceeding 3 logs CFU/g was also 36.1 times greater when the bag was located near the bottom of the pallets.

Table 2.4 Root Mean Square Errors and biases between observed pathogen growth and the Baranyi and McKellar model predictions.

Temp profiles	**Pathogen**	***E. coli* O157:H7**				***L. monocytogenes***	
	Model	**Baranyi**		**McKellar**		**Baranyi**	
	Errors (log CFU/g)	**RMSE**	**Bias**	**RMSE**	**Bias**	**RMSE**	**Bias**
Transport	A1	0.20	-0.14	0.44	0.37	0.33	0.16
	A2	0.44	0.04	0.49	0.18	0.17	0.04
	A3	N/A[a]	N/A	N/A	N/A	0.18	0.11
	A4	N/A	N/A	N/A	N/A	0.12	-0.04
	A5	N/A	N/A	N/A	N/A	0.14	0.05
Retail storage	B1	0.38	0.24	0.57	0.47	0.94	-0.03
	B2	0.35	0.10	0.72	0.58	0.86	-0.20
	B3	0.26	0.14	0.26	0.00	0.38	-0.27
	B4	0.54	0.16	0.43	0.20	0.44	-0.29
	B5	0.16	-0.09	0.35	-0.28	0.09	0.05
Retail display	C1	0.11	0.01	0.25	-0.18	0.17	0.11
	C2	0.18	0.11	0.34	0.25	0.93	-0.54
	C3	0.13	-0.08	0.15	-0.10	0.23	0.15
	C4	0.14	0.05	0.15	0.00	0.25	0.12
	C5	0.13	0.00	0.45	0.37	0.12	-0.02

[a]N/A - Growth of *E. coli* O157:H7 was not assessed.

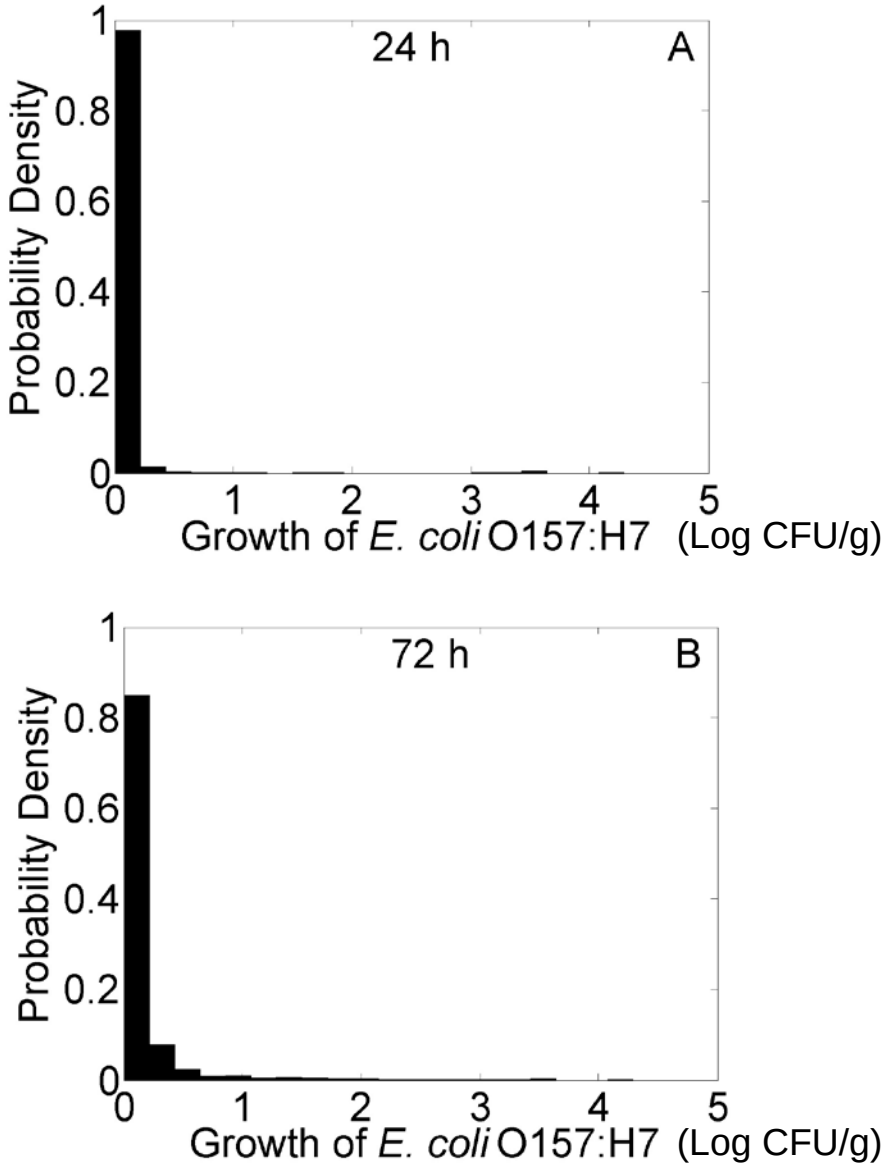
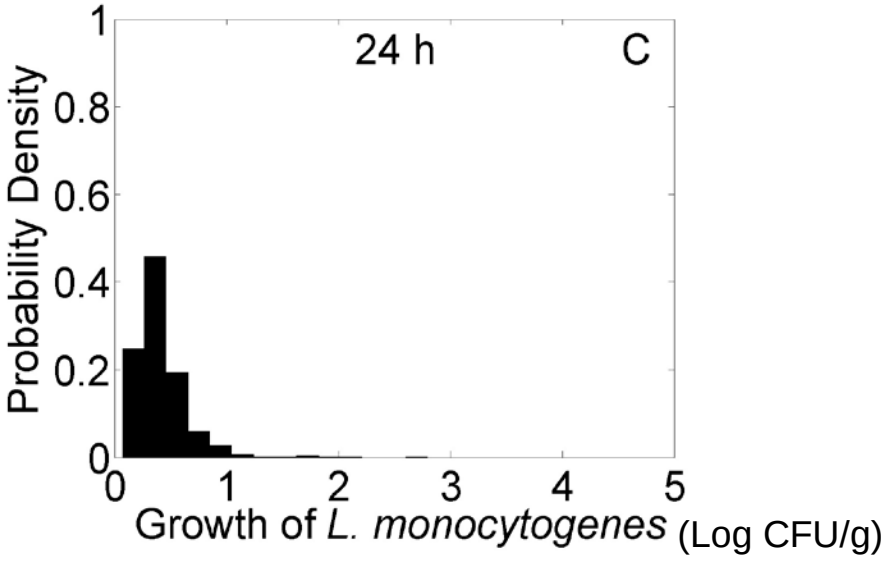
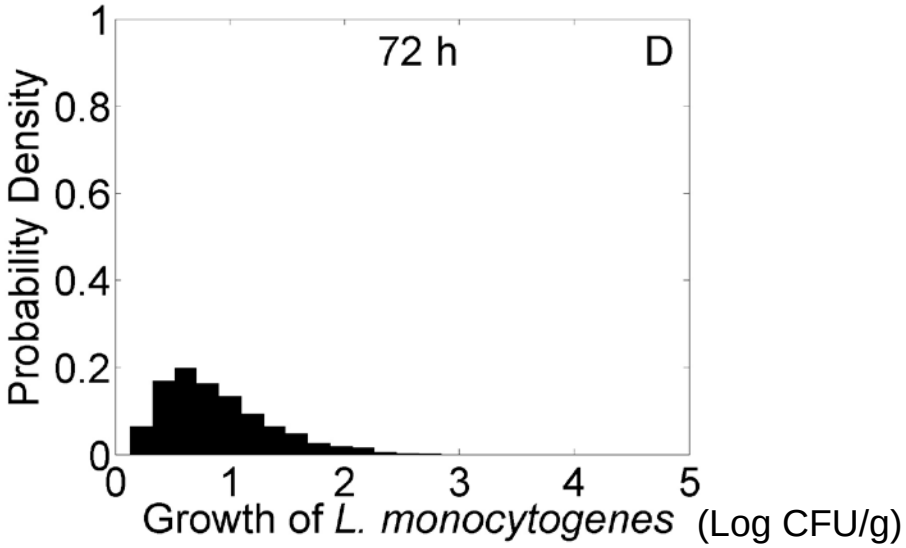


Figure 2.3 Probability distributions for *E. coli* O157:H7 (A, B) and *L. monocytogenes* (C, D) from transport to retail storage with 24 and 72 h of retail storage, respectively.

Figure 2.3 (cont'd)





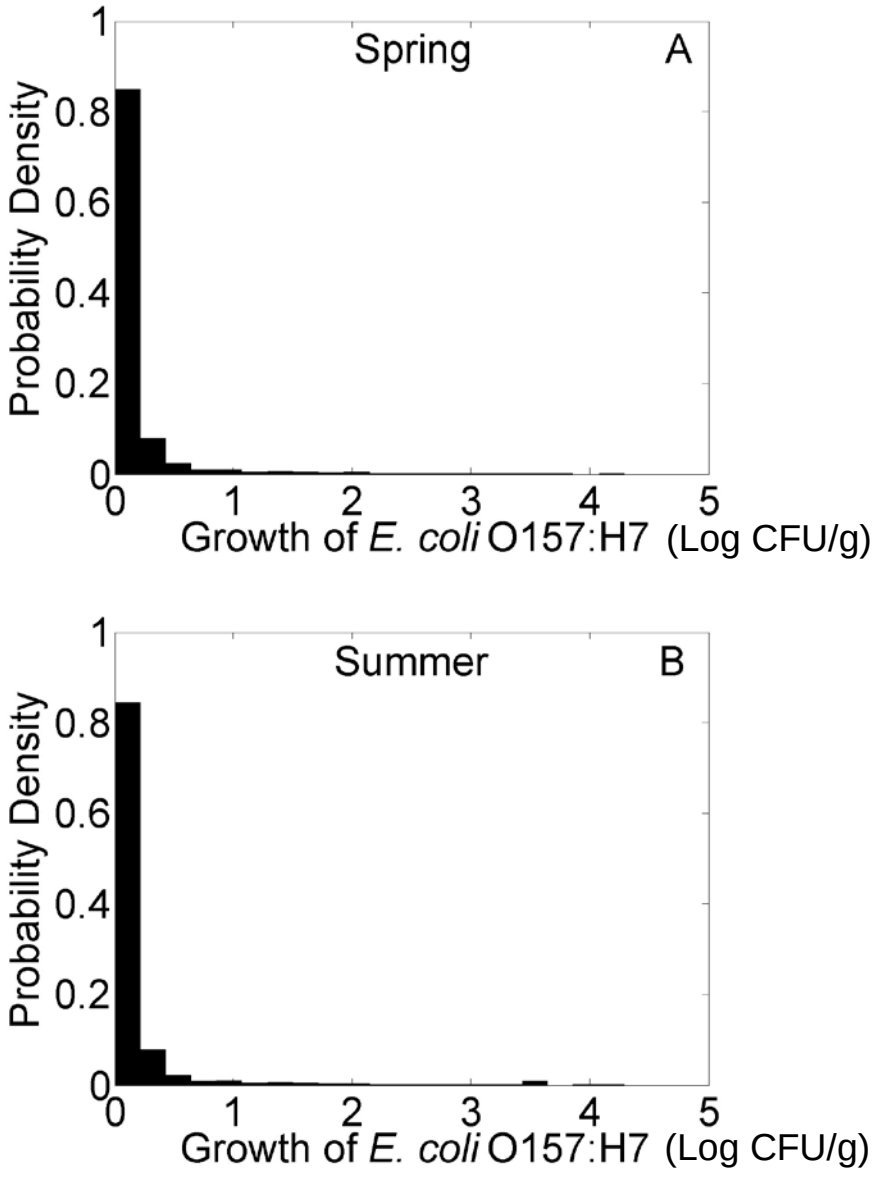
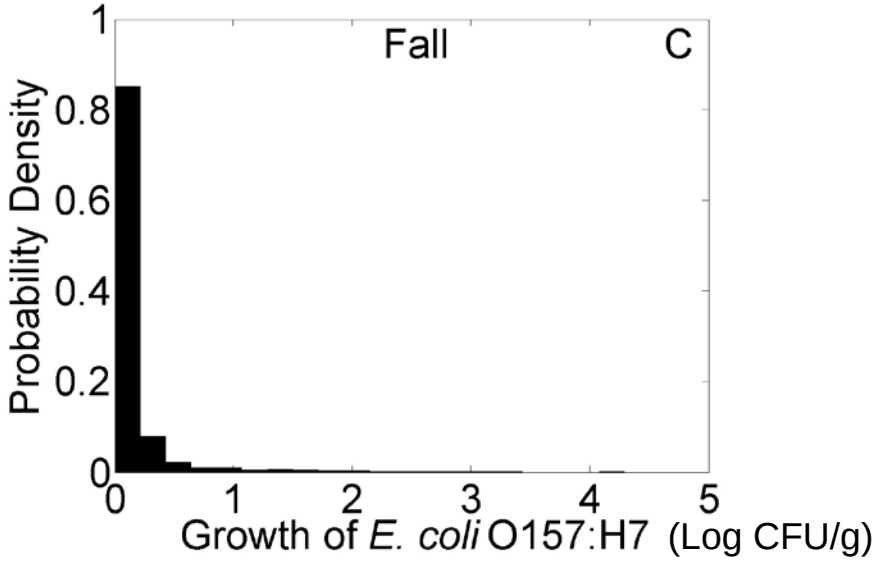


Figure 2.4 Probability distributions for *E. coli* O157:H7 (A, B, C, D) and *L. monocytogenes* (E, F, G, H) growth in romaine mix transported during spring, summer, fall and winter.

Figure 2.4 (cont'd)



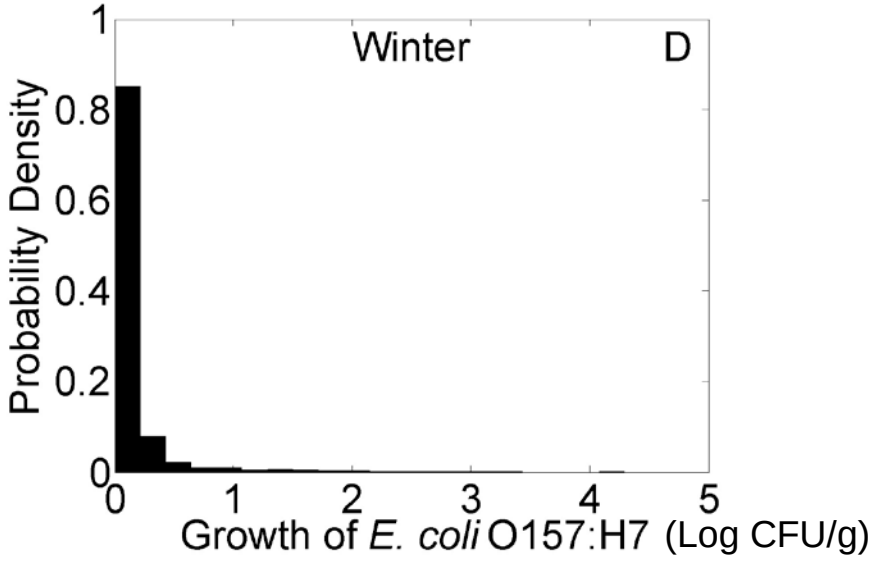
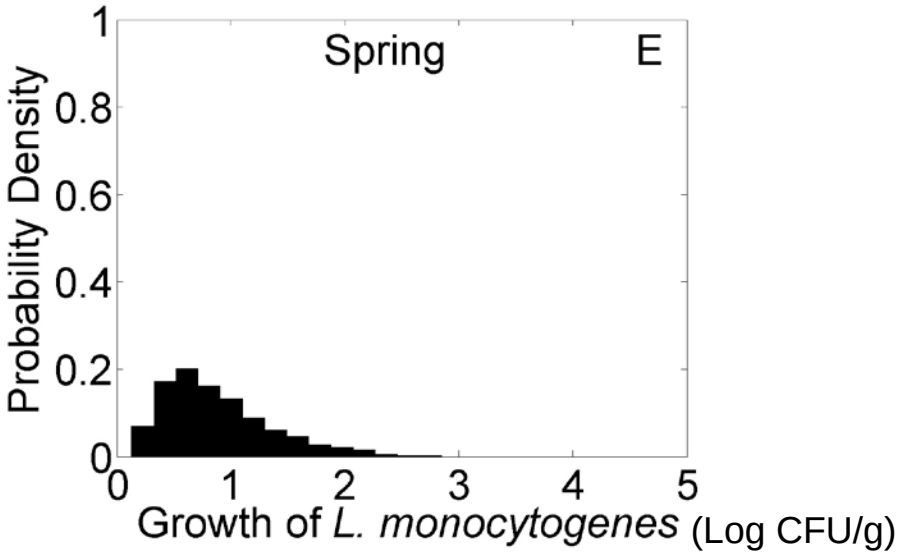


Figure 2.4 (cont'd)



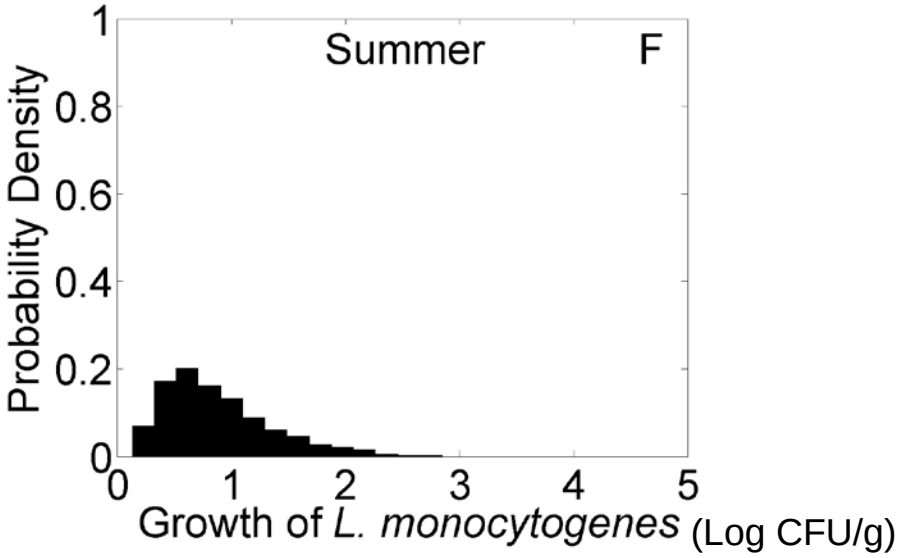
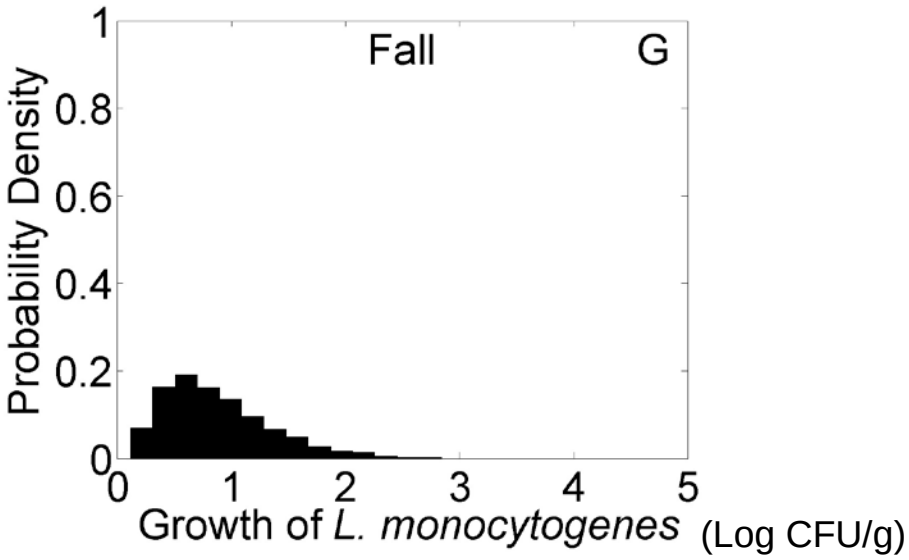
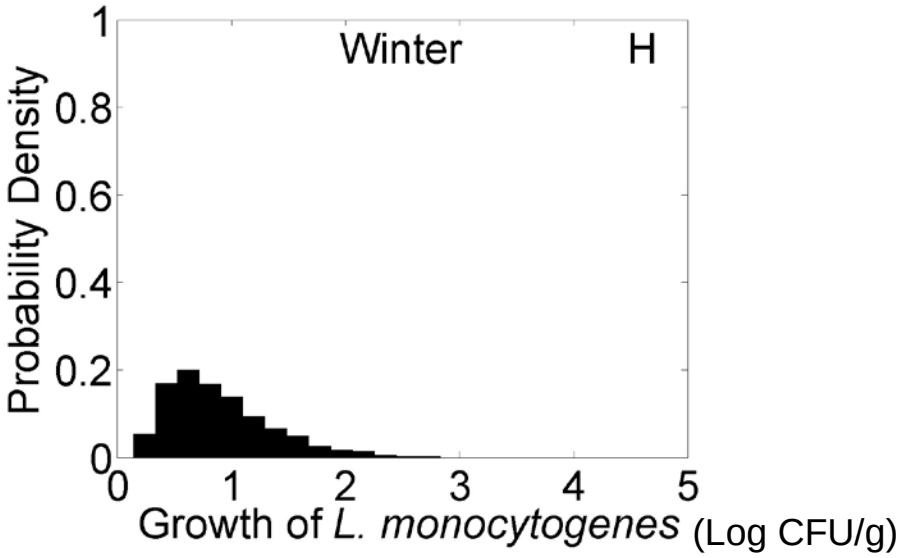


Figure 2.4 (cont'd)





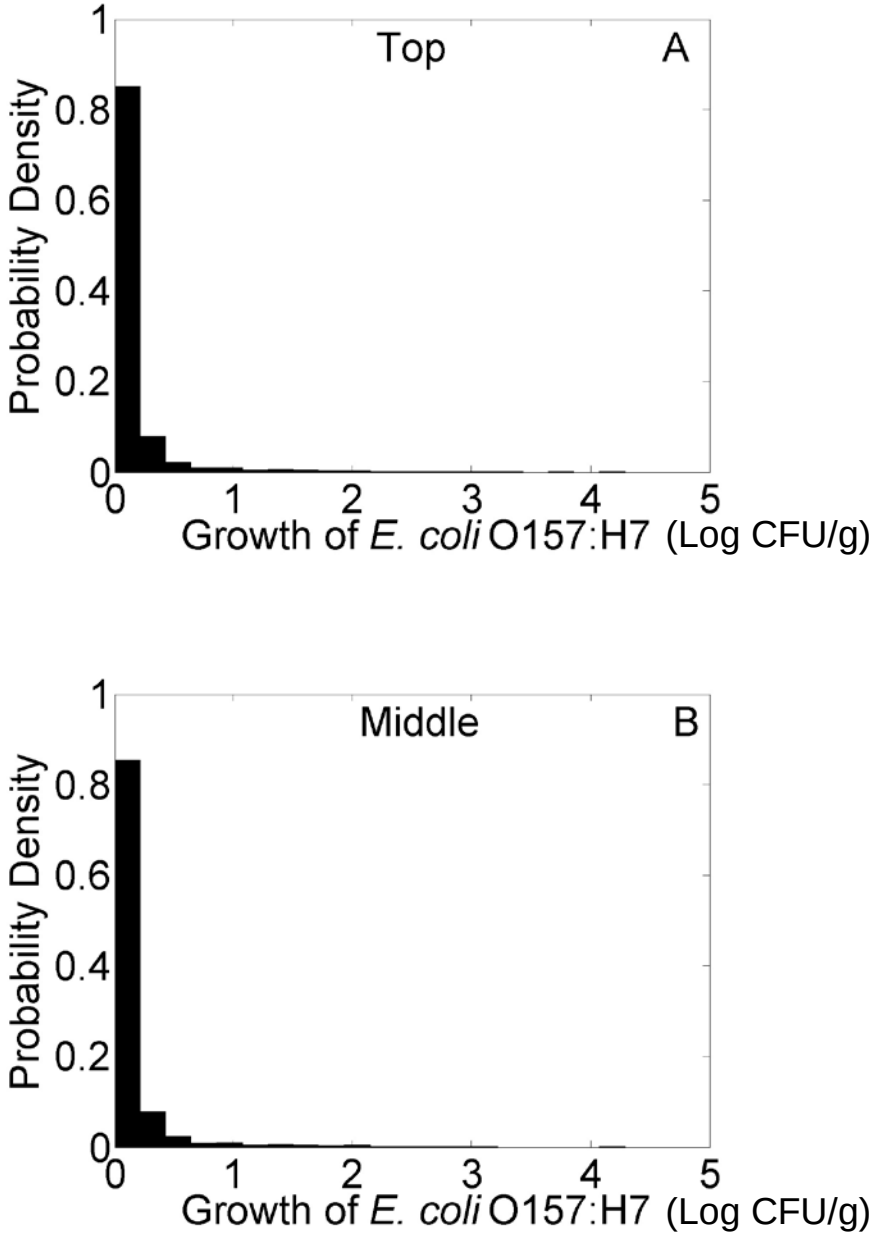
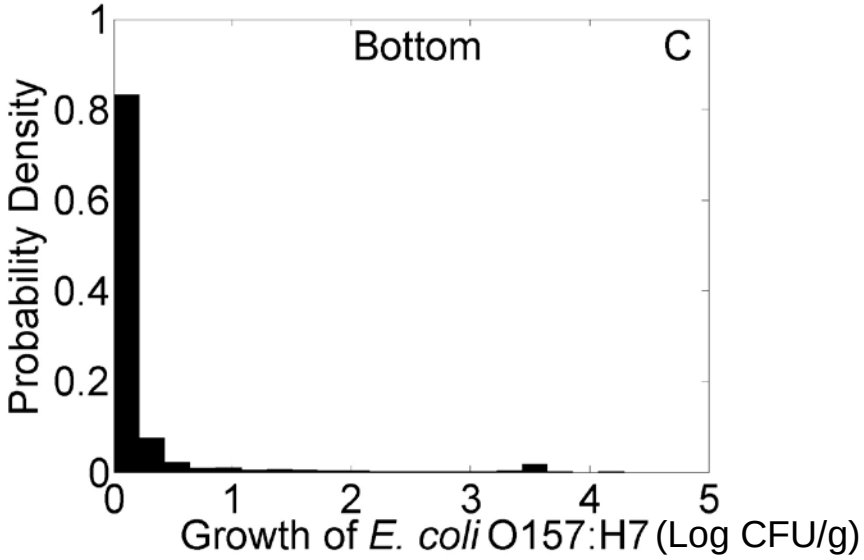


Figure 2.5 Probability distributions for *E. coli* O157:H7 (A, B, C) and *L. monocytogenes* (D, E, F) growth in romaine mix at the top, middle, and bottom of the truck during transport.

Figure 2.5 (cont'd)



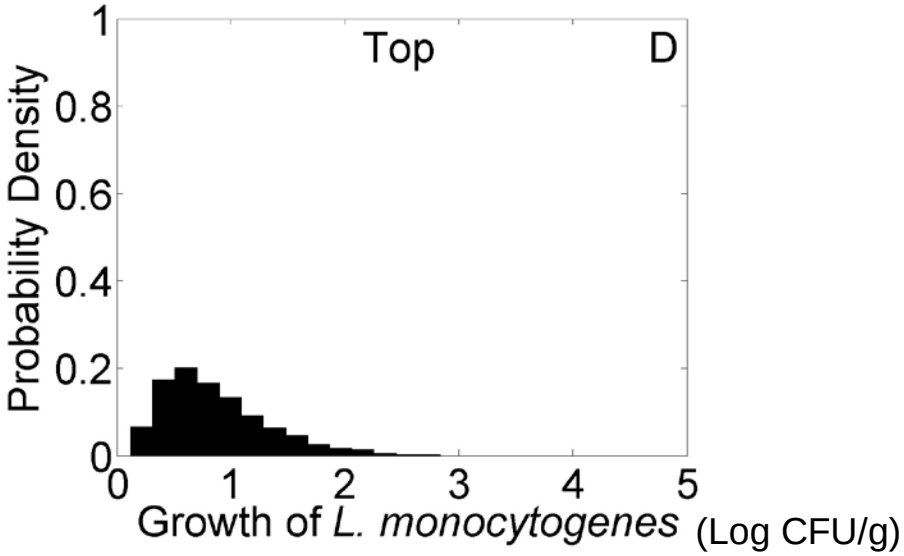
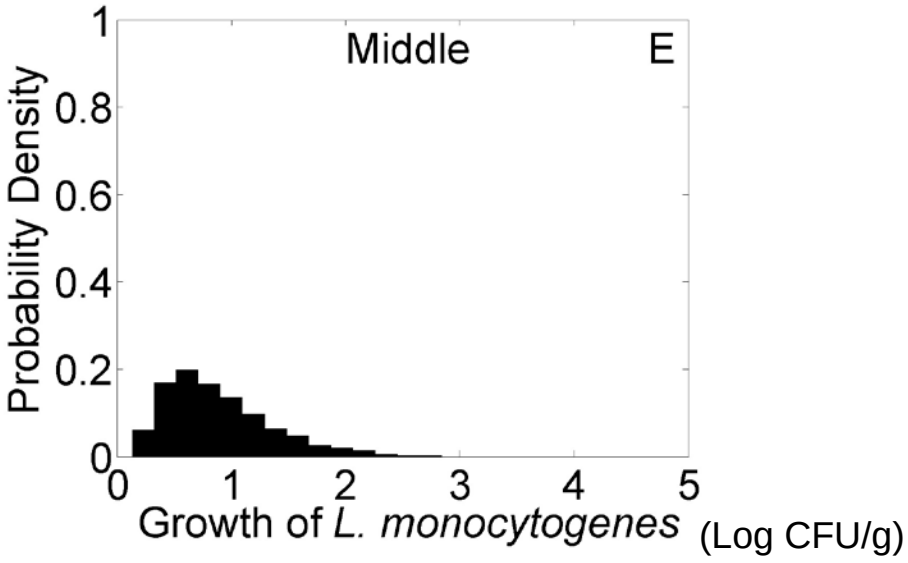
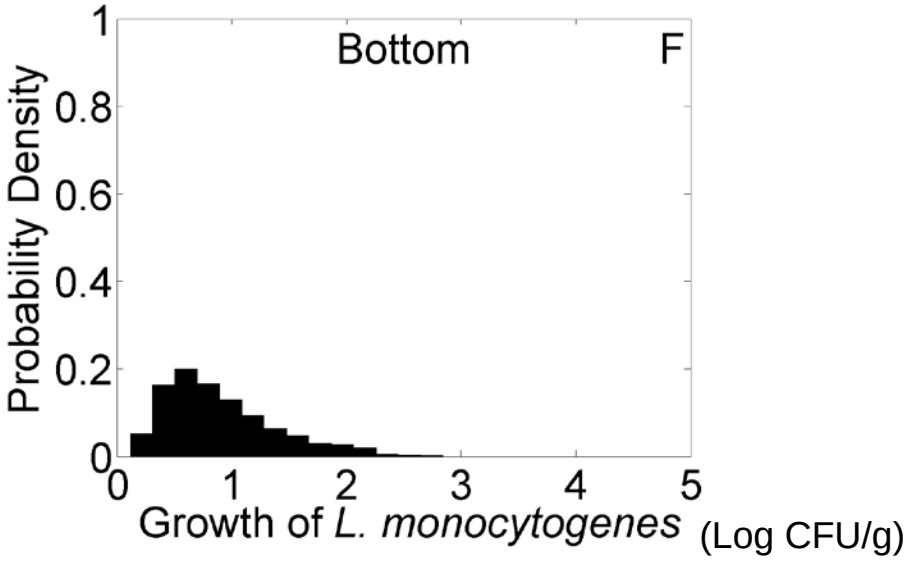


Figure 2.5 (cont'd)





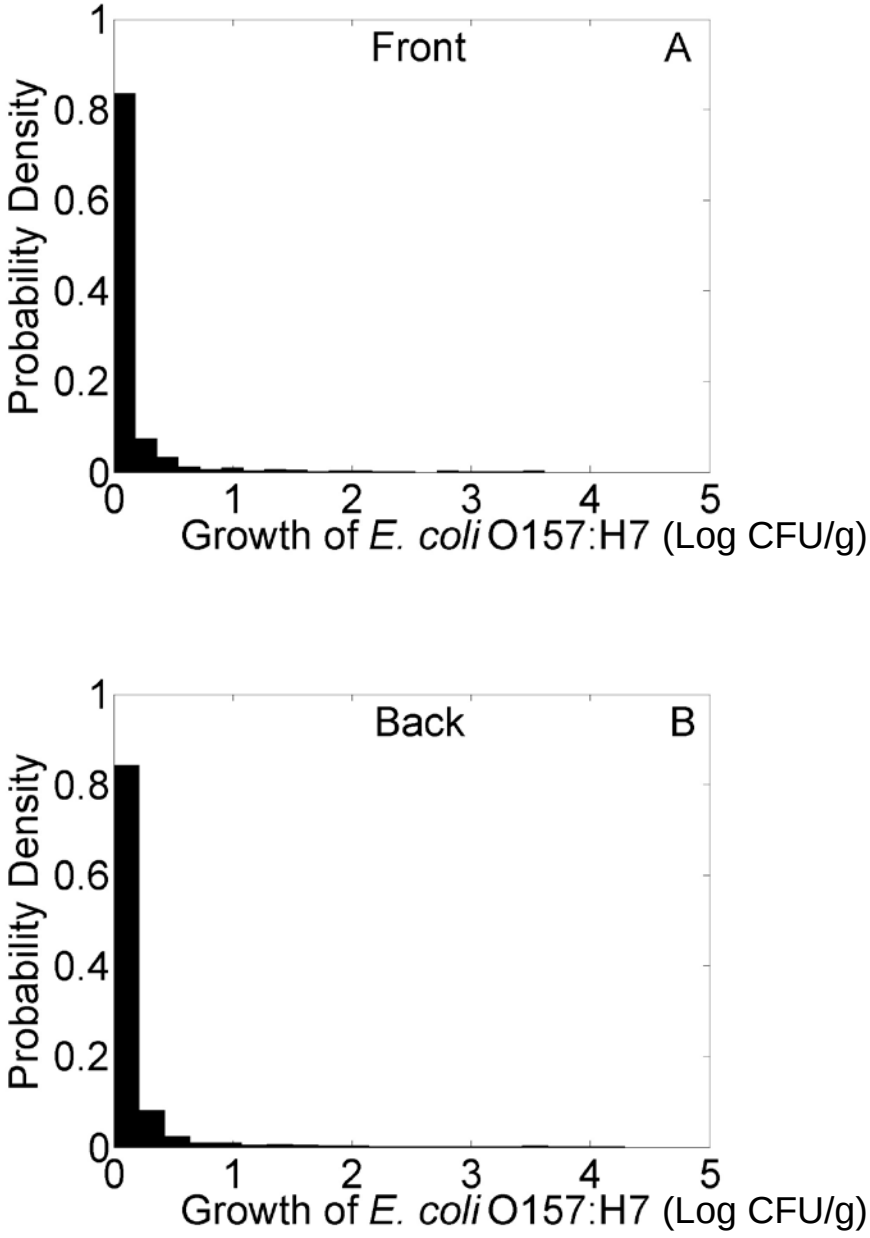
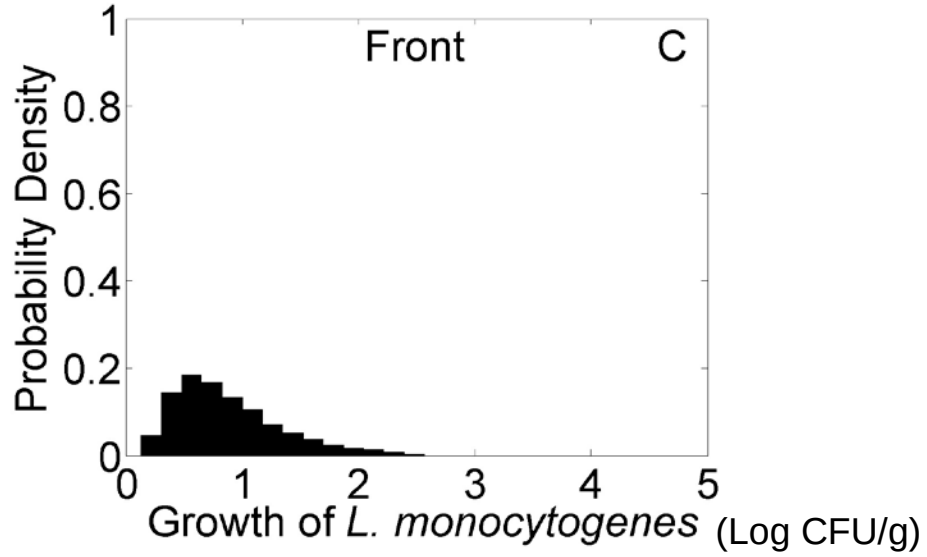
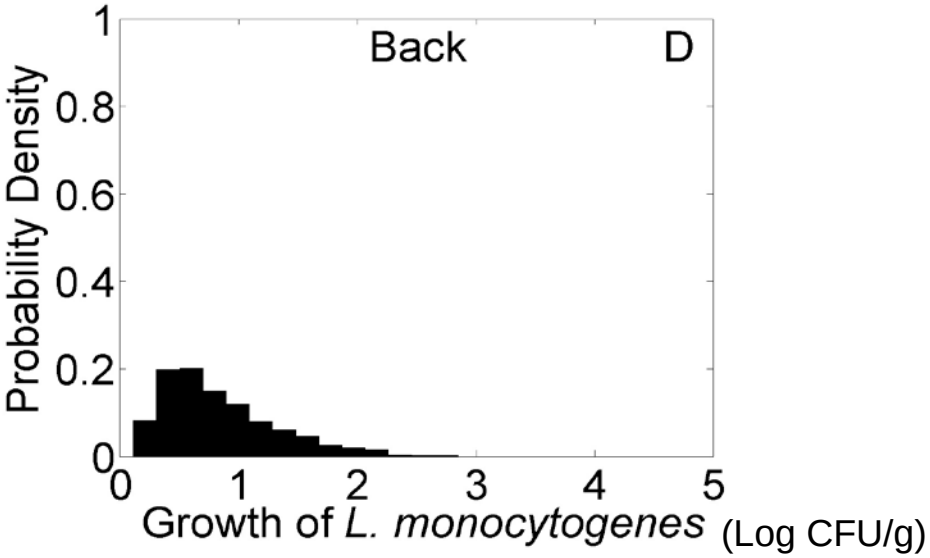


Figure 2.6 Probability distributions for *E. coli* O157:H7 (A, B) and *L. monocytogenes* (C, D) growth in romaine mix at the front and back of the retail display.

Figure 2.6 (cont'd)





2.5 Discussion

Temperature abuse has the potential to increase the chance for *E. coli* O157:H7 and *L. monocytogenes* growth in bagged salad greens during transport and subsequent handling. To our knowledge, this is the first large-scale study in the United States using commercial time/temperature profiles to assess the microbial risk of leafy greens during transport, retail storage, and display. The impact of season on temperature abuse during transport and product location in the trucks and retail display cases was also considered. In general, the commercial cold chain distribution system in the U. S. is well-controlled with most temperatures under 6°C - the minimum growth temperature for *E. coli* O157:H7 (Delaquis and others 2007). Properly maintained time/temperature profiles also have been reported during cold chain transport in Canada (McKellar and others 2012) and Belgium (Rediers and others 2009). However, temperature abuse during transport did exist. Working in Florida, Nunes and others (2009) measured the mean surface temperatures for bagged salads of 6.8, 7.7 and 8.1°C upon delivery to three retail stores. These temperatures were higher than our truck temperatures and those from other studies, probably due to the warm ambient environment. Moreover, a small proportion of non-commercial trucks have also been reported to carry unrefrigerated perishable foods when headed to grocery stores and restaurants (Segall 2011).

Our findings and those of others (McKellar and others 2012; Rediers and others 2009) demonstrate that temperature abuse of commercially produced fresh-cut salad greens is most likely to occur during retail storage, such as when the refrigeration system is temporarily shut down during cleaning and sanitizing of the cold room. Storage duration typically varies between 1 and 3 days, depending on the size of the supermarket, supply chain distribution pattern, and

consumer sales activity (Danyluk and Schaffner 2011). When combined with varying retail storage times, these short-term periods of temperature abuse can lead to significant microbial growth.

Storing bags of romaine mix for 72 h significantly increased the growth probabilities for both *E. coli* O157:H7 and *L. monocytogenes*. Our storage temperatures were similar to the retail display temperatures recorded by Nunes and others (2009), with temperatures fluctuating from -1.2 to 19.2°C. Although temperature variations due to seasonal differences and sensor location did not generally impact microbial populations, increased growth of *E. coli* O157:H7 was observed for summer temperatures when products were located near the floor of the truck during transport. In our study, the 432, 4867, and 3799 time/temperature profiles collected during transport, retail storage and display, respectively, were handled separately due to logistics. While impractical, continuous monitoring from transport through home storage would be most beneficial for risk assessments.

In our study, *E. coli* O157:H7 and *L.* monocytogenes populations increased a maximum of 3 logs, which is consistent with the results from Koseki and Isobe (2005a) who used similar temperatures. However, more growth of *E. coli* O157:H7 was observed throughout cold chain distribution compared to McKellar and others (2012) who used colder temperatures during winter monitoring in Canada. In most cases however, *E. coli* O157:H7 populations remained relatively stable during 3 days of simulated transport at well-controlled refrigeration temperatures. Several researchers also reported slight decreases in *E. coli* O157:H7 numbers during 4 to 12 days of incubation at temperatures below those needed for sustained growth (McKellar and others 2012; Jones and others 2005). Visvalingam and others (2012) showed that

E. coli cells in culture at 6°C became elongated but did not divide with cells losing viability at similar rates.

The effect of sudden temperature changes on the microbial lag phase was previously assessed by Swinnen and others (2005). Lag phase changes for *E. coli* are typically not seen when the temperature fluctuates from 20 to 37°C. However, temperature changes outside this range will lead to a period of adaptation. Therefore, at the low temperatures encountered in the cold chain, the temperature spikes caused by normal refrigeration cycles should not impact *E. coli* O157:H7 growth.

Using the time/temperature profiles for storage at 9 to 16°C (B1, B2, and B4), *L. monocytogenes*, which is psychrotrophic, generally grew better than *E. coli* O157:H7. However, *E. coli* O157:H7 and *L. monocytogenes* exhibited similar growth rates ($P > 0.05$) at temperatures below 9°C or above 16°C with growth of the background microflora similar to the two pathogens. Greater growth of the mesophiles and psychrotrophs was seen during retail storage with these average temperatures higher than those observed during transport and display. Koseki and Isobe (2005a) also indicated that the background bacterial flora did not affect the growth rate of *E. coli* O157:H7 or *L. monocytogenes*.

In this study, laboratory-generated growth data were also used to validate two currently available predictive models by Baranyi and McKellar for growth of *E. coli* O157:H7 or *L. monocytogenes* in romaine mix at fluctuating temperatures. While both models were able to predict microbial growth, Baranyi's model yielded slightly smaller RMSEs and biases. Therefore Baranyi's model was used for Monte Carlo simulation. When combined with various secondary models, Baranyi's model can also reliably predict *E. coli* O157:H7 and *L. monocytogenes* growth in bagged salads as a function of temperature and relative humidity or carbon dioxide

concentration (Carrasco and others 2008; Ding and others 2012). However, Danyluk and Schaffner (2011) and McKellar and Delaquis (2012) both showed that Baranyi's model overestimated the rate of *E. coli* O157:H7 growth. But most of these growth studies have been conducted at constant temperatures (Chang and Fang 2007; Delaquis and others 2002; Lee and Baek 2008; McEvoy and Luo 2009; Theofel and Harris 2009). McKellar and others (2011) recently developed a growth-death model based on previously published data with lab validation of their prediction errors needed for future comparison. In one study modified atmosphere packaging (MAP) was shown to inhibit the growth of *L. monocytogenes* in fresh-cut lettuce (Carrasco and others 2008). These lower experimental counts could be due to MAP since Koseki and Isobe (2005a) did not consider MAP for parameter determination of Baranyi's model. More studies and model parameter optimizations are needed to better predict the behavior of pathogens under MAP conditions at fluctuating temperatures.

Retail storage duration had the greatest impact on *E. coli* O157:H7 and *L. monocytogenes* growth. Our probability distributions demonstrate that the simulated populations of *E. coli* O157:H7 and *L. monocytogenes* increased < 2 log CFU/g in most cases. However, slight growth does not necessarily equate to a safe product. Low levels of contamination in a large number of servings are presumed to be responsible for most cases of foodborne illnesses (Danyluk and Schaffner 2011). In addition, profiles of long term temperature abuse and high average temperature over time rather than short time temperature abuse such as the defrost cycle of the refrigerated unit lead to greater probability distributions of *E. coli* O157:H7 and *L. monocytogenes* growth.

Tracing distribution temperatures during commercial transport and retail sale of leafy greens and mathematically predicting the potential for growth and/or survival of *E. coli* O157:H7

and *L. monocytogenes* have both been identified as major data gaps in current risk assessments (Carrasco and others 2010; Danyluk and Schaffner 2011). In a recent quantitative microbial risk assessment of leafy greens from farm-to-table, the effect of temperature abuse during transport was not considered (Danyluk and Schaffner 2011). The findings from this study should help fill a vital data gap in the current *E. coli* O157:H7 and *L. monocytogenes* risk assessments being developed to better define the risks associated with consumption of ready-to-eat bagged salad products in the U. S.

APPENDICES

Appendix A

Matlab code for *Escherichia coli* O157:H7

a) Baranyi dynamic model

```
function out = Baranyi_dyn(Tempvec, secmod, primod)
 % time - time vector in MINUTES
% temp - temperature vector in degrees Celsius
% Secondary model: mu = a(T-Tmin)^2. (1-exp(b(T-Tmax)))
% a, b, Tmin, and Tmax are parameters of the secondary model
% Secondary model: mu = a(T-Tmin)^2
% a, Tmin, are parameters of the secondary model
% a = b^2 from Koseki
 % xo - initial innoculum value in log(CFU/g)
% xmax - maximum microbial population in log (CFU/g): equation function of
% T was adopted from Koseki
% h - paremeter in the Baranyi model
close all
clc;
TIME_min = Tempvec(:, 1);
Temp_C = Tempvec(:, 2);
Tmin_C = secmod(1);
a = secmod(2);
x0 = primod(1);
xmax_const1 = primod(2);
```

```
xmax_const2 = primod(3);
q0 = primod(4);
% clear the screen
clc;
% define time step
dt_min = 5;
dth = dt_min/60;
% Create time vector - user input (will be in hours now)
% it is assumed in uniform time steps.
t = TIME_min/60;
nt = length(t);
tt = [t(1):dth:t(nt)];
csT = spline(t,Temp_C);
TT = ppval(csT,tt);
n = length(TT);
tfinal = tt(n);
% SOLVE DYNAMIC BARANYI'S MODEL USING RUNGE-KUTTA 4th ORDER
% Spline interpolation of temperature to obtain midpoints of each time interval
% Oversampling the time and temperature vectors by a factor of two for
% calculation purposes
csT = spline(tt,TT);
t1 = [t(1):dth/2:tfinal];     % new time vector containing midpoint values
T1 = ppval(csT,t1);           % new temperature vector containing midpoint values
nn = length(t1);
% Discretized Secondary Model
```

```
% secondary model predicts growth rate as a function of temperature
% determine the growth rate for the oversampled temperature vector.
% initialize growth rate vector
mumax = [];
xmax = [];
for i = 1:length(T1)
     xmax(i)=xmax_const1*T1(i)+xmax_const2; % dynamic xmax
    if T1(i) >= Tmin_C
        mumax(i) = a*(T1(i) - Tmin_C)^2;
    elseif T1(i) < Tmin_C
       mumax(i) = 0;
    end
 end
% Numerical Solution of dQ/dt using RK4
% Define Initial Condition
% the "work to be done", constant for a given microorganism at a given
% substrate
%      %h0 = 1.75;
%      alpha = exp(-h0);           % initial physiological state of cells
%      q0 = 1/(exp(h0)-1);      % initial bottleneck substance
 q = [];
 q(1) = q0;            % initial condition for dq/dt
 % Runge-Kutta algorithm
 i = 1;
    for j = 2:n
```

```
        % PREDICTION
         k1 = dth*(mumax(i))*q(j-1);
         k2 = dth*(mumax(i+1))*q(j-1);
         k3 = dth*(mumax(i+1))*q(j-1);
         k4 = dth*(mumax(i+2))*q(j-1);
         i = i+2;
        % CORRECTION
         q(j) =   q(j-1) + ((1/6)*(k1 + (2*k2) + (2*k3) + k4));
end
% Numerical Solution of dy/dt using RK4
 % Define Initial Condition
 x = [];
 x(1) = x0;     % initial condition for dy/dt - user input
 % Define Maximum Cell Population - constant for a given microorganism at a given
 % substrate
 % xmax = xmax;
% Spline interpolation of q to obtain midpoints of each time interval
 % Oversample or increase the length of q vector by a factor of two
csq = spline(tt,q);
t1 = [t(1):dth/2:tfinal];      % new time vector containing midpoint values
qq = ppval(csq,t1);        % new q vector containing midpoint values
% Runge-Kutta algorithm
i = 1;
    for j = 2:n
        % PREDICTION
```

```
        k1 = dth*mumax(i)*(qq(i)/(1+qq(i)))*(1-(x(j-1)/(xmax(i))))*x(j-1);

        k2 = dth*mumax(i+1)*(qq(i+1)/(1+qq(i+1)))*(1-((x(j-
1)+(k1/2))/(xmax(i+1))))*(x(j-1)+(k1/2));

        k3 = dth*mumax(i+1)*(qq(i+1)/(1+qq(i+1)))*(1-((x(j-
1)+(k2/2))/(xmax(i+1))))*(x(j-1)+(k2/2));

        k4 = dth*mumax(i+2)*(qq(i+2)/(1+qq(i+2)))*(1-((x(j-
1)+(k3))/(xmax(i+2))))*(x(j-1)+(k3));

        i = i+2;

    % CORRECTION

        x(j) = x(j-1) + ((1/6)*(k1 + (2*k2) + (2*k3) + k4));

end

xpred = x;

out = [tt' xpred'];

% figure, plot (t', y, 'b');

% figure, plot(t', T);

figure, plotyy(tt,xpred,tt,TT);

[AX,H1,H2] = plotyy(tt,xpred,tt,TT,'plot');

 grid on

 xlabel('Time (h)')

set(get(AX(1),'Ylabel'),'String','log(CFU)/mL')

set(get(AX(2),'Ylabel'),'String',' Temp (C)')

title ('Time (h), Temp (C), log (CFU)/mL')
```

b) Bacterial growth predicted from Baranyi/Ratkowsky model

```
% read Excel file and corresponding tab.

% CHANGE DEPEDING ON WHAT FILE YOU WANT TO READ!
```

```
data = xlsread('sept 15.xls','Sheet1');
 t_hr = data(:,1);
%transform t to minutes for model solving
t = t_hr*60;
T = data(:,2);
% define t/T vector
Tempvec = [t,T];
% primary model parameters
primod = [6.91, 0.218, 10.320, 0.059322];%2.63 8.35, 1.7788 % E. coli
% primod = [7.53, 0.037, 12.434, 0.077586];%2.63 8.35, 1.7788 % LM
% x0, xmax_const1, xmax_const2, q0
% secondary model parameters
secmod = [4.54,0.001089]; %2.8988, 0.00164 % E. coli
% secmod = [-4.26,0.000256]; %2.8988, 0.00164 % LM
% Tmin_C, a
% solve/run program!
out = baranyi_dyn(Tempvec, secmod, primod);
maxgrowthlog = out(length(out),2);
```

c) Monte Carlo simulation for *E. coli* O157:H7

```
clear;
% how many random points to collect
randomSelectLength=100000;
% use random seed by time: for better random
% rng('shuffle');
```

```
format long g

[dates,routes,departs,arrvs,tags,locTrucks,locPallets,seasons,incrEC, incrLM,tmp] =
textread('input\phase1.csv','%s %n %s %s %s %s%s%s%f %f%s','delimiter',',');

% first 1D array to save the 1st phase end value; to serve as the init value

% of the 2nd phase;

phase1Res=incrEC;

% phase1Res=incrLM;

phase1ResLen=length(phase1Res);

% secondary model parameters: const

secmod = [4.54,0.001089]; %2.8988, 0.00164 % E. coli

% secmod = [-4.26,0.000256]; %2.8988, 0.00164 % LM

% Tmin_C, a

% Generate file lists for type B & compute file numbers

phase2_list = dir('phase2_data_output_all_3days/*.csv');

phase2ResLen = length(phase2_list);

% Generate file lists for type C & compute file numbers

phase3_list = dir('phase3_data_output_all_3days_front/*.csv');

phase3ResLen = length(phase3_list);

% Flattened: for drawing chart:  Pre-allocation result space:  rows and 1 column

allCaseLength = phase1ResLen * phase2ResLen * phase3ResLen;

% save all random selected result: for the final value.

randomSelectAllPhaseCases = nan(randomSelectLength,1);

randomCaseIndex=1;

for rand_iter = 1 : randomSelectLength

randomPhase1Index= randi(phase1ResLen);
```

```
% Load phase 1 result: note that this value needs to be changed to base n

% (log) from base 10

phase2init= ( phase1Res(randomPhase1Index) +3.0)* log(10);

% phase2init =log( 10.^( phase1Res(iter)+3.0 ));

% Load phase 2

randomPhase2Index=randi(phase2ResLen);

phase2CSVFileName = ['phase2_data_output_all_3days/'
phase2_list(randomPhase2Index).name];

[phase2Time,phase2Temp,dateTimePhase2] =
textread(phase2CSVFileName,'%n%f%s','delimiter',',');

phase2Vec = [phase2Time,phase2Temp];

% now to put the variables into the model to compute the increased

% b number in log

% primary model parameters: need to use the 1st phase result to

% serve as the initial value of the 2nd phase

phase2Primod = [phase2init, 0.218, 10.320, 0.059322];%2.63 8.35, 1.7788 % E. coli

%phase2Primod = [phase2init, 0.037, 12.434, 0.077586];%2.63 8.35, 1.7788 % LM

phase2IncrResultMatrix = Baranyi_dyn(phase2Vec, secmod, phase2Primod);

phase2IncrResult = phase2IncrResultMatrix(end,2);

% just use this log base n to be the init value of the next phase:

% phase 3

phase3init = phase2IncrResult;

% enter phase 3

randomPhase3Index=randi(phase3ResLen);

% Load phase 2 result: note that this value must be changed to base n

% (log) from base 10
```

```
phase3CSVFileName = ['phase3_data_output_all_3days_front/'
phase3_list(randomPhase3Index).name];

[phase3Time,phase3Temp,dateTimePhase3] =
textread(phase3CSVFileName,'%n%f%s','delimiter',',');

phase3Vec = [phase3Time,phase3Temp];

% now to put the variables into the model to compute the increased

% b number in log

% primary model parameters: need to use the 2nd phase result to

% serve as the initial value of the 3rd phase

phase3Primod = [phase3init, 0.218, 10.320, 0.059322];%2.63 8.35, 1.7788 % E. coli

%phase3Primod = [phase3init, 0.037, 12.434, 0.077586];%2.63 8.35, 1.7788 % LM

phase3IncrResultMatrix = baranyi_dyn(phase3Vec, secmod, phase3Primod);

phase3IncrResult = phase3IncrResultMatrix(end,2);

% change to base 10 for final result

phase3IncrResult=phase3IncrResult / log(10) - 3.0;

randomSelectAllPhaseCases(rand_iter) = phase3IncrResult;

if( mod(rand_iter,10)==0)

disp( ['Reaching case No. ', num2str(rand_iter), '.'])

   end

end

% Distribution plot for "result"

clf, hist(randomSelectAllPhaseCases,20);

[n,myHist]=hist(randomSelectAllPhaseCases,20);

   bar(myHist,n/randomSelectLength,1);

   myFont=30;

   xlabel('Growth of \it{E. coli} \rm{O157:H7}','fontsize', myFont);
```

```
    ylabel('Probability Density','fontsize', myFont);
    ylim([0 1.0]);  xlim([0 5.0]);
%x = normplot(randomSelectAllPhaseCases);
%y = histfit(randomSelectAllPhaseCases);
%data = (randomSelectAllPhaseCases);
%[mu,sigma,muci,sigmaci] = normfit(data);
```

Appendix B

Matlab code for *Listeria monocytogenes*

Most codes for *L. monocytogenes* are the same as the codes for *E. coli* O157:H7 except parameters xmax_const1=0.037, xmax_const2=12.434, and q0=0.077586.

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