ABSTRACT

Title of Document: DEVELOPMENT AND APPLICATION OF PREDICTIVE MODELS FOR SURVIVAL, GROWTH, AND DEATH OF ENTERIC PATHOGENS IN LEAFY GREENS SUPPLY CHAIN

Abhinav Mishra, Doctor of Philosophy, 2016

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Nutrition and Food Science

Leafy greens are essential part of a healthy diet. Because of their health benefits, production and consumption of leafy greens has increased considerably in the U.S. in the last few decades. However, leafy greens are also associated with a large number of foodborne disease outbreaks in the last few years. The overall goal of this dissertation was to use the current knowledge of predictive models and available data to understand the growth, survival, and death of enteric pathogens in leafy greens at pre- and post-harvest levels.

Temperature plays a major role in the growth and death of bacteria in foods. A growth-death model was developed for *Salmonella* and *Listeria monocytogenes* in leafy greens for varying temperature conditions typically encountered during supply chain. The developed growth-death models were validated using experimental dynamic time-temperature profiles available in the literature. Furthermore, these

growth-death models for *Salmonella* and *Listeria monocytogenes* and a similar model for *E. coli* O157:H7 were used to predict the growth of these pathogens in leafy greens during transportation without temperature control.

Refrigeration of leafy greens meets the purposes of increasing their shelf-life and mitigating the bacterial growth, but at the same time, storage of foods at lower temperature increases the storage cost. Nonlinear programming was used to optimize the storage temperature of leafy greens during supply chain while minimizing the storage cost and maintaining the desired levels of sensory quality and microbial safety.

Most of the outbreaks associated with consumption of leafy greens contaminated with *E. coli* O157:H7 have occurred during July-November in the U.S. A dynamic system model consisting of subsystems and inputs (soil, irrigation, cattle, wildlife, and rainfall) simulating a farm in a major leafy greens producing area in California was developed. The model was simulated incorporating the events of planting, irrigation, harvesting, ground preparation for the new crop, contamination of soil and plants, and survival of *E. coli* O157:H7. The predictions of this system model are in agreement with the seasonality of outbreaks. This dissertation utilized the growth, survival, and death models of enteric pathogens in leafy greens during production and supply chain.

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By

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List of Abbreviations

ANN	Artificial neural networks
ANOVA	Analysis of variance
APZ	Acceptable prediction zone
BSE	Bovine spongiform encephalopathy
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CI	Confidence interval
COP	Coefficient of performance
cwt	Centrum weight (100 pounds)
FSIS	Food Safety and Inspection Services
FSO	Food safety objective
GUI	Graphical user interface
IPMP	Integrated Pathogen Modeling Program
LGMA	Leafy Green Marketing Agreement
MPD	Maximum population density
MPN	Most probable number

NLP	Nonlinear programming
PE	Prediction error
PI	Prediction interval
PMP	Pathogen Modeling Program
QMRA	Quantitative microbial risk assessment
RTE	Ready-to-eat
SSE	Sum of squares of errors
TCS	Time-temperature control for safety
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
UV	Ultraviolet
WHO	World Health Organization

Chapter 1. Introduction

1.1 Leafy greens: Health benefits

Leafy greens are also called greens, vegetable greens, or salad greens. The common types of leafy green vegetables are arugula, Butter-head lettuce, cabbage, chard, chicory, escarole, Iceberg lettuce, kale, green leaf lettuce, red leaf lettuce, radicchio, Romaine lettuce, and spinach. Leafy green vegetables are important component of a healthy diet, providing important vitamins, minerals, and phyto-nutrients (1). They are also vital source of antioxidants and dietary fibers that are very beneficial for weight loss. Many leafy green vegetables such as spinach are also high in carotenoids (2). Recent prospective epidemiologic studies have shown that green leafy vegetables are among the foods most protective against coronary heart disease and ischemic stroke (3). The U.S. Department of Agriculture recommends that adults should consume at least three cups of dark green vegetables each week (4).

1.2 Increasing production and consumption

Over the last years, production and consumption of leafy greens has increased dramatically in the U.S., which is in good agreement with the increasing trend in the number of farms and area harvested with lettuce in the U.S. over the past few decades (**Figure 1.1**). In 1984, total production of Iceberg, Romaine, and leaf lettuce in the U.S. was 72,103,000 cwt (1 cwt = 100 pounds), which increased to 90,488,000 cwt in 2009. California has been the largest producer of leafy greens, producing about

72,173,000 cwt Iceberg, Romaine, and leaf lettuce in 2009 (5). **Figure 1.2** represents the amount of Romaine and leaf lettuce produced in the U.S. (1984-2009).

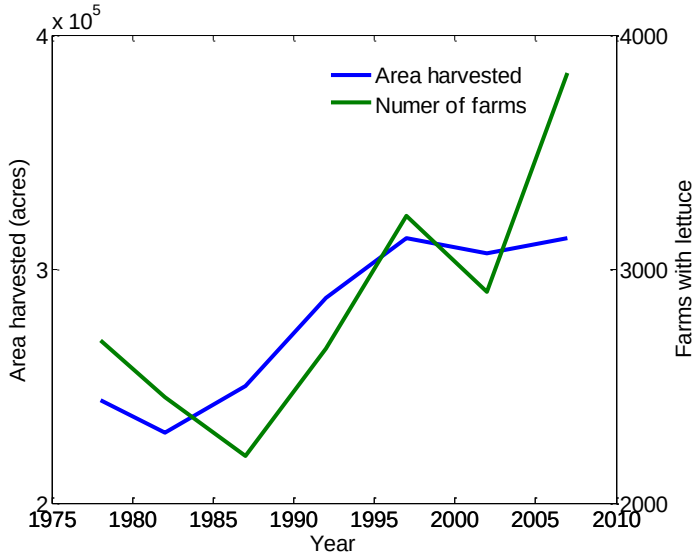

Figure 1.1. U.S. census with number of farms with lettuce and area harvested.

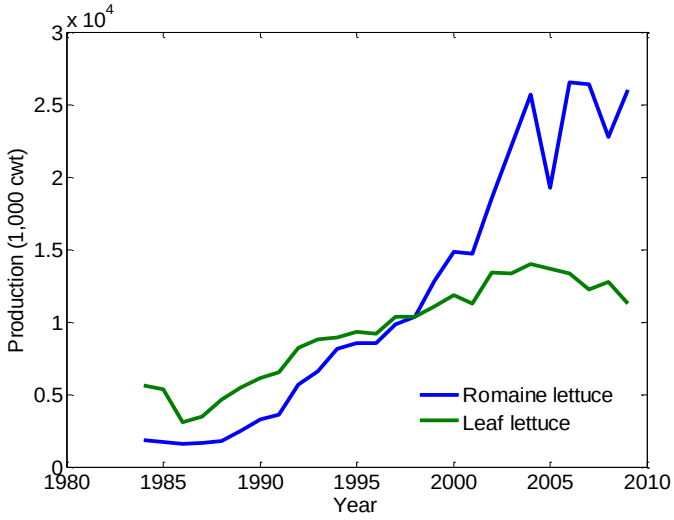

Figure 1.2 Romaine and leaf lettuce production (1984-2009); 1 cwt (centrum weight) = 100 pounds.

1.3 Risks associated with leafy greens

Recent foodborne disease outbreaks in the U.S. have suggested associations between several pathogens and leafy green vegetables such as lettuce and spinach. Leafy green vegetables carry the potential microbiological contamination from following sources:

- Application of inappropriate organic fertilizers or inadequately composted manure
- Application of untreated irrigation water or sewage
- Contact with humans
- Contact with domestic animals, wildlife or insects
- Contact with contaminated field and harvesting equipment
- Contact with bio aerosols drifting from adjacent contaminated land, and
- Other sources that can occur anywhere from farm to fork such as inappropriate handling and storage during harvesting, transport, storage, processing, packaging, marketing, restaurant services and at home.

It is estimated that foodborne illnesses costs the United States $152 billion per year, out of which produce related foodborne illnesses cause $39 billion per year (6). **Table 1.1** shows the number of outbreaks, illnesses and hospitalizations that occurred in the U.S. during 1973-2012. Out of 12,714 foodborne disease outbreaks with at least one food item implicated during 1973–2012, 606 (5%) had a leafy vegetable implicated (162 outbreaks with a simple leafy vegetable as the vehicle and 444

outbreaks with a leafy vegetable-based salad as the vehicle), resulting in 20,003 illnesses, 1,030 hospitalizations, and 19 deaths. Of the 272 confirmed single etiology outbreaks reported, norovirus was the most common (149 outbreaks), followed by *E. coli* (48 outbreaks), and *Salmonella* (29 outbreaks) (7). In addition to these common pathogens, *Listeria monocytogenes* is of particular concern because of its wide distribution in the environment and its ability to grow in refrigeration conditions. While cases of listeriosis involving leafy greens are few, eight recalls have been issued since 2010 for *L. monocytogenes* contaminated leafy greens, thus legitimizing concern for this pathogen in lettuce (8).

Table 1.1 Leafy vegetable associated outbreaks, illnesses, and hospitalizations in the United States during 1973-2012

	Etiology	**Confirmed etiology**	**Suspected etiology**	**Total (%)**
Outbreaks	Single etiology	272	124	396 (66)
	Multiple etiology	--	--	3 (0)
	Unknown	--	--	207 (34)
	Total	272	124	606 (100)
Illnesses	Single etiology	11644	2402	14046 (70)
	Multiple etiology	--	--	60 (0)
	Unknown	--	--	5897 (30)
	Total	11644	2402	20003 (100)
Hospitalizations	Single etiology	947	31	978 (95)
	Multiple etiology	--	--	0 (0)
	Unknown	--	--	52 (5)
	Total	947	31	1030 (100)

1.4 Project overview

Leafy greens can be contaminated with pathogens at any step during their production and supply chain. Fresh leafy greens also have a short shelf life and are exposed to conditions that can destroy their superior quality especially during transport and at retail. Temperature conditions primarily determine the growth rate of any contaminating bacteria and the rate of sensory quality degradation which can determine acceptability by consumers (9).

The overall goal of this study was to develop and use predictive models for the behavior (growth, survival, and death) of enteric bacteria (*E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*) and for deterioration of sensory quality attributes in leafy greens during production and supply chain. Specific objectives were:

(1) **To develop growth and death models for *Salmonella* and *Listeria monocytogenes* during non-isothermal time-temperature profiles in leafy greens.** Despite being two major pathogens of concern, no growth-death model is available that can predict growth and death of *Salmonella* and *Listeria monocytogenes* in leafy greens during non-isothermal time-temperature profiles.

(2) **To predict the growth of *Escherichia coli* O157:H7, *Salmonella,* and *Listeria monocytogenes* in leafy greens without temperature control.** Temperature of leafy greens begins to increase when taken out of

refrigeration. When non-refrigerated storage is of sufficient duration, there is risk of growth of pathogens present in leafy greens.

(3) **To optimize the temperature during the supply chain of leafy greens using nonlinear programming.** Refrigeration of leafy greens meets the purposes of increasing their shelf-life and mitigating the bacterial growth, but at the same time, storage of foods at lower temperature increases the storage cost. There is a need for optimization of the temperature of leafy greens considering the aspects of food safety, product quality, and economy.

(4) **To develop a system model to understand the role of animal feces as a route of contamination of leafy greens before harvest.** Most of the reported *E. coli* O157:H7 outbreaks associated with leafy greens produced in the Salinas and adjacent valleys in California (a major producing region of leafy greens in the U.S.) occurred during July-November. Currently, limited research has been conducted on development of a dynamic system model to understand the behavior of pathogens in a biological system, such as the production field.

These four objectives collectively depict the behavior of pathogens in leafy greens at pre- and post-harvest levels. The growth-death models developed in objective 1 were used to predict the growth of pathogens in objectives 2 and 3. Objective 4 is important in providing information for a better understanding of pre-

harvest factors potentially responsible for foodborne outbreaks associated with the consumption of contaminated leafy greens.

Chapter 2. Review of literature

2.1 Introduction

Fresh produce is an important part of a healthy and nutritious diet (10). Over the last few decades, production and consumption of fresh-cut or minimally-processed vegetables has increased dramatically in the U.S. (11). The U.S. fresh fruit and vegetable-produce industry is very diverse, including over 300 products, each with a specific system of production and handling. The final value of fresh produce sold in the U.S. through all marketing channels was estimated to be over $122.1 billion in 2010 (12). While the value of produce sold through foodservice channels such as restaurants and salad bars has been growing to 42% of total sales, the retail channel still predominates with approximately 57% of sales; the expanding direct-to-consumer channel is estimated to account for less than 2% of total sales (12). There were 3.2 million acres of fresh fruits and vegetables harvested in 2010, producing 99.9 billion pounds, with a farm gate value of $21.8 billion (12). Consumption of fresh fruits and vegetables was 313 pounds per capita in 2010, increased by 27% since 1976, due to growing awareness of the health benefits of fresh produce and greater year-round availability from rising imports (12). Leafy vegetables in particular have become very popular in the last few decades in the U.S. due to their high fiber and micronutrients content. Common examples of leafy greens are arugula,

cabbage, chard, endive, escarole, kale, lettuce (iceberg, red, baby leaf, green leaf, and romaine lettuce), and spinach.

Because leafy greens are consumed raw without cooking steps involved, any contaminated leafy vegetables with pathogens have the potential to cause foodborne diseases. Surveys have revealed presence of human enteric pathogens (such as *E. coli*, *Salmonella*, and *L. monocytogenes*) (5, 13-15) and experimental works have reported the ability of these pathogens to colonize crops and to grow in contaminated pre-harvest environment (16). Thus, while farm animal products are traditionally considered to be at high risk of contamination, leafy greens are now seen as an emerging pathogen carriers (17). This is not surprising as leafy greens are grown in open fields where accidental contamination may occur. **Figure 2.1** shows the possible sources of contamination in leafy greens during different steps in their production and supply chain. During recent years a growing number of foodborne illnesses have been associated with the consumption of leafy greens. Leafy greens associated outbreaks in the U.S. accounted for an increasing proportion of all reported foodborne outbreaks rising from 0.7% in the 1970s to 6% in the 1990s (15). More recent data from the Centers for Disease Control and Prevention database revealed that leafy greens outbreaks accounted for 22.3% of foodborne outbreaks causing about 46% of the diseases in the US between 1998 and 2008 (18). Some of the recent outbreaks of leafy greens in the U.S. are shown in **Table 2.1**.

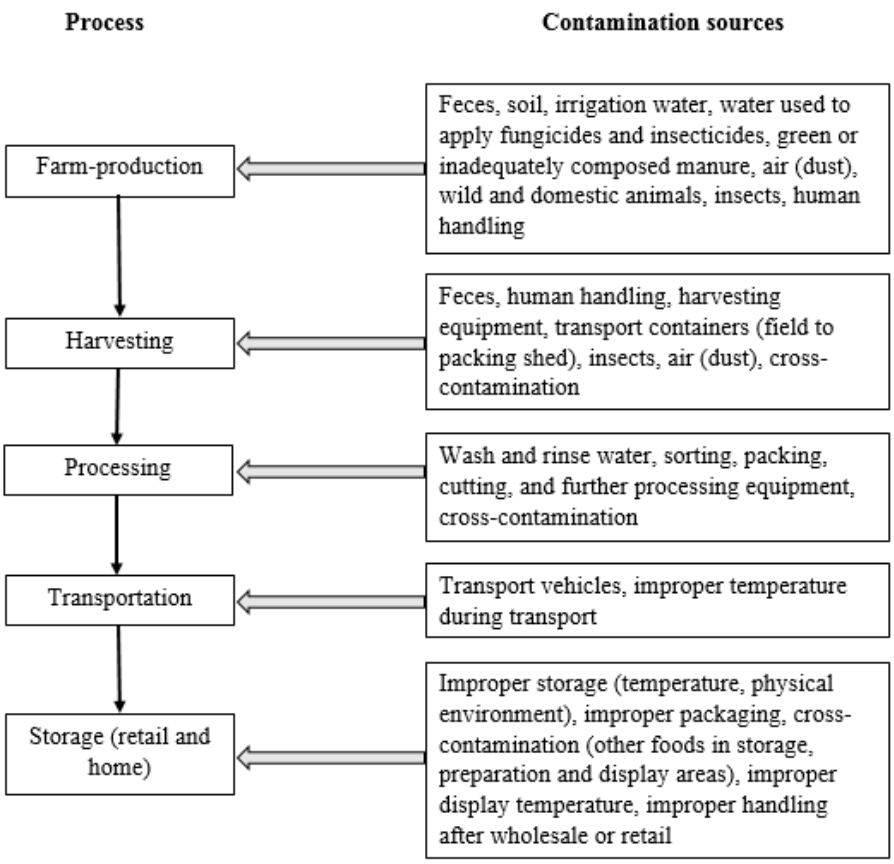

Figure 2.1 Flow-chart of farm to fork processes of leafy greens with possible contamination sources.

Mathematical models are pre-requisite to predict the risk of human illness caused by consumption of foodborne pathogens. Different types of mathematical models (for example, quantitative microbial risk assessments, data collection frameworks such as field surveys, controlled experiments, and epidemiological studies) have been used to define the behavior of pathogens under different conditions. Quantitative microbial risk assessment (QMRA) is an important approach for food safety in which risk and factors that influence food safety are identified. The

goal is to provide an estimate of the level of illness that a pathogen can cause in a given population (19). Thus, the goal of this review is evaluate the available data on contamination sources and pathogen ecology, predictive microbial models, and quantitative risk assessment models for different pathogens in leafy greens in the farm-to-table continuum.

2.2 Microbial contamination of leafy greens

Leafy greens have been implicated with several disease outbreaks in the world (7, 15, 20). Microbial contamination can occur in leafy greens at any stage from farm to fork, which can be broadly categorized into two groups: pre-harvest contamination, and post-harvest contamination.

2.2.1 Pre-harvest contamination

There are many potential pre-harvest contamination sources including contaminated manure, manure compost, irrigation water, runoff water from livestock operations, exposure to waste products from wild and domestic animals, and interactions between plants and plant foragers like birds, mammals, and insects (22-30).

Table 2.1 Recent vegetable associated outbreaks in the United States during 2010-2014 (30)

Year	State	Genus Species	Hospitalizations (deaths)	Food
2010	New York	Norovirus unknown	30 (2)	Leaf lettuce
2010	Florida	Norovirus Genogroup II	4	Cucumber, leafy green, onion, tomato
2010	Colorado	Norovirus unknown	26	Guacamole
2010	Minnesota	Norovirus Genogroup II	10	Vegetables (unspecified)
2010	Minnesota	Clostridium perfringens	19	Vegetables (unspecified)
2010	New York	Norovirus unknown	45 (2)	Green salad
2010	Minnesota	Norovirus Genogroup I	38	Salad (unspecified)
2010	Washington	Unknown	4	Vegetables (unspecified)
2010	New York	Norovirus unknown	13	Vegetable-based salads
2010	Ohio	Norovirus Genogroup I	8	Vegetable dip, unspecified
2010	California	Norovirus unknown	26 (2)	Sandwich, vegetable-based
2010	Wyoming	Norovirus Genogroup II	33 (1)	Multiple salads, mixed vegetables
2010	California	Norovirus unknown	18	Vegetable-based salads, pasta-based salads
2011	Pennsylvania	Norovirus unknown	57 (1)	Vegetable (unspecified)
2011	New York	Unknown	15	Vegetable platter
2011	New York	*Bacillus cereus*	3	Vegetable (unspecified)
2011	Washington	Norovirus unknown	6	Vegetables (unspecified)
2012	Illinois	Norovirus Genogroup II	196 (2)	Coleslaw, green beans, vegetables
2012	California	Unknown	3	Vegetable (unspecified)

2012	Ohio	Unknown	4	Vegetable (unspecified)
2012	Ohio	*Clostridium perfringens*	70	Soup, vegetable-based
2012	California	*Escherichia coli*	12 (1)	Vegetable-based salads
2012	Michigan	Unknown	6	Salad (unspecified)
2012	Kansas	Unknown	32	Vegetable platter
2012	Multistate	*Escherichia coli*	33 (13)	Prepackaged leafy greens
2012	Wisconsin	*Salmonella enterica*, *Campylobacter jejuni*	21 (5)	Beef, intestine soup with vegetables;
2013	Multistate	*Escherichia coli*	14 (10)	Prepackaged leafy greens
2013	California	Unknown	41	Mixed vegetables
2013	Connecticut	*Escherichia coli*	34	Salad, unspecified, vegetable tray
2013	Illinois	Unknown	3	Vegetable, lettuce based salads
2013	Pennsylvania	*Escherichia coli*	15 (10)	Prepackaged leafy greens, lettuce
2013	Wisconsin	*Salmonella enterica*	75 (5)	Vegetables (unspecified)
2014	Massachusetts	*Salmonella*	11 (2)	Leaf lettuce
2014	Ohio	Unknown	2	Vegetable (unspecified)
2014	Hawaii	*Salmonella enterica*	24 (1)	Vegetables (unspecified)

2.2.1.1 Contaminated manure

Vegetable plants can become contaminated with pathogens before harvest when grown in fields fertilized with fresh or inadequately composted manure (31, 32). Both conventional and organic vegetable producers commonly apply animal manure as fertilizer to fields where crops are grown (33). Healthy cattle sporadically harbor *E. coli* O157:H7 in their gastrointestinal tract and shed the pathogen asymptomatically in their feces (34, 35). In the northern United States, the prevalence of *E. coli* O157:H7 carriage by cattle ranged from 6 to 9%. Recent surveillance data indicate that prevalence rates of *E. coli* O157:H7 in cattle are much higher than those estimated several years ago (36).

2.2.1.2 Irrigation water

Irrigation water could potentially carry and spread pathogen contamination to a large portion of a crop. Currently, no U.S. regulation mandates monitoring, protecting, or treating irrigation waters. In order to minimize the risk of crop contamination associated with irrigation waters, the voluntary California Leafy Green Marketing Agreement (LGMA) adopted in 2008 established water quality criteria for irrigation waters applied to leafy green crops (37). The initial standards included in the LGMA are as follows:

- water sample should not exceed 235 most probable number (MPN) of *E. coli* per 100 ml for overhead irrigation of foliar surfaces;

- water sample should not exceed 576 MPN *E. coli* per 100 ml for drip irrigation of roots; and
- for either overhead or drip irrigation, *E. coli* concentrations should not exceed a geometric average of 126 CFU or MPN *E. coli* per 100 ml among five samples taken over 30 days.

In general, while the LGMA standards provide a useful starting point for discussion they do not account for the specific contamination and pathogen ecology mechanisms associates with different crop, different water sources, and conveyance systems, irrigation practices and scheduling, and local environmental conditions.

Another issue of concern is irrigation of food crops with reclaimed water, which is a risk to human health arising from infectious diseases (19). A fecal coliform limit of 1,000 organisms per 100 ml has been advised by WHO as a bacteriological standard for irrigation water (28). Approximately 20 million hectare of land is being irrigated using raw, treated and/or partially treated wastewater worldwide (38). The U.S. EPA has set a goal that all water from surface sources should not pose a risk of infection from waterborne pathogens greater than 1:10,000 per year (39). While this value was intended for drinking water, it can also be used to evaluate the level of risk associated with the use of reclaimed waste-water for the purpose of food crop irrigation (19).

2.2.1.3 Irrigation methods

Sprinkler, furrow, and drip irrigation are three main types of irrigation methods used for leafy greens. Furrow irrigation and subsurface drip irrigation can minimize contact of crops with contaminants present in irrigation water, whereas for sprinkler irrigation, the edible portions of plants are exposed directly to irrigation water (40). Out of the three irrigation methods (sprinkler, furrow and drip) tested, only leaves from sprinkler-irrigated plots were positive for *E coli*, one-day after the irrigation event (41). Overhead sprinkler irrigation produced larger amount of background microflora regardless of the water potential level. It is possible that the direct contact of water with the leaf surface alter the bacteria kinetic, phyllosphere ecology, and related plant physiology (41). The furrow irrigation method utilizes the application of water to the soil surface of the field, which may result in direct contact between the aboveground portions of the plant with the irrigation water. In subsurface drip irrigation system, on the other hand, irrigation water is introduced directly to the root system of the plant (28). For this reason it has been suggested that subsurface drip irrigation reduces health risks from the use of reclaimed wastewater for irrigation (42). Even if the irrigation water is not contaminated, sprinkler irrigation can result in contamination of plants as pathogens present in the soil can be transferred from soil to plant through splashes created by irrigation water (43). Water with a bigger droplet size will have a higher kinetic energy, and will maximize the erosive forces of irrigation water on contaminated soil or feces (43).

Erickson et al. (24) proposed that, even though the risk of contamination in subsurface irrigation is lower than that of furrow and sprinkler irrigation, it is still not completely negligible. Through growth chamber and hydroponic system, pathogens can be internalized into the roots at lateral root junctions of vegetable plants (44, 45), and in some cases, translocated to aerial tissues (32, 46, 47).

2.2.1.4 Pathogen kinetics in soil

For a contamination level of 2 log CFU/ml in irrigation water, *E. coli* O157:H7 could not be detected in the soil after 14 days. *E. coli* O157:H7 populations in the soil decreased by an additional 2 to 3 log CFU/g 7 days following application of the 4-6 log CFU/ml doses of *E. coli* O157:H7 in contaminated irrigation water. In contrast, *E. coli* O157:H7 populations decreased by only 1 to 2 log in soil during the first 7 days when compost served as the vehicle of contamination. *E. coli* O157:H7 could only be detected after 3 or 7 weeks when contaminated irrigation water or contaminated compost, respectively, was applied on the day of transplantation (23). In the study of Islam et al. (48), however, *E. coli* O157:H7 populations averaged 2 to 3.5 log CFU/g in the soil 7 weeks after being initially contaminated with 5.5 to 6 log CFU/g. The differences in the results might be attributed to the variation in temperature, rain encountered during the study, types of plants grown in the soil and soil moisture. Increases in populations of fecal bacteria and *Salmonella* may be associated with rainfall after a relatively dry period (49). Similarly, Iovieno and Baath (50) observed that bacterial growth increased to levels twice that of moist soil 24

hours after rewetting. Contamination of land with trace metals is also common in urban and semi-urban areas due to past and present industrial activity and the use of fossil fuels (51, 52).

2.2.1.5 Pathogen internalization through roots

The internalized *E. coli* O157:H7 could not be detected (with a few exception) in the spinach, lettuce, or parsley roots sampled at 2-50 days after transplantation of leafy greens into compost-contaminated soil with the contamination level of up to 6 log CFU/ml (24). The exceptions may be attributed to low temperatures (< 4.4°C) that occurred in the 2 days prior to sampling. Such low temperatures may temporarily reduce plant defensive activities. Similar results were obtained in two studies of Zhang et al. (53, 54), who grew lettuce in the soil contaminated with 6 log CFU/g concentration of *E. coli* O157:H7. The results were also in close agreement with Johannessen et al. (55), who grew lettuce in soil contaminated with *E. coli* O157:H7 at levels of 4 log CFU/g. On the other hand, when roots were exposed either to higher doses of pathogens in soil (7 to 8 log CFU/g) (46, 47, 56) or to pathogens in pasteurized soil (57), internalization was detected. Internalization of *E. coli* O157:H7 into roots was detected at lower pathogen doses either when the inoculum was placed directly on the roots (45) or when plants were grown in hydroponic systems (58).

2.2.2 Post-harvest contamination

While pre-harvest period of leafy green vegetables is the main concern in terms of foodborne pathogens, there are several postharvest opportunities during transportation (inappropriate temperature), processing (cross-contamination, immersion in water and cutting or slicing steps), packing (improper packaging, packing equipment), distribution, storage (improper temperature or very long duration) at market, retail, or home. All of these factors have the potential to contaminate the leafy green vegetables and their RTE salads with pathogens and to enhance growth of any pathogens already present in leafy greens (1).

2.2.2.1 Harvesting and processing

During harvesting and minor manipulation (e.g., removal of outer leaves and coring) or direct packing in the growing field or packinghouse, leafy vegetables and herbs may be at risk of the introduction of microbial contamination. A key characteristic of harvesting operations is that they involve considerable contact of fresh produce with workers (handler), different types of tools and equipment surfaces, water, and the field environment.

Postharvest contamination and subsequent spread of pathogens can occur during shredding, conveying, fluming, and dewatering of fresh-cut leafy greens. During processing, leafy vegetables and herbs may be exposed to microbial contamination and microorganisms may persist and grow. However, to a large extent,

the microflora of leafy vegetables and other fresh produce reflect the species present at the time of harvest. Of particular concern during processing is the contact between the leafy vegetables and the multiple surfaces in the factory environment, the microbiological status of water, and the potential for tissue injury during primary preparation. In terms of the wet equipment surfaces, greatest transfer was seen to the interior walls, basket carrier, and drain of the dewatering centrifuge, with the centrifugation water also yielding *E. coli* O157:H7 populations that were 1 to 2 log higher than the processing water. In addition to the wet surfaces, direct transfer of *E. coli* O157:H7 was also seen between the product and product contact surfaces of the shredder and conveyor belt. After processing iceberg and romaine lettuce, the shredder and conveyer belt generally yielded higher *E. coli* O157:H7 counts than the flume tank, shaker table, or centrifugal dryer (59).

Some processes have the potential to reduce microbial risks (e.g., disinfection), control microbial growth (e.g., chilling) and protect the product from further exposure (e.g., packaging). However, current technologies or practices do not effectively eliminate any hazard acquired during post-harvest processing or packaging of fresh and fresh-cut leafy vegetables and herbs. According to industry experience, and from extrapolation of laboratory experiments, only a slight risk reduction appears possible. The main food safety aim of post-harvest handling is prevention of increasing risk (60).

2.2.2.2 Changes in microbial contamination during storage

Produce temperatures are known to fluctuate during distribution and storage, and individual steps (transportation, retail storage, retail display, and home storage) may be highly variable in duration (61), thus modeling the changes in microbial contamination is a complex process. The most common way to estimate the parameters of growth and attenuation models is through controlled laboratory experiments that grow an organism in a specific medium and under specific environmental conditions. The number of microorganisms is recorded over time, and the various models are fitted to these data to derive the parameter values for this combination of microorganism/ growth medium/ environmental conditions (62).

Refrigeration storage temperature and storage time are the key factors affecting the growth/reduction kinetics of pathogens. The risk can be mitigated by the use of recommended home refrigeration storage temperatures and product storage time (63). Temperature is one of the most important environmental parameters affecting both food quality and food safety. The temperature of fresh produce should be maintained below approximately 5°C to reduce the proliferation of spoilage organisms and human pathogens. Temperature abuse was identified as the most important contributing factor in foodborne disease outbreaks, responsible for more than 32% of the total number of outbreaks (64). According to a quantitative risk assessment study conducted in 2003 by the USFDA in collaboration with the USDA and Food Safety and Inspection Services (FSIS), temperature of 40°F (4°C) or below

and storage time of less than 8 days could reduce the risk of illness from *L. monocytogenes* by more than 50% (64).

Pouillot et al. (63) fitted the distributions of time to first and last consumption of salad using the results of the national survey of U.S. adults to characterize home storage and refrigeration practices conducted by RTI International, Tennessee State University, and Kansas State University. The average time of first consumption was 2-3 days from the date of purchase for deli and bagged salad, whereas the average time for last consumption was 6 days from the date of purchase. The storage period of 6 days may result in considerable growth of pathogens if the food is not stored at proper temperature.

2.2.2.3 Use of antimicrobial agents and irradiation

Bacterial attachment and biofilm formation on the surfaces of leafy greens are two key factors that negatively impact the removal of microorganisms during conventional washing (59, 65). Some of the common antimicrobial agents are peroxyacetic acid, mixed peracid, sodium hypochlorite, mercuric chloride, calcium lactate (59, 66). However, elimination of pathogens from the surface of vegetables by disinfection is limited and unpredictable (67, 68). Moreover, it has also been shown that high chlorine concentration does not necessarily kill bacteria, and after removing chlorine some can recover during the rinsing step (69). Furthermore, recently, it has been suggested that *E. coli* O157:H7 could become internalized into plant tissues

reducing the effectiveness of disinfection treatments (70). In the light of these facts, it seems clear that disinfection treatments can fail resulting in the presence of the pathogens in leafy greens at the time of consumption.

Recently, the FDA approved the use of gamma irradiation on vegetables allowing irradiation levels up to 4 KGy (71). However, sensory characteristics in irradiated vegetables can be affected at irradiation levels above 0.5 kGy (72), and thus, only lower irradiation levels could be used practically (73, 74).

2.3 Quantitative microbial risk assessments (QMRA) and mathematical models for pathogens in leafy greens

A number of QMRA and mathematical models for pathogens in leafy greens have been developed in the past. Some of these focus on specific processes, whereas others deal with contamination from farm-to-fork as a holistic approach (**Table 2.2**).

2.3.1 Risk due to contaminated soil

In their study, Gale (27) developed a quantitative risk assessment model to predict the number of humans in the UK infected through consumption of root crops grown on agricultural land to which treated sewage sludge had been applied. The risk assessment was based on the source-pathway-receptor approach for *Salmonella*, *L. monocytogenes*, *E. coli* O157, *Cryptosporidium parvum*, *Giardia*, and enteroviruses. The model confirmed that the risks to humans from consumption of vegetable crops were very low.

Table 2.2 Quantitative microbial risk assessment of pathogens in leafy greens

Author(s)	Country	Crop(s)	Pathogen(s)	Focus of study
Bouwknegt et al. (75)	Netherlands	Leafy greens and berry fruits	Norovirus and hepatitis A virus	Production and processing
Carrasco et al. (76)	Spain	Lettuce	*L. monocytogenes*	Processing to consumption
Danyluk and Schaffner (77)	United States	Leafy greens	*E. coli* O157:H7	Farm to consumption
Ding et al. (78)	Korea	Lettuce	*L. monocytogenes*	Farm to consumption
Franz et al. (16)	Netherlands	Lettuce	*E. coli* O157:H7	Manure-amended soil
Franz et al. (16)	Netherlands	Leafy greens consumed at salad bars	*E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*	Temperature fluctuations in cold chain
Gale (27)	United Kingdom	Vegetable crops	*Salmonella*, *L. monocytogenes*, *E. coli* O157, *Cryptosporidium parvum*, *Giardia*, and enteroviruses	Harvest intervals
Gale and Stanfield (79)	United Kingdom	Vegetable crops	Bovine spongiform encephalopathy (BSE) agent	Sewage sludge to agricultural land
Hamilton et al. (19)	Australia	Broccoli, cabbage, cucumber, lettuce	Enteric viruses	Non-disinfected secondary treated reclaimed water
Kokkinos et al. (80)	Greece	Leafy greens	Human adenovirus and human pathogenic virus, and hepatitis A virus	Production, processing, and point of sale

Liu et al. (81)	China	Vegetables	Heavy metals	Industrial and waste mining sites near field
Mota et al. (82)	Mexico	Fresh produce	*Cryptosporidium* and *Giardia*	Contaminated irrigation water
Nabulo et al. (52)	Uganda	Leafy greens	Heavy metals	Irrigation with wastewater, effluent discharge from industry and dumping of solid waste
Ottoson et al. (83)	Sweden	Lettuce	*E. coli* O157	Survival of bacteria as a function of temperature and light intensity during production
Park et al. (84)	South Korea	Leafy greens, stalk and stem vegetables	Pesticide residues	Production
Petterson et al. (85)	Australia	Lettuce	Viruses	Wastewater irrigation
Puerta-Gomez et al. (86)		Baby spinach	*Salmonella*	Temperature during harvest, washing, and irradiation
Sant'Ana et al. (87)	Brazil	Leafy greens	*Salmonella* and *L. monocytogenes*	Retail to consumption
Shuval et al (88)	Israel	Ready-to-eat vegetables	Hepatitis A virus and rotavirus	Wastewater irrigation
Stine et al. (28)	United States	cantaloupe, iceberg lettuce, and bell peppers	*E. coli*, *Salmonella*, hepatitis A virus	Contaminated irrigation water
Szabo et al. (89)	Australia	Iceberg lettuce	*L. monocytogenes*	Antimicrobial washing agents

2.3.2 Risk due to irrigation water

A risk assessment was conducted by Shuval et al. (88) to estimate the risk by irrigating the crops with recommended wastewater irrigation microbial health guidelines of the WHO and the USEPA for unrestricted irrigation of vegetables normally eaten uncooked. The study indicated that the annual risk of a virus disease from regularly eating vegetables irrigated with effluent meeting WHO guidelines (1,000 fecal coliform/100mL) was negligible and of the order of 10^{-6} to 10^{-7}. For WHO guidelines, the risk of the more infectious, but less serious, rotavirus was estimated as 10^{-5} to 10^{-6}. The USEPA considers an annual risk of 10^{-4} to be acceptable for microbial contamination of drinking water. The health benefit that might result from a further reduction of risk gained by adhering to the USEPA Reuse Guidelines (39), which require no detectable fecal coliforms/100mL, was found to be insignificant in relation to the major additional costs associated with the expensive technology required to treat effluent to such a rigorous standard (88).

The timing of the last irrigation also critically affects the postharvest microbial population of leafy greens (28). Hamilton et al. (19) conducted a risk assessment to estimate the effect of decay in pathogen concentration due to days elapsed between last irrigation and harvest. Across various crops (cucumber, broccoli, cabbage, and lettuce), and virus contaminations levels (based on the available data on enteric virus concentrations in non-disinfected secondary effluent from five sewage treatment plants in California), the annual risk of infection ranged from 10^{-3} to 10^{-1}

when reclaimed-water irrigation ceased one day before harvest and from 10^{-9} to 10^{-3} when it ceased 2 weeks before harvest.

Petterson et al. (85) evaluated the potential health risk from viruses associated with the consumption of lettuce crops spray irrigated with secondary-treated municipal effluent. Predicted infection rates were much more sensitive to the decay rate of viruses than occasional high virus numbers. The median and 99th percentile risks of infection from the overall model were 0.10 and 0.51 per 10,000 lettuce consumers, respectively, indicating possible human health concern.

2.3.3 Processing

Buchholz et al. (59) estimated the transfer of *E. coli* O157:H7 from leafy greens to different processing equipment during processing. During processing, up to 90% of the *E. coli* O157:H7 transferred to the wash water. After processing, *E. coli* O157:H7 populations were highest on the conveyor and shredder, followed by the centrifugal dryer, flume tank, and shaker table. Similar results were obtained in another study by Buchholz et al. (90), where transfer of *E. coli* O157:H7 from equipment surfaces to fresh-cut leafy greens during processing in a pilot-plant production line with sanitizer-free water was studied. Initially the greatest *E. coli* O157:H7 transfer was seen from inoculated lettuce to the shredder and conveyor belt, and later the *E. coli* O157:H7 concentration on all equipment surfaces decreased by 90 to 99% after processing 90.8 kg of uncontaminated product. After processing

lettuce containing 6 or 4 log *E. coli* O157:H7 CFU/g followed by uninoculated lettuce, *E. coli* O157:H7 was quantifiable throughout the entire 90.8 kg uncontaminated product. At an inoculation level of 2 log CFU/g, *E. coli* O157:H7 was consistently detected in the first 21.2 kg of uninoculated lettuce.

2.3.4 Storage conditions

The growth-death models to predict the change in the concentration of pathogens can be divided into three categories: (i) primary models, that describe changes in microbial numbers with time (for example three-phase linear model (91), and Baranyi model (92, 93), (ii) secondary models, that describe changes in parameters of primary models to changes in environmental conditions (for example, square-root model (94) and response surface polynomial models), and (iii) tertiary models, which are user-friendly software or expert systems (for example, ComBase Predictor, and USDA Pathogen Modeling Program).

Franz et al. (16) conducted a quantitative microbial risk assessment for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* infection from consumption of leafy green vegetables in salad from salad bars in The Netherlands. Pathogen growth was modeled using time-temperature profiles in the chilled supply chain and one particular restaurant with a salad bar. The temperature in the studied cold chain was well controlled below 5°C. Growth of *E. coli* O157:H7 and *Salmonella* was minimal (17 and 15%, respectively), whereas, growth of *L. monocytogenes* was considerably

greater (194%). The ranges of the average number of annual cases were estimated as 42 to 551 for *E. coli* O157:H7, 81 to 281 for *Salmonella*, and 0.1 to 0.9 for *L. monocytogenes*. Szabo et al. (89) evaluated the growth potential of *L. monocytogenes* on lettuce packaged in a gas-permeable film and stored at 4 or 8°C for 14 days. The results of this study showed that under storage at either 4 or 8°C for 14 days in the packaging film, the lettuce was no longer acceptable. To ensure that the food safety objective is met, the initial *L. monocytogenes* level in the processed lettuce must not exceed 0.1 log CFU/g, provided that the storage time is limited to 7 days and the maximum temperature is not greater than 8°C.

2.3.5 Intervention methods for decontamination of pathogens

Washing is the most common decontamination strategy for fresh produce. When the contamination level of bacteria such as *L. monocytogenes* is more than 3 log CFU/g, washing under running tap water is not enough and dipping with or without bleach is necessary. However, when the contamination level is 6 or 9 log CFU/g, none of the studied treatments were found effective (66). Keeratipibul et al. (95) investigated the efficiency of hypochlorous and peracetic acids in reducing coliforms and *Escherichia coli* levels on lettuce leaves using artificial neural networks (ANN). Hypochlorous acid could reduce the level of viable coliforms and *E. coli* on lettuce leaves by up to 1-log CFU/g. When peracetic acid was used, a maximum reduction of about 2-log CFU/g was observed.

The equation representing the reduction of *L. monocytogenes* in lettuce due to washing under running tap water was fitted by Doménech et al. (66) as Equation 1.

$$\log(N/N_o) = -0.28\ln(t) - 0.0103 \quad \text{...(1)}$$

Where, N_o is the initial number of cells (CFU/g); N the number of survivals after washing treatment; and t is duration (seconds) of washing.

The levels of *L. monocytogenes* obtained after dipping lettuce in sodium hypochlorite were estimated according to Equation 2 (66, 96).

$$\log(N/N_o) = \left(-\frac{C}{0.35} + 0.65\ C\right)\ t^{-0.44+0.54C^{-0.008}} \quad \text{…(2)}$$

Where, N_o is the initial number of cells (CFU/g), N the number of survivals after washing treatment, t is duration (seconds) of washing and C is the concentration (ppm) of sodium hypochlorite. The efficacy of chlorinated water is also dependent on surface accessibility, pH, the concentration of available free chlorine, temperature, duration of treatment, organic matter, and produce type (97).

High calcium lactate concentrations (3%) can produce a reduction in the respiration rate of the salad-cut lettuce during storage, but their application may also result in loss of luminosity and greenness. Another factor in washing that affects quality retention is water temperature. The use of high temperatures causes a positive effect on enzymes related to quality maintenance. It reduces the activity of the

browning-related enzymes polyphenol oxidase and peroxidase but it increases the activity of pectin methyl esterase, an enzyme involved in the maintenance of texture. Therefore, the use of high temperatures (up to 50°C) and intermediate calcium lactate concentrations (1.5%) is an optimum washing treatment to maintain the quality of salad-cut lettuce over 10 days storage (98).

Many other treatments have been proposed for the decontamination of fresh produce, including single or combined application of chemical sanitizers, detergents, organic acids, UV light, ozonation, pulsed UV light, high pressure, ionizing irradiation, vaporized ethyl pyruvate and several other treatment methods (97, 99).

Puerta-Gomez et al. (86) developed the inactivation model for the inactivation of *Salmonella* in baby spinach leaves (Equation 3).

$$S_{irr} = \frac{N}{N_o} = e^{-12.121D} \quad \ldots(3)$$

where, N_o is the initial number of microorganisms, N is the number of remaining microorganisms after exposure to dose D (in kGy).

The International Commission on Microbiological Specifications for Foods has introduced the concept of food safety objective (FSO) (100). The FSO concept translates public health risk into a definable goal: a specified maximum frequency or concentration of a (microbiological) hazardous agent in a food at the time of consumption that is deemed to provide an appropriate level of health protection.

2.4 Conclusions

Increased global production, distribution, and consumption of leafy greens in conjunction with more intensive production methods and inconsistent application of good agricultural practices explain the high incidence of foodborne illness linked to this food category. However, environmental factors, both during pre- and post-harvest play significant roles as sources of foodborne pathogens. It is clear that pathogens can survive in soil as well as on fresh produce for long periods. Bacterial harborage on plant surfaces complicate efforts to consistently sanitize contaminated produce. It is evident that current options in commercial use to sanitize leafy greens are prone to failure and thus emphasis must continue to be placed upon prevention of produce contamination.

Chapter 3. Development of growth and death models for *Salmonella* and *Listeria monocytogenes* during non-isothermal time-temperature profiles in leafy greens

3.1 Abstract

Leafy greens contaminated with *Salmonella enterica* have been linked to large number of illnesses in many countries in recent years. *Listeria monocytogenes* is also a pathogen of concern for leafy greens because of its prevalence in the growing and processing environment and its ability to grow at refrigeration temperatures. Experimental data for the growth and survival of *S. enterica* and *L. monocytogenes* under different conditions and storage temperatures were retrieved from published studies. Predictive models were developed using the three-phase linear model as a primary growth model and square-root model to calculate specific growth rate at different temperatures. The square-root model for *Salmonella* was calculated as $R = (0.020(Temperature+0.57))^2$. The square-root model for *L. monocytogenes* was fitted as $R = (0.023(Temperature-0.60))^2$. The growth-survival model for *Salmonella* and growth model for *L. monocytogenes* were validated using several dynamic time-temperature profiles during the production and supply chain of leafy greens. The models from this study will be useful for future microbial risk assessments and predictions of behavior of *Salmonella* and *L. monocytogenes* in the leafy greens production and supply chain.

3.2 Introduction

Over the last few decades, production and consumption of leafy greens has increased dramatically in the U.S. (5). In 1984, total production of Iceberg, Romaine, and leaf lettuce in the U.S. was 72,103,000 cwt (3.66 billion kg), which increased to 90,488,000 cwt (4.59 billion kg) in 2009 (USDA, 2011). The convenience and benefits of cut, prewashed, and packaged leafy greens have created a demand for high quality products (101, 102). On the other hand, leafy vegetables can be contaminated during production from many sources, such as contaminated manure, irrigation water, animals, birds, and insects (103, 104, 105). Following production, processes such as harvesting, washing, cutting, packaging, and shipping can create additional contamination (59, 90, 104). Since the minimal processing associated with fresh and fresh-cut leafy greens has few intervention steps that kill microorganisms, contaminated leafy greens are more likely to cause outbreaks due to enteric pathogens (106).

The incidence of foodborne infections caused by bacterial pathogens in leafy greens continues to be a problem in developed and developing countries (107). Surveys have suggested the presence of human enteric pathogens (such as *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes*) on produce (13, 15, 108). From 1973 to 2012, 396 foodborne illness outbreaks were linked with the consumption of leafy greens in the U.S., causing 14,046 illnesses and 978 hospitalizations (7). *S. enterica* was linked with 10 outbreaks associated with the consumption of leafy greens in the

U.S. during 1998-2008 (109). Thus, while animal products have traditionally been considered to have a greater risk of being a vehicle for *S. enterica*, in recent years, leafy greens have emerged as a major contributor to the incidence of salmonellosis (110). *L. monocytogenes* is also concerning because of its ubiquitous nature and ability to grow at refrigerated temperatures. In 2011, an outbreak linked with cantaloupe contaminated with *L. monocytogenes* caused 147 cases of listeriosis resulting in 33 deaths and one miscarriage (CDC, 2012). There are limited cases of listeriosis caused by *L. monocytogenes* on leafy greens, but since 2010 there have been 8 recalls issued due to possible contamination in leafy greens (8). These recalls indicate a considerable risk of contaminated leafy greens entering the supply chain and eventually causing a large scale outbreak (112).

Based on the assumption that the responses of bacteria in a defined environment are reproducible, predictive microbiology models are used to predict a bacterial population's size according to the initial contamination and the food environment (113). In the last few years, few predictive models for growth of *S. enterica* and *L. monocytogenes* in leafy greens have been developed and reported (114-118). However, these models are based on temperature data much higher than the temperatures reported in the surveys for leafy greens during transportation and storage (8, 112). Also, there is considerable variability in the growth rates reported in the reported models. For example, Koseki & Isobe (114) reported growth rates higher than other models (114-116, 118) predicting the growth at similar temperatures.

In the published literature substantial amount of growth and survival data for *S. enterica* and *L. monocytogenes* in leafy greens are available. However, there is a need to compile these data to develop a generic growth-survival model and to address the variability in the growth rates. Based on the available growth and survival data in literature, McKellar & Delaquis (61) developed a model for *E. coli* O157:H7 in leafy greens. However, similar studies for *S. enterica* and *L. monocytogenes* have not been compiled to develop growth-survival models. Thus, this study developed a growth-survival model for *S. enterica* and *L. monocytogenes* in leafy greens.

3.3 Materials and methods

3.3.1 Data

Data for growth and survival of *S. enterica* and growth of *L. monocytogenes* were taken from different studies described in **Table 3.1**, **Table 3.2**, and **Table 3.3**. Eight studies and seventeen studies were identified for growth data of *S. enterica* (**Table 3.1**) and *L. monocytogenes* (**Table 3.3**), respectively. Six studies were found for survival of *S. enterica* (**Table 3.2**). Growth and survival curves which included any active intervention step to affect the growth or survival rate of microorganism, such as application of chlorine washing (119), and alkaline electrolyzed water (116) were excluded. After exclusion of such data, 35 curves were identified for growth of *S. enterica*, 16 curves for survival of *enterica*, and 118 curves for growth of *L. monocytogenes* (**Tables 3.1-3.3**). Since *L. monocytogenes* is known to be a psychrotroph bacteria which can tolerate refrigerated temperatures $\leq 3^{o}C$, no studies

showing reduction of this pathogen at lower temperatures were identified with a few exceptions. For example, there was a decline in the level of *L. monocytogenes* at 3°C and 1°C in iceberg lettuce treated with modified atmosphere, and chlorine washing, respectively (120, 121). Thus, only growth data were considered for model development of *L. monocytogenes.* Data were acquired from tables, graphs, text, or personal communications. Graphs were digitized using PlotDigitizer software (http://plotdigitizer.sourceforge.net/). Validation data for dynamic time-temperature profiles were taken from available studies (8, 114, 115). **Table 3.4** shows different time-temperature profiles used for model validation.

Table 3.1 Growth data for *Salmonella*

Authors	**Temperature (°C)**	**Product**	**Data Source**	**No of curves**
Brandl and Amundson (122)	28	Romaine lettuce	Digitized	3
Brandl and Mandrell (123)	22, 24, 26, and 30	Cilantro	Digitized	8
Chang and Fang (124)	22	Iceberg lettuce	Table	1
Koseki and Isobe (114)	10, 15, 20, and 25	Iceberg lettuce	Digitized	4
Ma et al. (125)	12 and 21	Cilantro	Digitized	2
Oliveria et al. (101)	25	Shredded romaine lettuce	Digitized	3
Puerta-Gomez et al. (117)	10, 20, 30, and 37	Baby spinach	Digitized	8
Sant'Ana et al. (118)	7, 10, 15, 20, 25, and 30	Lettuce	Personal communications	6

Table 3.2 Survival data for *Salmonella*

Authors	Temperature (˚C)	Product	Data Source	No of curves
Chang and Fang (124)	4	Iceberg lettuce	Table	1
Kakiomenou et al. (126)	4	Lettuce	Digitized	2
Ma et al. (125)	4	Cilantro	Digitized	1
Oliveria et al. (101)	5	Lettuce	Digitized	3
Vandamm et al. (127)	4	Fresh-cut celery	Table	8
Weissinger et al. (128)	4	Shredded lettuce	Table	1

Table 3.3 Growth data for *Listeria monocytogenes*

Authors	Temperature (˚C)	Product	Data Source	No of curves
Carlin et al. (129)	10	Endives	Table	6
Carlin et al. (130)	3 and 10	Endives	Table	16
Carrasco et al. (131)	5 and 13	Shredded iceberg lettuce	Digitized	2
Ding et al. (116)	4, 10, 15, 20, 25, 30 and 35	Iceberg lettuce	Digitized	7
Farber et al. (132)	4 and 10	Coleslaw and Rutabaga leaves	Digitized	6
Francis and O'Beirne (120)	8	Shredded iceberg lettuce	Digitized	6
Francis and O'Beirne (133)	8	Lettuce and Rutabaga leaves	Digitized	6

Francis and O'Beirne (134)	8	Lettuce and Rutabaga leaves	Digitized	8
Francis and O'Beirne (135)	8	Lettuce and coleslaw mix	Digitized	15
García-Gimeno et al. (136)	4	Mixed salad	Digitized	1
Gleeson and O'Beirne (137)	8	Butterhead and iceberg lettuce	Digitized	5
Jacxsens et al. (138)	7	Chicory, endives, and iceberg lettuce	Digitized	2
Kaminski et al. (139)	4, 7 and 10	Celery	Digitized	3
Koseki and Isobe (114)	5, 10, 15, 20, and 25	Shredded iceberg lettuce	Digitized	5
Li et al. (119)	5 and 15	Shredded iceberg lettuce	Digitized	20
Oliveria et al. (101)	5 and 25	Shredded romaine lettuce	Digitized	12
Sant'Ana et al. (118)	7, 10, 15, 20, 25, 30	Shredded lettuce	Personal communication	6

Table 3.4 Non-isothermal time temperature profiles for leafy greens supply chain

Profile	**Pathogen in consideration**	**Description**	**Reference**
Profile 1	*S. enterica, L. monocytogenes*	Farm to retail (initial temperature ~15°C)	(114)
Profile 2	*S. enterica, L. monocytogenes*	Farm to retail (initial temperature ~25°C)	(114)
Profile 3	*S. enterica*	Slow cooling	(117)
Profile 4	*L. monocytogenes*	Transportation to retail	(8)
Profile 5	*L. monocytogenes*	Retail storage	(8)
Profile 6	*L. monocytogenes*	Retail display	(8)

3.3.2 Primary models

3.3.2.1 Growth model

Three-phase linear model was used as a primary growth model because of the simplicity of this model. The three-phase model fits lag-phase, log-phase and stationary-phase as straight lines (91). A major advantage of using the three-phase linear model is unlike other models such as Baranyi and Gompertz model, it does not require initial concentration of pathogens to predict the growth. The Equations (Eq. 1) of the three-phase linear model were fitted on the growth data using the Integrated Pathogen Modeling Program (IPMP), version 2013 (http://www.ars.usda.gov/Main/docs.htm?docid=23355).

$$\log N_t = \log N_o \qquad \text{for } t \leq t_{lag}$$

$$\log N_t = \log N_o + \frac{\mu}{2.303}\left(t - t_{lag}\right) \qquad \text{for } t_{lag} < t < t_{max} \qquad \ldots(1)$$

$$\log N_t = \log N_{max} \qquad \text{for } t \geq t_{max}$$

Where, N_t= cell concentration (CFU g^{-1}) at time t; N_o= initial cell concentration (CFU g^{-1}); N_{max}= maximum cell concentration (CFU g^{-1}); t = time (h); t_{lag} = lag time (h); t_{max} = time required for maximum growth (h); μ = growth rate (ln CFU $g^{-1}h^{-1}$).

3.3.2.2 Death model

A log-linear death model (61) was used for inactivation of *Salmonella* at lower temperatures (≤ 5°C). IPMP software was used for linear regression of *Salmonella* inactivation. Equation 2 was used as the inactivation/death model for *Salmonella*.

$$log(N_t) = \log(N_o) - \frac{k}{2.303} \times t \quad \ldots(2)$$

Where, k is death rate parameter in ln CFU g^{-1} h^{-1}.

3.3.3 Secondary models

3.3.3.1 Growth model

The growth temperature data were fitted to the square-root model (140) using MATLAB software (Mathworks, ver. 2013b):

$$\sqrt{\mu} = b(T - T_{min}) \quad \ldots(3)$$

In Equation 3, μ is specific growth rate (ln CFU h^{-1}) mentioned in Equation 1; b is the temperature coefficient, T is the temperature (°C) and T_{min} is the notational minimum temperature (°C) for growth of the bacterium. Regression line was calculated using least-square method and 95% confidence interval (CI) and 95% prediction interval (PI) for growth rate μ were calculated using Equations 4-7:

$$CI = y^* \pm t^*_{n-2}\sigma\sqrt{\frac{1}{n} + \frac{\left(x^* - \bar{x}\right)^2}{(n-1)s_x^2}} \quad \ldots(4)$$

$$PI = y^* \pm t^*_{n-2}\sigma\sqrt{1 + \frac{1}{n} + \frac{\left(x^* - \bar{x}\right)^2}{(n-1)s_x^2}} \quad \ldots(5)$$

$$\sigma = \sqrt{\frac{SSE}{n-2}} \quad \ldots(6)$$

$$SSE = \sum(y_i - y^*)^2 \quad \ldots(7)$$

Where, CI is confidence interval, y* is the predicted value of dependent variable, n is the total number of samples, t* is student's t-value, σ is standard deviation of residual of y, x* is the value of independent variable, $\bar{x}$ is the mean of independent variable values in the data-set, s_x^2 is standard deviation of x, and SSE is the sum of square of errors.

3.3.3.2 Death model

The values of death coefficient 'k' in Equation 2 were fitted for several distributions using @RISK 6.0 software (Palisade Corporation, Ithaca, NY). The survival data for *Salmonella* from different studies were used (**Table 3.2**).

3.3.4 Tertiary model

Because leafy greens are exposed to non-isothermal time-temperature profiles in the supply chain, a model was developed that could predict the change of levels of *Salmonella* and *L. monocytogenes* with respect to the varying temperatures. Therefore, a tertiary model was developed in MATLAB (ver. 2013b) to combine the growth and death models for *Salmonella* and *L. monocytogenes*. Growth of *Salmonella* was modeled above 7°C, whereas it would decline below 5°C (Equation 9). *Salmonella* populations were modeled to remain constant between 5°C and 7°C. Being a psychrotroph, *L. monocytogenes* can grow at ≥3°C (Equation 10). The dynamic model for growth of *Salmonella* and *L. monocytogenes* and death of *Salmonella* was defined using Equation 8 (61).

$$\frac{dN_t}{dt} = Rate * N_t \quad \ldots(8)$$

For *Salmonella*,

$$Rate = \begin{cases} Growth\ (if\ T \geq 7) \\ 0\ (if\ 7 > T > 5) \\ Death\ (if\ T \leq 5) \end{cases} \quad \ldots(9)$$

$$Growth = b(T - T_{min})^2 \quad \ldots(9a)$$

$$Death = -k \quad \ldots(9b)$$

For *L. monocytogenes*,

$$Rate = (if\ T \geq 3, Growth, 0) \quad \ldots(10)$$

3.3.5 Model validation

3.3.5.1 Profiles

Tertiary models developed for *Salmonella* and *L. monocytogenes* were used to simulate the changes in bacterial concentration with respect to the dynamic time-temperature profiles from available literature (8, 114, 117) mentioned in **Table 3.4**. The 95% confidence interval of pathogen growth at different time intervals was also calculated using the 95% confidence interval of the square-root growth model (Equation 3). Model validation was performed using MATLAB (Mathworks, ver. 2013b).

3.3.5.2 Goodness of fit

The validation indices were calculated to assess accuracy (Equation 11) and the bias (Equation 12) of the models (141).

$$Accuracy\ factor\ (A_f) = 10^{\frac{\sum\left|\log\left(\frac{predicted}{observed}\right)\right|}{n}} \quad \ldots(11)$$

$$Bias\ factor\ (B_f) = 10^{\frac{\sum\log\left(\frac{predicted}{observed}\right)}{n}} \quad \ldots(12)$$

An ideal predictive model has A_f = B_f = 1, indicating the exact match between experimental observation and model predictions. Acceptable prediction zone (APZ)

analysis was also done to validate the models. The difference between the observed and predicted values of bacteria concentration was prediction error (PE) with the units log cfu g^{-1}. PE values below 0 are fail-safe, while PE values above zero were considered as fail-dangerous. The APZ was set between the PE values of −1.0 and 0.5 (61, 142).

3.4 Results

3.4.1 Growth models

3.4.1.1 *Salmonella*

Out of 35 growth curves for *Salmonella*, some data sets had as low as three data points, whereas some had as high as 15 data points. Thus, the three-phase linear model was selected because of its utility for fewer data points. For curves, which had less than 5 data points, it was difficult to judge whether there was any clear lag-phase or whether the maximum population density (MPD) was reached or not. Out of 35 growth curves, only 11 had a clear lag-phase, and only 26 had a clearly defined MPD.

The data from Koseki & Isobe (114) and Sant'Ana, Franco, & Schaffner (118) are examples where sufficient (up to 11) data points were available to distinguish lag time and MPD. On the other hand, data from Oliveira et al. (101) and Escalona et al. (143) are examples where data were not enough to identify the lag phase or MPD. Thus, the lag-phase data were excluded, and square-root model was fitted using the data points corresponding to exponential phase. The final concentration was taken as

MPD if MPD was not attained. Specific growth rates corresponding to different temperatures are shown in **Figure 3.1a**. Fitted parameters for square-root model for *Salmonella* and *L. monocytogenes* have been presented in **Table 3.5**.

3.4.1.2 *L. monocytogenes*

Out of 118 curves were collected for *L. monocytogenes*, some had as low as 4 data points (119, 132), whereas others had more than 10 points (114, 118). The exponential growth rates for *L. monocytogenes* are shown in **Figure 3.1b**.

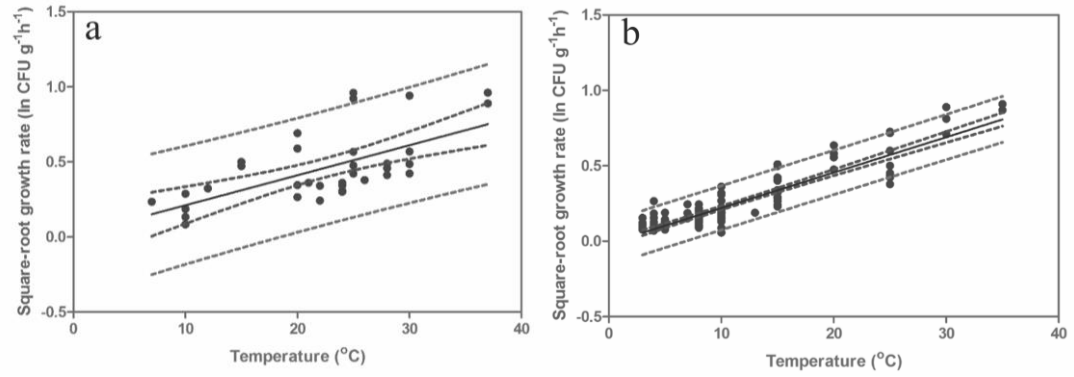

Figure 3.1 Square-root model for growth of (a) *Salmonella*, and (b) *Listeria monocytogenes*. Symbols represent experimental data from available literature, solid line represents mean square-root model, inner and outer band of dotted lines represent upper and lower 95% confidence intervals and prediction intervals, respectively.

Table 3.5 Fitted parameters for square-root model

	Salmonella			***L. monocytogenes***		
	Model	**2.5 percentile**	**97.5 percentile**	**Model**	**2.5 percentile**	**97.5 percentile**
b	0.020	0.011	0.028	0.023	0.021	0.025
$\mathbf{T_{min}}$	-0.571	-1.668	0.747	0.599	-1.691	0.338
$\mathbf{R^2}$	0.41			0.83		

3.4.2 Survival model

For *Salmonella,* 16 curves were collected showing the declining population in leafy greens corresponding to 4 and 5°C. The survival rate was calculated using the linear survival model in Integrated Pathogen Modeling Program (IPMP), ver. 2013 software. Log-logistic distribution was found to be best-fitting for the survival rate of *Salmonella* in leafy greens. The parameters are shown in **Figure 3.2**. A log-logistic distribution is defined in @Risk as Riskloglogistic (γ, β, α), where γ is continuous location parameter, β is continuous scale parameter, and α is continuous shape parameter. Mean of this distribution is calculated using Equation 13.

$$\text{Mean} = \beta\ \theta\ \text{cosec}\ (\theta) + \gamma \quad \ldots(13)$$

Where, $\theta = pi / \alpha$

For the values of parameters γ, β, and α given in **Figure 3.2**, the mean death rate was calculated as 0.013 ln CFU g^{-1} h^{-1}.

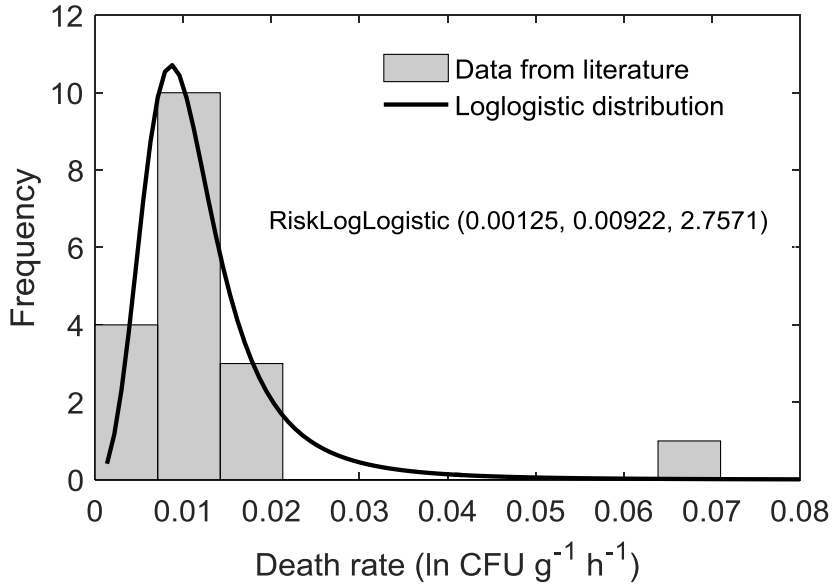

Figure 3.2 Distribution fitting for survival data of *Salmonella*.

3.4.3 Tertiary model

For growth, mean values of parameters *b* and T_{min} in **Table 3.5** were used. The notational minimum temperatures (T_{min}) were calculated as -0.57°C and 0.60°C for *Salmonella* and *L. monocytogenes*, respectively. Slopes for *Salmonella* and *L. monocytogenes* were calculated as 0.020 and 0.023, respectively. Variability, as measured by 95% confidence interval values, were also used in the tertiary models.

3.4.4 Validation of developed models using non-isothermal time-temperature profiles

Koseki & Isobe (114) had two temperature profiles with the higher maximum temperature (~25°C) and lower maximum temperature (~15°C), respectively for both *Salmonella* and *L. monocytogenes*. Puerta-Gomez et al. (117) had a cooling temperature profile for *Salmonella* with the temperature constantly dropping from 30°C to 2°C in about 5 hours. Zeng et al. (8) recorded several dynamic time-temperature profiles during transportation to retail, retail storage and retail display. One profile each from transportation, retail storage and retail display was selected. The predicted values from the developed tertiary model and observed values from published studies are presented in **Figure 3.3** and **Figure 3.4**.

3.4.5 Goodness of fit

Prediction error (PE) values for different non-isothermal time-temperature profiles were calculated, and the acceptable prediction zone (APZ) analysis are presented in **Figure 3.5**. In time-temperature profiles, all of the 28 observed points for *Salmonella* and the 41 observed points of *L. monocytogenes* profiles were found to be within the APZ. Out of 28 data points for *Salmonella*, 15 were in the fail-safe zone ($-1 \leq PE < 0$). Similarly, 21 out of 41 data points of *L. monocytogenes* were fail-safe.

Table 3.6 shows the A_f and B_f values for *Salmonella* and *L. monocytogenes* for different temperature profiles mentioned in **Table 3.4**. For all the temperature

profiles, the A_f values ranged from 1.017-1.059, whereas B_f value was in the range of 0.985-1.057. A_f and B_f values were also calculated from the predictions given for the respective time-temperature in the original studies. While Koseki & Isobe (114) and Puerta-Gomez et al. (117) had reported the graphs representing the predictions from their models, Zeng et al. (8) only gave the root mean squared error (RMSE), biases, and APZ results in a Table. A_f and B_f values reported were calculated by digitizing the prediction curves reported by Koseki & Isobe (114) and Puerta-Gomez et al. (117). A_f B_f values could not be calculated for Profiles 4-6 (8) because of the unavailability of prediction curves. The equation used by Zeng et al. (8) to calculate the biases was different from the bias factor reported in this study calculated in this study (Equation 12), and therefore, bias values were not calculated for Profiles 4-6. For Profile 1 in *Salmonella*, the values of A_f and B_f were the maximum, i.e., 1.059 and 1.057, respectively. This was because of systematic over-predictions of the values in this profile. It has been reported that models with B_f in the range of 0.9 to 1.05 can be regarded as good for describing a pathogen growth rate (141). The predicted values were in very close agreement with the observed values. The A_f and B_f reported in this study were consistently better than the values calculated from the respective original studies (Profiles 1-3) for the given time-temperature profiles, except the bias factor predicted for *L. monocytogenes* in Profile 2.

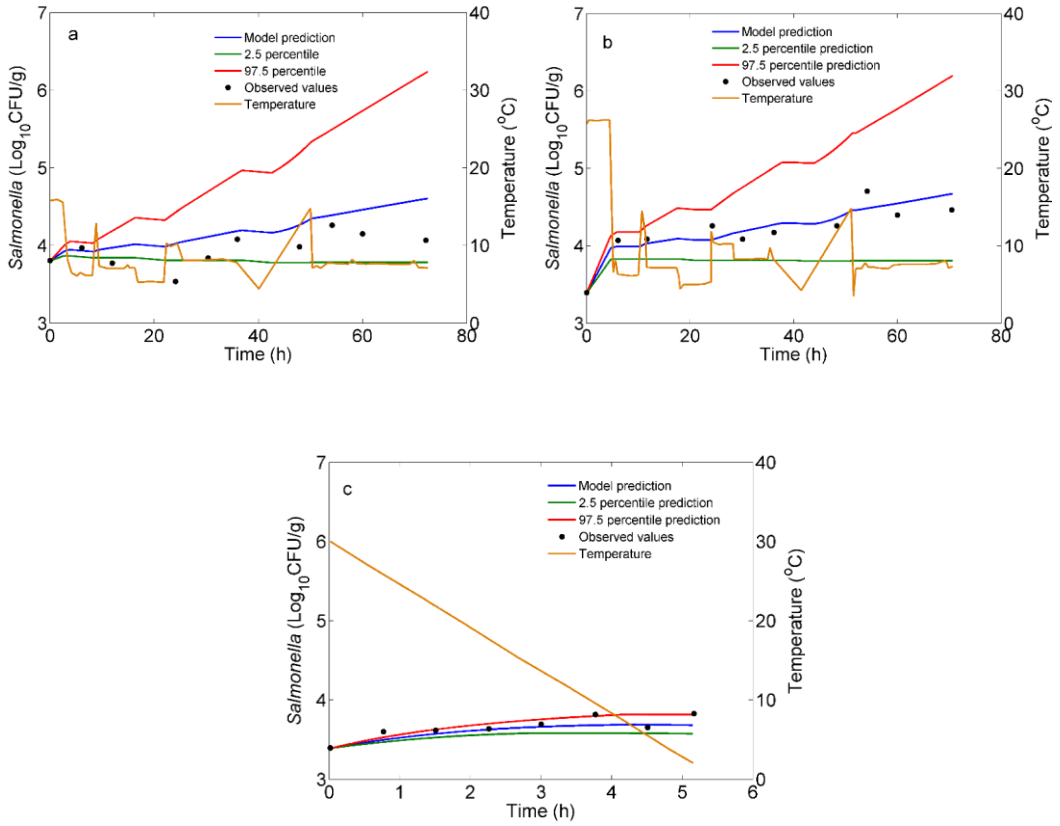

Figure 3.3 Validation for (a) *Salmonella* during Profile 1; symbols represent Koseki and Isobe (114) data points; (b) *Salmonella* during Profile 2, symbols represent Koseki and Isobe (114) data points; and (c) *Salmonella* during Profile 3, Symbols represent Puerta-Gomez et al. (117) data points.

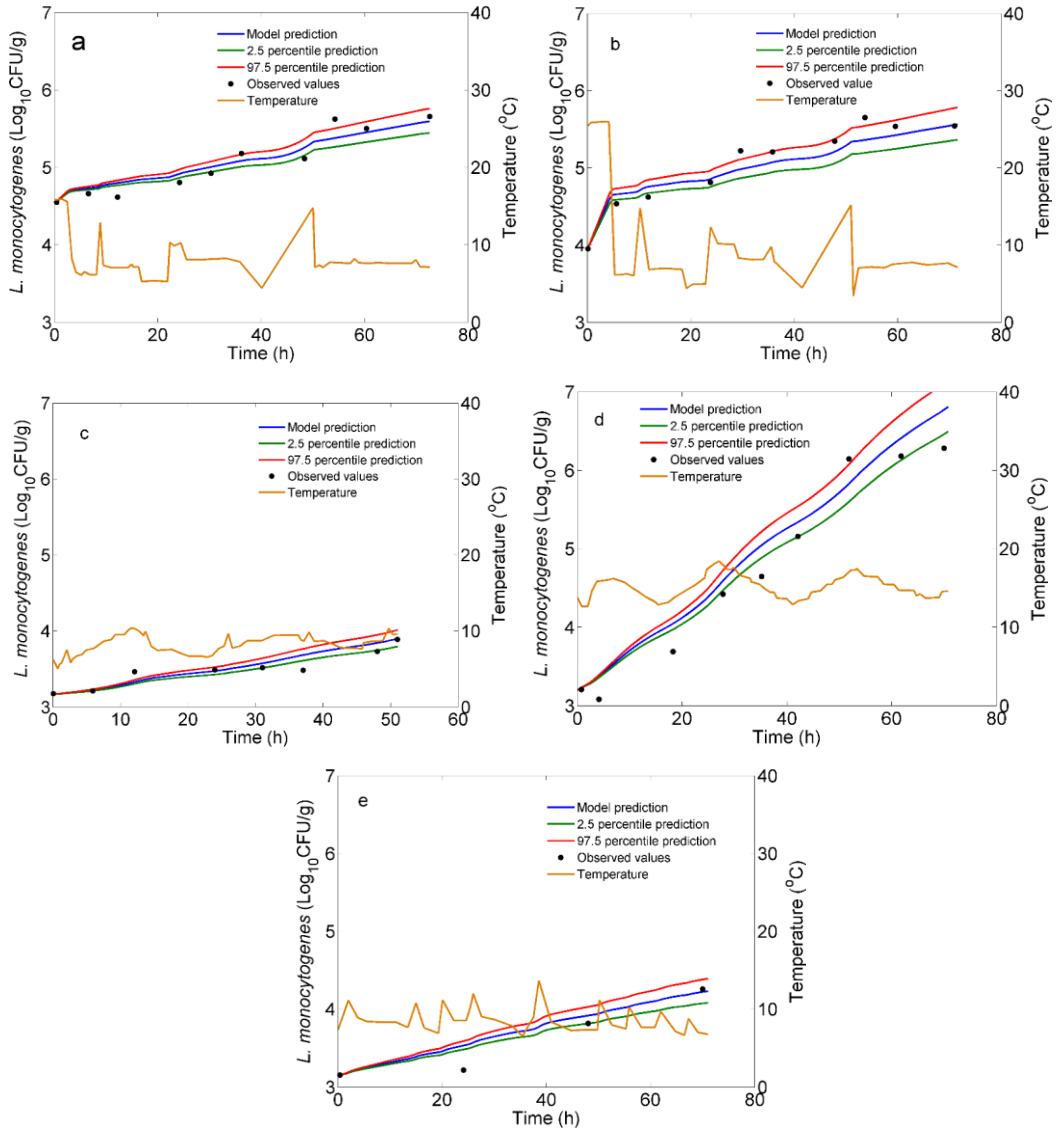

Figure 3.4 Validation for (a) *L. monocytogenes* during Profile 1; symbols represent Koseki and Isobe (114) data points; (b) *L. monocytogenes* during Profile 2, symbols represent Koseki and Isobe (114) data points; (c) *L. monocytogenes* during Profile 4 (transportation from processing to retail), symbols represent Zeng et al. (8) data points; (d) *L. monocytogenes* during Profile 5 (retail storage), symbols represent Zeng et al. (8) data points; (e) *L. monocytogenes* during Profile 6 (retail display), symbols represent Zeng et al. (8) data points.

3.5 Discussion

This study utilizes the available growth and survival data of *Salmonella* and *L. monocytogenes* in different leafy greens in order to develop growth-survival models for non-isothermal conditions. Growth and survival data were generated for different experimental conditions and treatments, such as type of leafy greens, temperature, gas composition, cutting etc. The models developed using growth and survival data from the studies conducted under variable conditions are robust and can accommodate the variations in parameters which would be expected under real processing and storage conditions.

Clearly, there was variability in the growth data of *Salmonella*, with the coefficient of determination value of 0.41 (**Table 3.5**). When these data were further analyzed, there was a very clear trend that at any particular temperature, it was found that the growth rates in cut leafy greens (101, 114, 118, 122, 124, 144) were higher than the growth rates in uncut leafy greens (117, 123, 125). This trend could be better understood by considering the information that all the experiments were carried out using surface inoculation. For uncut leafy greens, the pathogens are not easily internalized from the surface, and are exposed to the relative humidity of the air surrounding it. The growth rate in this case is affected by the air, which may not provide high water activity or relative humidity necessary for the growth of bacteria. On the other hand, once the leafy green is cut, the nutrients in the juices are available to bacteria. This means that after a leafy green is sliced, diced or shredded,

contamination could lead to high bacterial growth, particularly on the cut surfaces. Lettuce is one of few edible crops that produce latex (145). Upon cutting of lettuce stems, a large quantity of latex is released onto the cut surfaces (146). As early as 2 h and 4 h after its inoculation onto lettuce stem discs, population sizes of *E. coli* O157:H7 increased 5.6- and 11.1-fold. By 22 h of incubation, the *E. coli* O157:H7 population size on the stem discs increased >20,000-fold, suggesting that the surface of cut lettuce stems holds large quantities of substrates that allow for the multiplication of bacteria (146). After an outbreak of shigellosis was traced to shredded lettuce, rapid growth of *Shigella* sonnei was observed in shredded lettuce stored at room temperature (147, 148). For *L. monocytogenes*, there was relatively little variability in growth data, shown by coefficient of determination value 0.83. Growth data for *L. monocytogenes* were analyzed, and all the 17 studies were carried out using uncut leafy greens. This information suggests that in cut leafy greens, the growth of *L. monocytogenes* may be actually more than the values predicted by the growth-survival model presented in this study.

From the growth-data presented in **Table 3.1** and **Table 3.3**, the minimum temperature supporting the growth of *Salmonella* and *L. monocytogenes* are 7°C and 3°C, respectively. The notational minimum temperature (T_{min}) is often lower than the minimum temperature supporting the growth of bacteria (61). The results from this study comply with this fact where the T_{min} for *Salmonella* and *L. monocytogenes* were calculated as -0.57°C and 0.60°C, respectively.

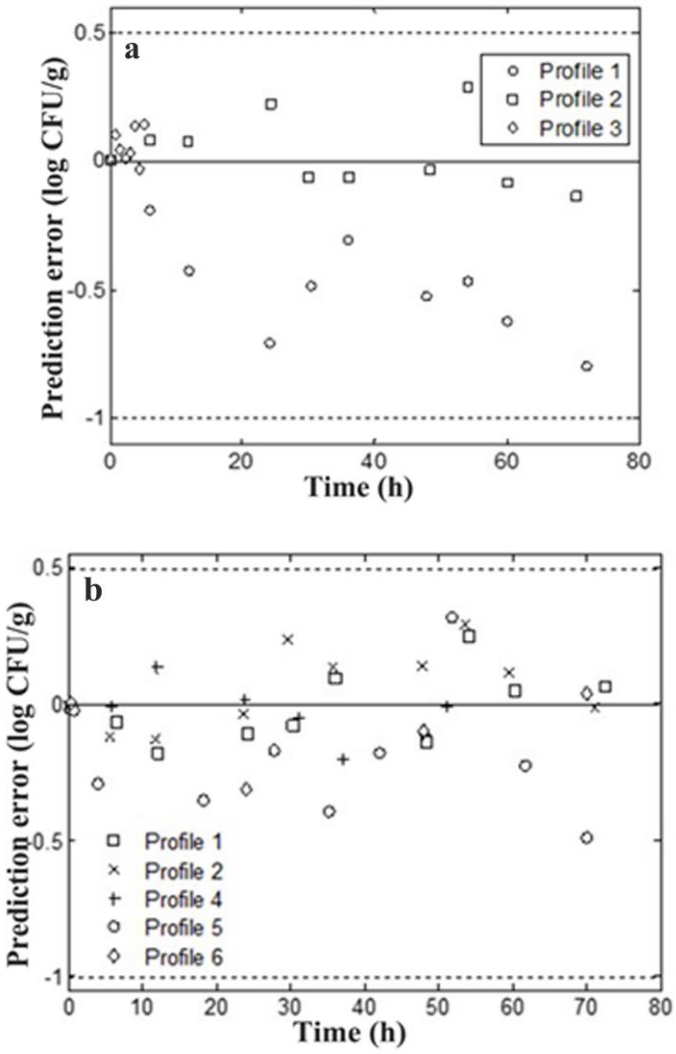

Figure 3.5 Acceptable Prediction Zone (APZ) analysis: (a) Data for time-temperature profiles for *Salmonella*, and (b) *L. monocytogenes*.

Table 3.6 A_f and B_f values for predicted growth of *Salmonella* and *Listeria monocytogenes* in leafy greens for different time-temperature profiles given in Table 3.4

	Salmonella				***Listeria monocytogenes***			
Profile	**A_f (This study)**	**A_f (Original study)**	**B_f (This study)**	**B_f (Original study)**	**A_f (This study)**	**A_f (Original study)**	**B_f (This study)**	**B_f (Original study)**
Profile 1	1.059	1.089	1.057	0.983	1.021	1.076	1.004	1.049
Profile 2	1.028	1.033	1.002	1.032	1.024	1.052	0.990	0.996
Profile 3	1.017	1.034	0.985	1.003	--	--	--	--
Profile 4	--		--		1.019	NA	1.007	NA
Profile 5	--		--		1.058	NA	1.045	NA
Profile 6	--		--		1.032	NA	1.028	NA

--: Pathogen growth/survival not studied in the original study; NA: Value not provided in the original study

3.6 Conclusions

There was more variability in the growth data of *Salmonella* as compared to the variability in the growth data for *L.* monocytogenes. A possible reason for this variability could be the use of different kinds of leafy greens (cut/shredded or uncut) in the experiments carried out for growth of *Salmonella*. There was a clear trend that the growth rates of *Salmonella* on cut leafy greens were more rapid than the growth rates for uncut leafy greens. All the studies for *L.* monocytogenes included in this study had data of growth of pathogen in uncut leafy greens. More studies need to be conducted to differentiate the growth of pathogens in cut and uncut leafy greens. This will greatly impact the results of future quantitative microbial risk assessments (QMRAs) of leafy greens.

Chapter 4. Modeling for growth of *Escherichia coli* O157:H7, *Salmonella,* and *L. monocytogenes* in leafy greens without temperature control

4.1 Abstract

A recent study by the Centers for Disease Control and Prevention reported that in between 1998 and 2008, leafy greens outbreaks accounted for 22.3% of foodborne outbreaks in the U.S. Several studies on growth of bacteria at different temperatures have been conducted; however, there is a need for more research on prediction of bacterial growth when leafy greens are transported without temperature control. Food products, when taken out of refrigeration, begin to undergo a temperature change and the rate of temperature change is proportional to the difference in the temperature of food and its surroundings. The objective of this study was to estimate the growth of *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes*, in leafy greens during transportation from retail to home at ambient temperatures ranging from 10°C to 40°C for up to 10 hours. Experiments were conducted to model the temperature increase in fresh spinach when these are taken from refrigeration temperature to ambient temperature. The growth of pathogens was predicted using the dynamic temperature profiles with the three-phase linear model as a primary model, and square-root model as the secondary model. The concentration of *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* increased by 3.12, 2.43,

and 3.42 log CFU at 40°C for the 10 hour time period, respectively when no lag phase was assumed. If leafy greens are not kept out of refrigeration for more than 3 hours, when the air temperature is 40°C or more, pathogen growth should be less than 1 log CFU. These results would assist in developing recommendations for food transportation without refrigeration.

4.2 Introduction

One of the most challenging tasks in today's food industry is controlling the product quality and microbial safety throughout the food supply chain (149). Microbial contamination can occur during any of the steps in the farm-to-table continuum (e.g. production, harvest, processing, retail storage, transportation, or household handling). Contamination can arise from environmental, animal, or human sources (99, 150). Temperature during transport can be controlled (e.g. from processing plant to retail store in a refrigerated trucks, or uncontrolled (e.g. transportation from retail store to home). Many studies on growth of pathogens at different temperatures have been conducted; however, there is a need for more research on prediction of bacterial growth when the food is transported without temperature control (151). The time-temperature profile during transportation has a critical impact on growth of pathogens in leafy greens. Leafy greens were added to the definition of potentially hazardous food requiring time-temperature control for safety (TCS) following 24 multi-state outbreaks between 1998 and 2008 in the United States (152). The pH, water activity, available moisture, and nutrients of leafy greens

support the growth of foodborne pathogens, and refrigeration at ≤ 41 °F (5 °C) inhibits growth of some pathogens such as *E. coli* O157:H7 and *Salmonella* (114, 124, 125, 127). Other studies suggest that psychrotrophic pathogens, like *L. monocytogenes* exhibit minimal growth in leafy greens ≤ 5°C (116, 129).

Food products begin to undergo a temperature change when taken out of refrigeration, and this process continues until the product temperature approaches ambient air temperature. The rate of temperature change of a food is proportional to the driving force (the temperature differential between the food temperature and surrounding environment) (153). This means that the rate of temperature change is greatest when the food is first placed in the environment and gradually slows as the food and environmental temperatures converge (151). This is also known as Newton's law of heating (154). Current federal regulations stipulate that food products be protected against microbial contamination and their growth during transportation and storage (155). The objective of this study was to characterize the dynamics of temperature change in spinach once removed from refrigeration and to understand the effect of this temperature change on growth of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*.

4.3 Materials and methods

4.3.1 Newton's law of heating:

The temperature rise in a food is a function of food temperature, ambient air temperature and the time that the food is out of refrigeration. Consider a lumped system analysis for unsteady state heat transfer, and assume a spinach bunch of irregular shape of mass *m*, volume *V*, surface area *As*, density ρ, and specific heat c_p initially at temperature T_o. At time $t = 0$, the food is placed into air temperature T_a, and heat transfer begins to take place between the food and its environment, with a heat transfer coefficient *h*.

$$hA_s(T_a - T)dt = mc_p dT \quad \ldots(1)$$

or, $\frac{d(T_a - T)}{T_a - T} = -\frac{hA_s}{\rho V c_p} dt \quad \ldots(2)$

Where, *dT* is the difference between air and food temperature, *dt* is change in time. The simplified form of Equation 2 can be written as Equation 3.

$$\frac{T_a - T(t)}{T_a - T_o} = e^{-Bt} \quad \ldots(3)$$

Where, $B = \frac{hA_s}{\rho V c_p}$

B is time constant (minute^{-1}), *t* is time (minutes), T_a is outside air temperature (°C), *T(t)* is product temperature (°C) at time *t*, and T_o is initial food temperature (°C).

4.3.2 Estimation of time constant 'B':

In Equations (1-3), the food temperature (T) is a function of time, ambient air temperature (T_a), initial temperature (T_o) and B. Experiments were conducted to estimate the value of B for fresh spinach, which was then used to predict the time-temperature profiles of spinach when removed from retail displays and transported without temperature control. Fresh bunches of unpackaged spinach (~ 0.75 pounds each) were purchased from a retail store in Maryland, placed in plastic bags, and maintained at 5°C for 24 hours, then transferred to the incubators with controlled temperatures of 10, 20, 30, and 40°C. Each bunch of spinach was transferred to a separate incubator without any forced air circulation. Temperature data loggers with K-type probes (Lascar Electronics, Erie, PA) were used to monitor ambient temperature and spinach temperature for each bunch of spinach. Temperature measurements were made using the probe located near the surface of the food (~ one half centimeter inside the outer leaves). Spinach temperatures were recorded at one minute interval for 10 hours. Experiments were repeated six times at each temperature (10, 20, 30, and 40°C). One-way ANOVA (V9.0, JMP, SAS, NC) was performed to test for significant differences among the values of B measured at temperatures 10, 20, 30, and 40°C, with the significance level set at 0.05 (156).

4.3.3 Primary growth model

The lag phase and log phase of the three-phase linear model (91) was used as the primary growth model because of its simplicity and wider application. The three phase linear model fits lag phase, log phase, and stationary-phase as straight lines (61). Equations. 4 and 5 represent lag and log phases of the three-phase linear model.

$$log\, N_t = log\, N_o \qquad \text{for } t \leq t_{lag} \qquad ...(4)$$

$$log\, N_t = log\, N_o + \frac{\mu}{2.303}(t - t_{lag}) \qquad \text{for } t_{lag} < t < t_{max} \qquad \ldots(5)$$

where N_t = cell concentration (CFU g^{-1}) at time t; t = time (h); t_{lag} = lag time (h); t_{max} = time required for maximum growth (h); and μ = specific growth rate (ln CFU h^{-1}).

E. coli O157:H7 and *Salmonella* populations are known to decline at temperatures lower than 5°C (8, 61, 101, 125, 127, 157). On the other hand, *L. monocytogenes* is known to be a psychrotroph pathogen, and it has the ability to survive even at 3°C (116, 130, 139). However, the survival model was not used in this study because the initial food temperature at retail display was assumed to be 5°C, and was modeled to rise after the food was taken out of retail storage.

4.3.4 Secondary growth model

Square-root model was selected as the secondary growth model (94).

$$\sqrt{\mu} = b(T - T_{min}) \qquad \ldots (6)$$

In Equation 6, μ is specific growth rate in Equation 5; b is the temperature coefficient, T is food temperature (°C) and T_{min} is the notational minimum temperature (°C). The values of b and T_{min} are dependent on the microbe and the food. Square root model parameters for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* were taken from the literature (**Table 4.1**). Minimum temperature for growth of pathogens has been reported as 5°C for *E. coli* O157:H7 (61), 7°C for *Salmonella* (118), and 3°C for *L. monocytogenes* (130).

Since temperature and growth rate μ change with respect to time, the modified square-root model equation can be written as:

$$\sqrt{\mu} = b((T_a - (T_a - T_o)e^{-Bt}) - T_{min}) \quad \ldots(7)$$

Table 4.1 Parameters for square-root model for exponential growth of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in leafy greens

Pathogen	**b**	**T_{min}**	**Reference**
E. coli O157:H7	0.023	1.20	(61)
Salmonella	0.020	-0.57	Chapter 3
L. monocytogenes	0.023	0.60	Chapter 3

4.3.5 Lag phase duration

The lag-time (t_{lag}) is also a function of food temperature. Lag-time data was obtained from the stand-alone software of the U.S. Department of Agriculture-Agricultural Research Service's Pathogen Modeling Program (PMP version 7.0). Since the food temperature gradually changes with respect to time when food is taken out of refrigeration, the expected lag-time is also assumed to be changing with respect to the changing temperature. The percentage of lag time elapsing in each minute interval was estimated by dividing the interval time by the lag time for the interval temperature and multiplying the resulting value by 100. The percentage of lag time contributed by each minute interval was accumulated until 100% of the time in the lag phase elapsed, as shown by Equation. 8 (158).

$$\%lag\ time\ elapsed = \sum_{i=1}^{n} (interval\ time\ (1\ minute) / lag\ phase\ for\ i^{th}\ minute) \times 100 \quad \ldots(8)$$

4.3.6 Prediction of bacterial growth

In this study, two scenarios were considered for each pathogen: with lag phase (i.e., because food is considered to be taken out of refrigeration and hence the food temperature is increasing with respect to time, it is assumed to undergo lag phase which is a function of food temperature) and without lag phase (i.e., the lag phase was assumed to be over when the food was refrigerated in retail). Following the calculation of lag time, growth of pathogens was calculated using Equation 9 at every

1 minute interval. Calculations for lag-time and growth of pathogens were conducted using MATLAB software (MathWorks, ver. 2015b).

$$Total\ growth = \sum_{i=1}^{n} exponential\ growth\ rate\ for\ interval\ i \times interval\ time\ i \quad \ldots(9)$$

4.4 Results

4.4.1 Lag phase duration

The lag-time values given by PMP fitted the power-law equation for *E. coli* O157:H7 and *Salmonella*; and exponential equation for *Listeria monocytogenes* (R^2 = 0.99). **Table 4.2** shows the fitted equations for predicting lag-time for these pathogens in leafy greens at different temperatures.

Table 4.2. Fitted equations for predicting lag-time for bacteria as a function of temperature

Pathogen	**Equation**	
E. coli O157:H7	$LT = 75444 \times Temp^{-3.119}$	(R^2=0.99)
Salmonella	$LT = 72088 \times Temp^{-3.027}$	(R^2=0.99)
L. monocytogenes	$LT = 85.916 \times \exp(-0.131 \times \mathrm{Temp})$	(R^2=0.99)

LT = Lag-time; Temp = Temperature

4.4.2 Value of time constant 'B'

The values of *B* were not significantly different at the four temperatures ($p > 0.05$), so the average value of *B* (0.017 min^{-1}) was used. **Figure 4.1** shows the predicted change in food temperature as a function of time and ambient temperature.

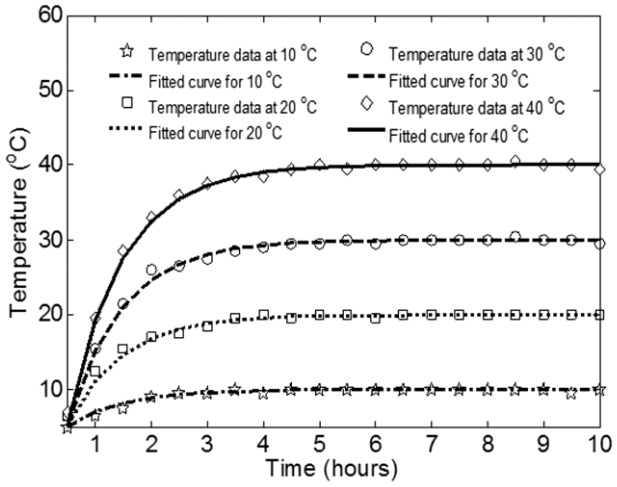

Figure 4.1 Predicted temperatures for fresh spinach as a function of ambient temperature and time out of refrigeration.

4.4.3 Prediction of pathogen growth

4.4.3.1 *E. coli* O157:H7 with lag phase

With lag phase consideration, it was predicted that the lag phase was not completed in the first 10 hours if the air temperature was less than 19°C. Expected lag phase duration for 19°C ambient temperature was 9.93 hours. As the air temperature increased, lag phase duration decreased and the growth of bacteria population

increased. The lag time for 25, 30, 35 and 40°C ambient temperatures for *E. coli* O157:H7 was estimated as 4.47, 2.75, 2.40, and 1.40 hours, respectively; whereas the predicted growth in the bacteria population for 25, 30, 35 and 40°C ambient temperatures was 0.66, 1.29, 2.00 and 2.81 log CFU g^{-1}, respectively at the end of 10 hours (**Figure 4.2a**).

4.4.3.2 *E. coli* O157:H7 without lag phase

When no lag phase was considered, the expected growth of *E. coli* O157:H7 at 10°C ambient temperature was 0.13 log CFU g^{-1}. For 20, 30 and 40°C ambient temperatures, the growth of the pathogen was 0.68, 1.68, and 3.12 log CFU g^{-1}, respectively (**Figure 4.2b**).

4.4.3.3 *Salmonella* with lag phase

When lag phase was considered, it was predicted that the lag phase was not complete in the first 10 hours if the air temperature was less than 21°C (**Figure 4.2c**). At the ambient temperature of 21°C, the lag phase duration was predicted as 9.03 hours. The lag time for 25, 30, 35 and 40°C ambient temperatures was estimated to be 5.48, 3.38, 2.30, and 1.70 hours, respectively; whereas the growth of *Salmonella* for these temperatures was 0.43, 0.93, 1.49 and 2.12 log CFU g^{-1}, respectively at the end of 10 hours.

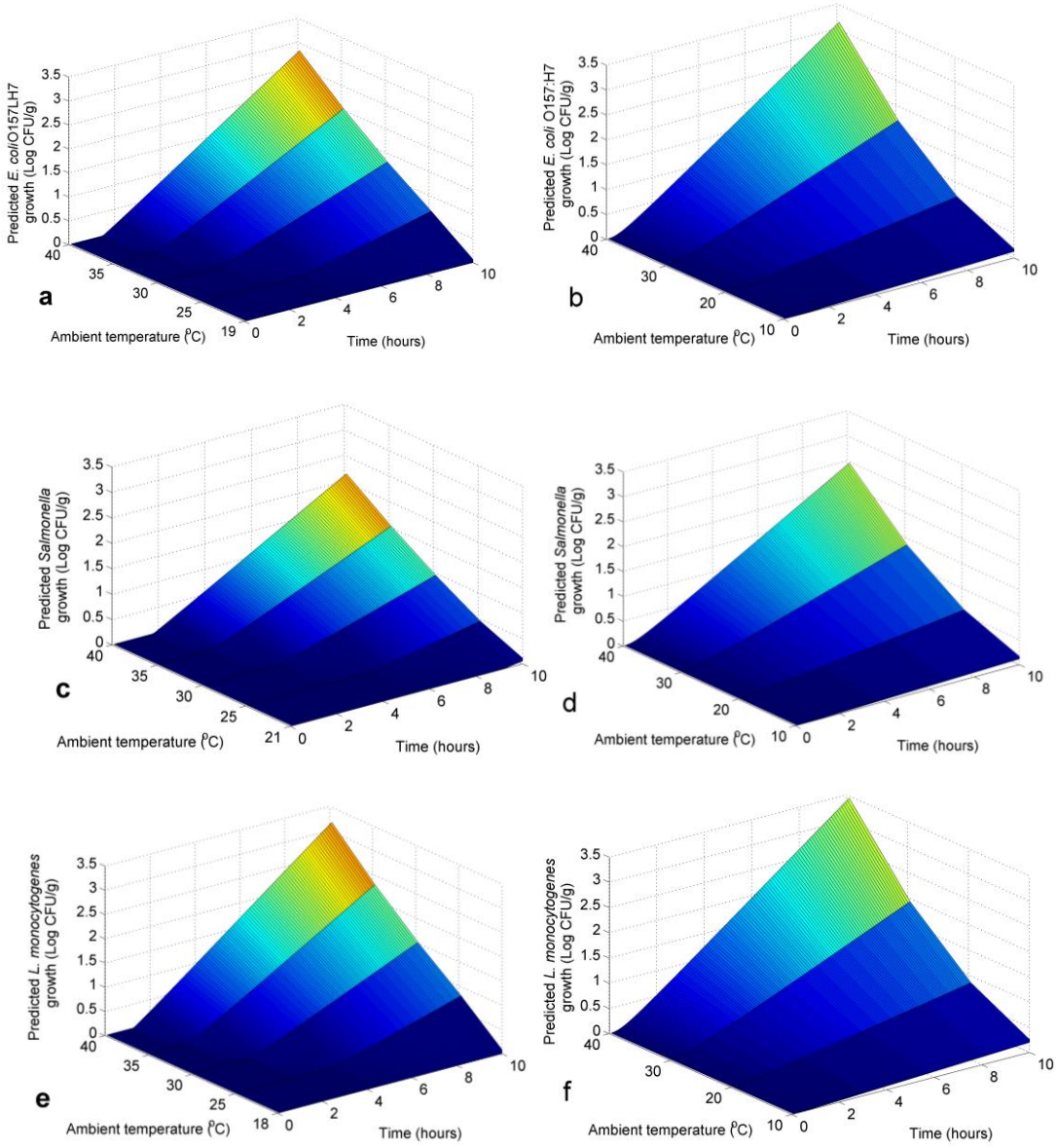

Figure 4.2 Predicted growth of (a) *E. coli* O157:H7 with lag phase, (b) *E. coli* O157:H7 without lag phase (c) *Salmonella* with lag phase, (d) *Salmonella* without lag phase, (e) *L. monocytogenes* with lag phase, and (f) *L. monocytogenes* without lag phase.

4.4.3.4 *Salmonella* without lag phase

At 10°C air temperature, the increase in *Salmonella* population was very minimal (0.09 log CFU g^{-1}) at the end of 10 hours. For 20, 30 and 40°C ambient temperatures, the growth of *Salmonella* in spinach was predicted as 0.62, 1.32, and 2.44 log CFU g^{-1}, respectively at the end of 10 hours (**Figure 4.2d**).

4.4.3.5 *Listeria monocytogenes* with lag phase

Lag phase duration was predicted more than 10 hours when the ambient temperature was less than 18°C. The expected lag time for *L. monocytogenes* in spinach was estimated as 9.80, 4.38, 2.63, 1.72, and 1.18 hours, respectively for 18, 25, 30, 35 and 40°C ambient temperature, respectively (**Figure 4.2e**). Growth in bacteria population at the end of 10 hours was estimated as 0.01, 0.78, 1.48, 2.27, and 3.16 log CFU g^{-1} for ambient temperatures of 18, 25, 30, 35, and 40°C, respectively.

4.4.3.6 *Listeria monocytogenes* without lag phase

When lag phase was not considered, the growth of *L. monocytogenes* was estimated as 0.20, 0.84, 1.90 and 3.43 log CFU g^{-1} at the end of 10 hours for ambient temperatures of 10, 20, 30 and 40°C, respectively (**Figure 4.2f**).

4.5 Discussion

When the ambient temperature was 20°C or below, the time needed for 1-log growth was more than 10 hours for no lag phase consideration for all three pathogens

in this study. For *E. coli* O157:H7 and *Salmonella*, 1-log growth was achieved in 6.18 and 7.68 hours at 30°C, and 3.57 and 4.41 hours at 40°C ambient temperature, respectively. For *L. monocytogenes*, this period was estimated as 5.50 hours at 30°C, and 3.28 hours at 40°C ambient temperature. Thus, based on 1-log growth time prediction without consideration of lag phase, leafy greens should not be kept out of refrigeration for more than 3 hours during summer afternoons, when the air temperature rises to 40°C or more in some parts of the world. These results are in close agreement with a U.S. Food and Drug Administration (FDA) position paper on quantitative risk assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods (64), which recommends that in order to limit the pathogen growth below 1-log, a conservative 4 hour limit for keeping foods without temperature control allows for a needed margin of safety if the temperature of the environment is higher than 24°C (75°F). The temperature of 24 °C (75 °F) was selected because it is a temperature at which mesophyllic and psychrotrophic pathogens will demonstrate growth (159). In addition, Schaffner (151) suggested a 0.6-log increase (two doublings) as a caution situation. Considering no lag phase, the time required for 0.6-log growth of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* was 8.83, 9.92, and 7.36 hours at 20°C, 3.93, 4.83, and 3.53 hours at 30°C, and 2.33, 2.85, and 2.15 hours at 40°C, respectively.

The starting retail temperature was considered to be 5°C (41°F) because the U.S. Food and Drug Administration Food Code requires that ready-to-eat fruits and

vegetables be refrigerated at 5°C or lower in order to minimize the growth of foodborne pathogens (152). In addition, in a large-scale U.S. study surveying 3799 time-temperature profiles, Zeng et al. (8) found that distributions of mean temperature during retail display was 4-6°C in more than 55% cases. In the EcoSure 2007 survey (160), the mean temperature for refrigerated products at retail was reported as 40°F.

The temperature of a food approaches the ambient temperature T_a exponentially. The temperature of the food changes rapidly at the beginning and slowly later on. A large value of *B* indicates that the food approaches the environment temperature in a short time. The larger the value of the *B*, the higher the rate of increase in temperature. *B* is proportional to the surface area, but inversely proportional to the mass and the specific heat of the food. This is not surprising since it takes longer to heat or cool a larger mass, especially when it has a large specific heat. The value of *B* was calculated for refrigerated food products in EcoSure 2007 report (160) takin the mean temperature at retail, and mean change in product temperature from store to home based on time out of refrigeration, and the calculated value of *B* was 0.0034 min^{-1}. The value of *B* for fresh spinach (0.017 min^{-1}) was higher than the reported results in EcoSure 2007 survey. Fresh-cut leafy vegetables and spinach have higher surface area to mass ratio than products like fresh meat and packaged deli, which were a part of the EcoSure 2007 survey. The experimental values in Schaffner (151) for ground beef and block cheddar cheese also suggest that

the temperature would not rise very rapidly in these foods, suggesting that these products have a lower value of *B.*

The assumption of the existence of lag time is a very critical one (161). The difference between the predicted growth of bacteria with and without consideration of lag time was less than 0.5 log at the end of 10 hours for all three pathogens considered in this study. Lag time plays a vital role in growth prediction for short time periods. Muñoz-Cuevas, Fernández, George, & Pin (162) found that when a food in lag phase is taken to a fluctuating temperature, the system is reset with a new lag phase. They concluded that the predictions were considerably more accurate when lag phase was included in the model. However, these authors also reported that there is a new lag phase when the fluctuations in the temperature are considerably large. On the other hand, prediction for bacterial growth without considering lag time could serve as providing conservative results.

4.6 Conclusions

Considering the lag phase for all three pathogens, it was estimated that lag phase for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* is more than 10 hours if spinach is kept in the ambient temperature of 18°C or lower. Considering no lag phase, the bacterial growth was not more than 3.5 log/g for 40°C (could represent the maximum temperature during summer). Considering the pathogens' 1-log growth/g without occurrence of lag phase, it could be recommended that leafy greens should not be kept out of refrigeration for more than three hours at ambient temperature of

40°C or more. The results of this study will be useful for estimating the risk to human health because of keeping leafy greens out of refrigeration for extended durations.

Chapter 5. Cost, quality, and safety: A nonlinear programming approach to optimize the temperature during supply chain of leafy greens

5.1 Abstract

Consumption of fresh and fresh-cut leafy greens in the United States has increased by more than 25% in the last 30 years. Leafy green vegetables are highly susceptible to microbial contamination because they are minimally processed. Pathogenic bacteria of concern include *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes*. Leafy greens are a highly perishable commodity, and in some cases have a postharvest shelf-life limited to one week. This study provides and approach to optimize storage of leafy greens in the supply chain, considering the cost of refrigeration, sensory quality parameters (i.e., fresh appearance, wilting, browning, and off-odor), and microbial safety using nonlinear programming (NLP). The coefficient of performance (COP) for refrigeration was considered in determining the cooling cost. The loss of sensory quality parameters was expressed as Arrhenius equations and pathogen growth were represented by three-phase linear (primary) and square-root (secondary) model. The objective function was refrigeration cost, which was to be minimized. The constraints were growth of pathogens (*E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*), and the loss of sensory characteristics. An interactive graphical user interface was developed in MATLAB. Pathogen growth is

of more concern than loss of sensory quality in fresh-cut iceberg lettuce when considering a shelf-life of up to two days, and the model indicates is difficult to maintain sensory qualities for longer shelf-life values. Browning is of maximum concern for fresh-cut iceberg and romaine lettuce, whereas off-odor is the biggest concern for fresh-cut chicory.

5.2 Introduction

Leafy greens are important sources of minerals, vitamins, antioxidants, and dietary fiber (163). Contamination of leafy greens with foodborne pathogens of particular concern because these foods are usually consumed raw without cooking or other interventions to kill any pathogens that might be present (164). The Center for Science in the Public Interest ranked leafy greens at the very top of the ‘‘FDA Top Ten’’ riskiest foods in 2009 (Buchholz et al., 2012). The annual number of produce-associated outbreaks reported has increased since surveillance for foodborne disease outbreaks began in 1973 (7). The production and consumption of leafy greens in the U.S. has increased steadily as well (12). In 12,714 documented foodborne outbreaks occurring in the U.S. during 1973–2012, 606 (about 5%) implicated a leafy vegetable, resulting in 20,003 illnesses, 1,030 hospitalizations, and 19 deaths (7). Between 1973-2012 Shiga toxin-producing *E. coli* O157:H7 and *Salmonella* were the most common bacteria implicated in foodborne outbreaks associated with leafy greens (7). *E. coli* O157:H7 accounted for 49 leafy vegetable-related outbreaks, 1,634 hospitalizations, and 450 deaths. *Salmonella* was associated with 32 leafy vegetable-related outbreaks,

1447 hospitalizations and 83 deaths during this period (7). *Listeria monocytogenes* can also be transmitted through raw fruits and vegetables, and has been isolated from packaged lettuce (165, 166, 167). The ability of *L. monocytogenes* to survive and grow under a wide range of environments and at low temperatures makes it also of concern in such foods (116). The CDC reported that of all tracked foodborne pathogens, *L. monocytogenes* had the second highest case fatality rate (21%) during 2009-2011 (168). Listeriosis almost always occurs in people considered to be at higher risk, such as the elderly and those who have a preexisting illness that reduces the effectiveness of their immune system (169, 170). The most recent multistate outbreaks in the USA, linked to consumption of whole cantaloupes also indicates that this pathogen may pose a serious microbiological hazard in other plant foods like leafy greens (171).

The limited shelf-life of fresh processed leafy greens is one of the greatest problems faced by commercial marketers (172). The shelf-life of leafy greens depends on type, cultivation process, maturity at harvest, environmental conditions after harvest, among others, but temperature is the most critical postharvest factor affecting shelf-life (163). The shelf-life of leafy greens ranges from less than a week to three weeks, depending upon variety and storage temperature (173). Poor temperature control during distribution from results in deterioration of a fresh appearance and odor, including browning, wilting, and off-odor (174). Tissue browning in leafy greens is a typical problem, easily detected by consumers, and is a

commonly studied defect (175). The color of leafy greens depends on many factors and when it is lost, chlorophyll and carotenoid degradation takes place, which results in browning. Browning of chopped surfaces is aesthetically unattractive and is due to oxidative reactions of phenolic compounds (176).

The specific objectives of this study were: (i) to estimate the upper limit of temperature to be maintained throughout the supply chain of leafy greens in order to minimize refrigeration cost, (ii) limit the microbial risk, and (iii) control the loss of sensory qualities. We also develop a modeling tool to integrate the results for different levels of microbial growth and sensory quality losses.

5.3 Materials and methods

5.3.1 Growth models

5.3.1.1 Primary models for growth

The exponential growth phase (log-phase) of the three-phase linear model (91) was used as the primary growth model because of its simplicity and wider application. The three phase linear model fits lag phase, log phase, and stationary-phase as straight lines. Equation 1 represents the log phases of the three-phase linear model. As a conservative approach, lag-phase was not considered in this study.

$$log\, N_t = log\, N_o + \frac{\mu}{2.303} \times t \qquad \ldots(1)$$

Where, N_t = cell concentration (CFU g^{-1}) at time t; N_o = initial cell concentration (CFU g^{-1}); t = time (h); μ = specific growth rate (ln CFU g^{-1} h^{-1}).

5.3.1.2 Secondary model for growth

Square-root model was selected as the secondary growth model (94).

$$\sqrt{\mu} = b(T - T_{min}) \quad \ldots(2)$$

In Equation 2, μ is specific growth rate mentioned in Equation 1; b is the temperature coefficient, T is food temperature (°C) and T_{min} is the theoretical minimum temperature (°C) for growth of pathogens. The values of b and T_{min} are dependent on the types of pathogens and food products. These parameters for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* were taken from different studies reporting the square-root models corresponding to the three-phase linear model (**Table 5.1**).

Table 5.1 Parameters for square-root model for exponential growth of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in leafy greens

Pathogen	**b**	**T_{min}**	**Reference**
E. coli O157:H7	0.023	1.20	(61)
Salmonella	0.020	-0.57	Chapter 3
L. monocytogenes	0.023	0.60	Chapter 3

5.3.2 Death model

A log-linear death model was used for gradual inactivation of *E. coli* O157:H7 and *Salmonella* that may occur at lower temperatures (≤ 5°C). Since *L. monocytogenes* is known to survive 3°C (116, 130, 139), it was modeled to grow at temperatures higher than 3°C, and survive (i.e. no change in concentration) in the temperature range of 0-3°C.

$$log\left(\frac{N_t}{N_o}\right) = -\frac{k}{2.303} \times t \quad \ldots(3)$$

Where, *k* is die-off coefficient in ln CFU h^{-1}. The mean die-off coefficient for *E. coli* O157:H7 was reported as 0.013 ln CFU/h below the storage temperature of 5°C (61). Die-off coefficient of *Salmonella* was reported as 0.0128 ln CFU/h at temperature below 5°C (Chapter 3).

5.3.3 Growth-death model

A dynamic growth-death model used by McKellar and Delaquis (61) and Zeng et al. (8) was also applied to simulate the growth of *E. coli* O157:H7. The model was used to predict the growth and death of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*.

$$\frac{dN_t}{dt} = Rate * N_t \quad \ldots(4)$$

For *E. coli* O157:H7,

$$Rate = (if\ T \geq 5, Growth\ (\mu), Death(k)) \quad \ldots(5)$$

For *Salmonella*,

$$Rate = (if\ T \geq 7, Growth\ (\mu), (\ if\ T > 5, 0, Death\ (k))) \quad \ldots(6)$$

For *L. monocytogenes*,

$$Rate = (if\ T \geq 3, Growth\ (\mu), 0) \quad \ldots(7)$$

5.3.4 Relative cooling cost

The cost of cooling during transportation and storage is directly related to the temperature. The coefficient of performance (COP) for refrigeration can be used to determine the cooling cost, as shown in Equation 8 (149):

$$COP = \frac{Desired\ output}{Required\ input} = \frac{Q_R}{W} = \frac{T_R}{T_A - T_R} \quad \ldots(8)$$

Where, Q_R is the heat transferred to a high temperature environment (air) from a lower temperature environment (refrigerator) (kWh), W is the input energy (kWh), T_A and T_R are higher and lower environmental temperatures (Kelvin or K), respectively. The refrigeration cost for 0°C refrigeration temperature was assumed to be one unit, i.e., 1. The costs for other refrigeration temperatures were calculated with

respect to this unit cost. For example, if the ambient temperature (T_A) is 293 K (20°C), refrigeration temperature (T_R) is 273 K (0°C), COP=273/(293-273)=13.65. For every unit of energy drawn from the electrical source, the coolant will absorb 13.65 units of heat from the refrigerator. We can calculate refrigeration costs for other temperatures on the basis of this unit cost. For example, the COP for 283 K (10°C) refrigeration temperature and 293 K (20°C) ambient temperature will be 283/(293-283)=28.3, and the relative cost will be 13.65/28.3=0.48.

5.3.5 Changes in sensory quality attributes

Major visual and quality changes that take place in leafy greens are loss of freshness in the general appearance and development of wilting, browning, and off-odor. One of the most commonly used models for sensory quality changes is the Arrhenius equation (9).

$$\pm\frac{dQ}{dt} = k_Q[Q]^n \quad \ldots(9)$$

Where, $k_{q(T)} = k_o exp\left(\frac{-E_a}{RT}\right)$ …(10)

Where Q is the score given for sensory quality attributes, t is time (in days), n is reaction order (n=1, i.e., the first order equation (9)), and k_q is quality change rate constant for the attribute. The pre-exponential factor (k_o) is the magnitude of the reaction rate independent of temperature and the activation energy (E_a) describes

temperature sensitivity of the reaction. The + sign refers to attributes with increasing values with respect to time (e.g. browning, off-odor and wilting), whereas, the – sign refers to decreasing values (e.g. quality of appearance).

Changes in appearance, wilting, browning and off-odor were predicted using the Arrhenius Equation. The information provided by Piagentini et al. (2005) was used to represent the changes in sensory quality attributes. Activation energy values for three fresh-cut leafy greens (fresh-cut iceberg lettuce, romaine lettuce and fresh-cut chicory) was as given by Piagentini et al. (2005) . Pre-exponential factors k_o were calculated in Microsoft Excel (Microsoft, 2010) for four sensory quality attributes for three kinds of leafy greens based upon the given activation energies and rate constants.

5.3.6 Nonlinear programming (NLP)

Nonlinear programming (NLP) is the process of solving an optimization problem defined by a system of equalities and inequalities, collectively termed constraints, over a set of unknown real variables, along with an objective function to be maximized or minimized, where some of the constraints or the objective function are nonlinear (74). The objective function minimized in this study was cost. The constraints were growth of pathogens (*E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*), and the loss of sensory characteristics of leafy greens.

5.3.7 Optimization of temperature for leafy greens

Two hypothetical standards (lenient and stringent), were set for sensory quality and microbial safety of leafy greens. The lenient standards were set to allow maximum 1 log CFU/g growth of any of three pathogens, and retention of at least 50% in all four sensory quality attributes. The limit of 1-log growth is consistent with recommendations from expert microbiologists (151, 159, 177). Less than a 1-log increase above the contamination level throughout the intended shelf life of the product and across replicate trials would be an appropriate acceptance criterion in determining whether a product supports growth of a pathogen throughout the supply chain. This level reflects the inherent variation that exists with enumeration of microorganisms (177). Retention of 50% sensory quality attributes as the end of useful shelf life was as suggested from Piagentini et al., (9). While a 50% quality loss does not mean leafy greens will not be consumable at this point, it is useful as a relevant metric. A more stringent standard was also considered for the three leafy greens under study (pathogen growth ≤ 0.3 log CFU g^{-1}, and sensory quality $\geq 75\%$ of original). A lower limit of 0°C was set for the optimized temperatures, as storage at temperatures lower than 0°C may result in freezing injuries to leafy greens.

5.3.8 Development of graphic-user interface (GUI) for calculations

A GUI named "SHELF" (**Figure 5.1**) was developed in MATLAB (MathWorks, 2015b). There were three components of SHELF: an input panel, a

result summary panel, and a panel for graphs for changes in sensory properties and microbial load with respect to time. The input panel records desired shelf-life (in days), the ambient temperature (°C), maximum allowed growth of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* (log CGU g^{-1}), and minimum percent retention of sensory quality parameters (general appearance, browning, wilting, and off-odor) from the user. Ambient temperature does not impact optimized temperature, but was used in cost calculations (Equation 4). As noted above, retention of sensory quality was as estimated by sensory scores from the published literature (9). Initial sensory score was set at 100%, and decline in sensory score was modeled using first order Arrhenius equation (Equations 9 and 10). Growth of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* were predicted using the growth and death models. The result summary panel shows the optimized temperature for the specified conditions, and the calculated refrigeration cost relative to the cost for refrigeration temperature of 0°C. This panel also gives the predicted change in pathogen concentration and decline in leafy greens' sensory properties at the end of the desired shelf-life. The graph panel depicts the predicted change in pathogen concentration and sensory attributes over time.

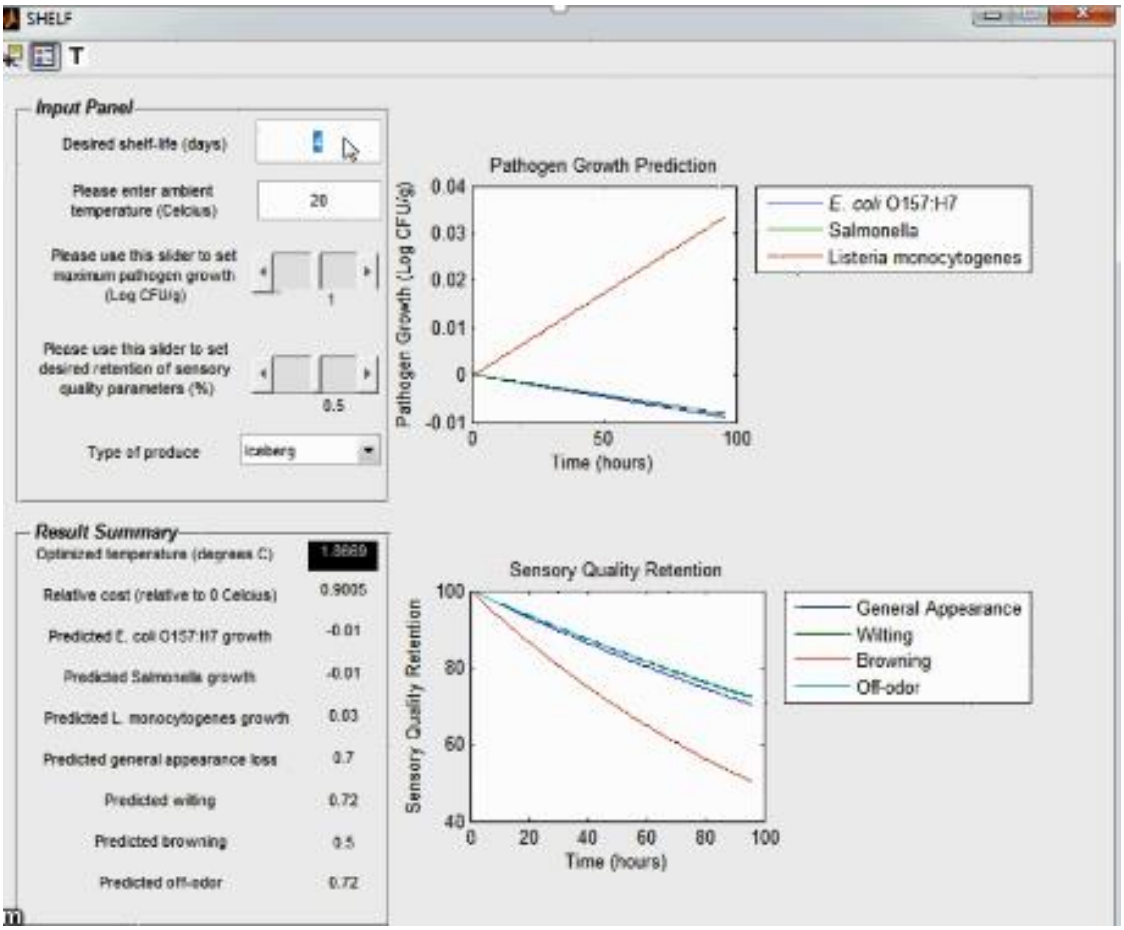

Figure 5.1 Different components of the graphic-user interface "SHELF" used for the calculations of optimum refrigeration temperature during leafy greens storage.

5.4 Results

5.4.1 Parameters for Arrhenius equation for sensory quality changes

The values for pre-exponential factor k_o and activation energy E_a are shown in **Table 5.2**. For fresh-cut iceberg and romaine lettuce, the E_a values for browning were lower than those for other sensory quality parameters. Lower activation energy for

sensory quality changes indicates the ease with which a food's sensory quality degrades (186). The overall rate of a foods sensory quality degradation is determined by the mixed effect of activation energy and pre-exponential factor.

Table 5.2 Pre-exponential factor (k_o) and activation energy (E_a) values for sensory quality attributes (general appearance, browning, wilting, and off-odor) for fresh-cut iceberg lettuce, fresh-cut romaine lettuce, and fresh-cut chicory.

	Fresh-cut Iceberg lettuce		**Fresh-cut Romaine lettuce**		**Fresh-cut chicory**	
Parameter	**k_o**	**E_a**	**k_o**	**E_a**	**k_o**	**E_a**
General appearance	3.03×10^{12}	71.1	1.35×10^{12}	69.6	1.21×10^{11}	65.7
Wilting	4.43×10^{11}	66.9	1.39×10^{11}	65.1	1.42×10^{16}	92.9
Browning	1.91×10^{8}	47.5	4.22×10^{6}	39.6	8.89×10^{11}	69.0
Off-odor	6.58×10^{8}	65.3	6.58×10^{8}	51.4	3.38×10^{16}	81.2

k_o: pre-exponential factor

E_a: Activation energy (J mol^{-1})

5.4.2 Optimization of temperature for leafy greens

Optimized temperature and relative costs were calculated for fresh-cut iceberg, romaine lettuce, and fresh-cut chicory under (i) lenient, and (ii) standards. Ambient temperature was taken as 20°C (68°F) for calculation of relative costs at different refrigeration temperatures. As expected, the relative cost increased as the optimized temperature was close to zero (**Table 5.3** and **5.4**). Because leafy greens suffer from

freezing injuries at temperatures lower than -0.5°C (178), optimized temperatures below -0.5°C are not reported.

Table 5.3 predicts that fresh-cut iceberg lettuce will meet the lenient standards for up to four days. A 1-log increase in the concentration of *L. monocytogenes* was the constraining variable for 1 and 2 days shelf-life of fresh-cut iceberg lettuce, whereas browning was the constraint for three and four day shelf-life of fresh-cut iceberg lettuce. The models predict that fresh-cut romaine lettuce can be stored for up to six days. Growth of *L. monocytogenes* would be the key constraint for the shelf-life of fresh-cut romaine lettuce with 1 and 2 day shelf life. *Salmonella* growth was predicted to be the key constraint for fresh-cut romaine lettuce with a target shelf-life of three and four days. Browning becomes the key concern for fresh-cut romaine lettuce with a target shelf-life of five days. The models predict that fresh-cut chicory can be kept under the lenient standards for up to 11 days. The limiting constraints fresh-cut chicory are the same as those for fresh-cut romaine for a desired shelf-life of up to four days. Off-odor was predicted to be the primary shelf-life concern for fresh-cut chicory with a target shelf life from five to eleven days.

Predictions for shelf life under the more stringent standards are shown in **Table 5.4**. Fresh-cut iceberg lettuce could not be stored for more than one day, and growth of *Salmonella* would be the primary concern for one-day storage (**Table 5.4**). Fresh-cut romaine lettuce could be stored for up to two days and still meet the more stringent standards. As with fresh-cut iceberg lettuce, growth of *Salmonella* is the

limiting constraint for one day of storage for romaine lettuce, whereas browning would be of primary concern for storage of two days for romaine lettuce. Fresh-cut chicory was predicted to meet the stringent standards for up to four days of storage. *Salmonella* and *L. monocytogenes* were the limiting constraints for chicory under the stringent standards for one and two days, respectively, while off-odor was the limiting constraint for three or four days.

5.5 Discussion

Our models predict that iceberg and romaine lettuce can be stored for up to four and six days, respectively, under the more lenient standards and the results are in close agreement with the studies previously conducted on the shelf-life of minimally processed leafy greens (165, 179, 180). Browning was predicted as the sensory quality attribute of maximum concern for iceberg and romaine lettuce at the longer shelf lives. Low temperatures are assumed to preserve sensory quality by slowing down all leaf metabolism. Color changes might be due to senescence (process of deterioration with age). The senescence usually leads to leaf browning as observed in leafy vegetables (181, 182).

Table 5.3 Optimized temperature (°C) for leafy greens for lenient standards (maximum permissible pathogen growth = 1 log CFU/g; minimum sensory quality retention = 50% of original)

Type of leafy green	Desired shelf life (days)	Optimized temperature (˚C)	Relative cost	Pathogen growth (log CFU/g)			Sensory quality retention (% of original)			
				E. coli O157:H7	*Salmonella*	*L. monocytogenes*	General appearance	Wilting	Browning	Off-odor
Fresh-cut iceberg lettuce	1	14.4	0.27	0.92	0.90	**1.00**	71	75	65	75
	2	10.3	0.47	0.88	0.96	**1.00**	64	68	53	68
	3	5.7	0.70	0.33	0.00	0.42	66	69	**50**	69
	4	1.9	0.90	-0.01	-0.01	0.00	70	72	**50**	72
Fresh-cut romaine lettuce	1	14.8	0.27	0.92	0.90	**1.00**	75	82	77	75
	2	10.3	0.47	0.88	0.96	**1.00**	69	77	66	66
	3	8.4	0.56	0.85	**1.00**	0.99	63	72	58	58
	4	7.2	0.62	0.78	**1.00**	0.94	58	68	51	52
	5	3.9	0.79	-0.01	-0.01	0.29	62	71	**50**	53
	6	1.0	0.95	-0.01	-0.01	0.00	66	73	**50**	55

Fresh-cut chicory	1	14.4	0.27	0.92	0.90	**1.00**	88	84	78	69
	2	10.3	0.47	0.88	0.96	**1.00**	84	82	73	64
	3	8.4	0.56	0.85	**1.00**	0.99	80	80	67	58
	4	7.2	0.62	0.78	**1.00**	0.94	77	78	63	54
	5	6.3	0.67	0.70	0.00	0.87	74	76	59	**50**
	6	4.8	0.75	-0.01	-0.01	0.58	74	76	58	**50**
	7	3.6	0.80	-0.02	-0.01	0.34	73	77	58	**50**
	8	2.6	0.87	-0.02	-0.02	0.00	72	77	57	**50**
	9	1.7	0.91	-0.02	-0.02	0.00	72	77	56	**50**
	10	0.8	0.95	-0.02	-0.02	0.00	71	78	56	**50**
	11	0.1	0.99	-0.02	-0.02	0.00	71	78	55	**50**

Table 5.4 Optimized temperature (°C) for leafy greens for stringent standards (maximum permissible pathogen growth = 0.3 log CFU/g; Minimum sensory quality retention = 75% of original)

Type of leafy green	Desired shelf life (days)	Optimized temperature (˚C)	Relative cost	Pathogen growth (log CFU/g)			Sensory quality retention (% of original)			
				E. coli O157:H7	*Salmonella*	*L. monocytogenes*	General appearance	Wilting	Browning	Off-odor
Fresh-cut iceberg lettuce	1	8.1	0.58	0.25	**0.30**	0.29	84	86	76	86
Fresh-cut romaine lettuce	1	8.1	0.58	0.25	**0.30**	0.29	86	90	84	84
	2	4.5	0.76	0.00	0.00	0.16	82	86	**75**	77
Fresh-cut chicory	1	8.1	0.58	0.25	**0.30**	0.29	93	93	88	84
	2	5.9	0.69	0.24	0.00	**0.30**	89	90	82	77
	3	3.4	0.82	-0.01	-0.01	0.12	88	90	79	**75**
	4	1.1	0.94	-0.01	-0.01	0.00	87	90	79	**75**

Our predicted shelf-life of leafy greens was similar to that predicted by Piagentini et al. (9) which is not surprising, since we used their data. For instance, we predicted maximum possible shelf-life of fresh-cut iceberg, romaine lettuce and fresh-cut chicory as 4, 6, and 11 days, respectively (assuming lenient standards), as compared with 6.4, 7.2 and 9.2 days, respectively predicted by Piagentini et al. (9).

Fresh-cut chicory was predicted to have a considerably longer shelf-life as compared with fresh-cut iceberg and romaine lettuce, which is not surprising as the behavior of different leafy greens varies in terms of their response to temperature abuse (9). For example, Allende et al. (165) reported spinach samples with shelf-life of two days at 20°C, four days at 16°C, six days at 12°C, eight days at 8°C, and ten days at 1°C.

Product's shelf life stage significantly affects its response to temperature. Quality deterioration proceeded more rapidly when temperature abuse occurred in late, as opposed to early, shelf-life stage (183, 184). The observed acceleration of the detrimental effect of temperature abuse occurring at late shelf life stage may be associated with the physiological condition of the products. In the late stages of product shelf life (after 6 d of storage), the product has already partially senesced, stored carbohydrates have been consumed, cell wall disassembly has progressed, thus the leaves become fully senesced more rapidly once exposed to elevated temperatures (183).

The Arrhenius equation we used has also been widely used by others as the basis for predicting loss of food quality or storage life in many processed foods as a function of storage temperature (9, 149, 185). The primary assumption made in using the Arrhenius equation is that the rate of quality loss is an exponential function of the reciprocal of absolute storage temperature (185). In the Arrhenius equation, as shown in equation 10 above, the pre-exponential factor is the magnitude of reaction rate independent of temperature and the activation energy describes temperature sensitivity of the reaction.

The FDA recommends that leafy greens should be maintained at 5°C or below in transport, storage and retail display in the 2013 Food Code (152, 184). There is also a practical lower temperature limit since storage of leafy greens at temperatures that are too low may result in freezing injuries. Freezing injury might result from transport in cold geographical regions, incorrect thermostat settings, refrigeration break-down or a lack of air circulation in refrigerated storage. One common symptom of freezing injury is the presence of water soaked damaged leaves. Leafy vegetables do not have as many dissolved sugars as fruits, so leafy vegetables freeze at -0.5°C, compared to fruits which can freeze between -2°C and -5°C (178). Therefore our report only includes predictions with an optimized temperature of -0.5°C and above.

5.6 Conclusions

There is considerable variability in the shelf-life of different leafy greens stored at the same temperature. Fresh-cut iceberg lettuce has the shortest shelf-life

among the leafy greens studied, followed by fresh-cut romaine lettuce and fresh-cut chicory. Pathogen growth is of greater concern than loss of sensory quality for fresh-cut iceberg lettuce with a target shelf-life of up to two days. Conversely, browning is a greater concern for fresh-cut iceberg lettuce with a desired shelf-life of more than two days. Pathogen growth is a greater concern than loss of sensory quality for fresh-cut romaine lettuce and fresh-cut chicory, with a desired shelf-life of up to four days. Browning and off-odor development are major concerns for fresh-cut romaine and chicory, respectively when target shelf-life is five days or more.

Chapter 6. Development of a system model to understand the role of animal feces as a route of contamination of leafy greens before harvest

6.1 Abstract

Leafy vegetables have been identified as the fresh produce commodity group of highest concern from a microbiological safety perspective. A majority of foodborne outbreaks in the U.S. associated with the consumption of leafy greens contaminated with *E. coli* O157:H7 have been reported during July-November. A dynamic system model consisting of subsystems and inputs to the system (soil, irrigation, cattle, wild pig, and rainfall) simulating a hypothetical farm was developed. The model assumed two crops of lettuce in a year, and simulated planting, irrigation, harvesting, ground preparation for the new crop, contamination of soil and plants, and survival of *E. coli* O157:H7. The concentrations of *E. coli* O157:H7 in the crops harvested in different months as predicted by the baseline model for conventional fields estimated that 11 out of 221 (4.98%) first crops harvested in July will have at least one plant with more than 1 CFU of *E. coli* O157:H7. The maximum *E. coli* O157:H7 concentration in a plant was higher in second crop (150 CFU) than in first crop (113 CFU), with the probability of having at least one plant with more than 1 CFU of *E. coli* O157:H7 in a crop predicted as 21/253 (8.3%), 4/333 (1.2%), 11/307 (3.58%), and 6/105 (5.71%) in August, September, October, and November,

respectively. For organic fields, the probabilities of having at least one plant with more than 1 CFU of *E. coli* O157:H7 in a crop (3.9%) were predicted to be higher than those for the conventional fields (2.65%).

6.2 Introduction

Total production of iceberg, romaine, and leaf lettuce in the U.S. was 72,103,000 cwt (3.66 billion kg) in 1984, which increased to 90,488,000 cwt (4.59 billion kg) in 2009 (5). California is the largest producer of leafy greens, producing about 72,173,000 cwt (3.67 billion kg) iceberg, romaine, and leaf lettuce in 2009 (5). A large majority of the salad greens consumed in the U.S. are grown within the Salinas Valley region in California, west of the San Joaquin Valley and south of San Francisco Bay. Lettuce, spinach, tomatoes, and strawberries are the dominant crops in this region and other crops include broccoli, cauliflower, wine grapes, and celery (187). This region has also been associated with production of leafy greens implicated in several *E. coli* O157:H7 outbreaks in recent years, causing a reported 395 illnesses during 1999-2008. Most (90.9%) of the outbreaks associated with consumption of leafy greens produced in the Salinas Valley during 1999-2008 and contaminated with *E. coli* O157:H7 were reported in only 5 months (July-November), except one outbreak in May (104, 188, 189, 190, 191).

The pathogen pathway in a pre-harvest leafy greens production system is complex, but may be understood using a system approach. A systems approach envisions a “system” as comprised of various subsystems which interact with each

other continuously (192). A typical system model for leafy greens farm may include various subsystems, such as soil, irrigation water, manures, fertilizers, and plants. The inputs to this system that can affect some of these subsystems are climatic factors (precipitation, temperature, wind speed, wind direction, interaction of domestic domestic/wildlife animals, birds, insects, and humans with the crop). Recently, a review by Liu et al. (193) discussed the impact of climatic factors (temperature and precipitation) on contamination sources (manure, soil, surface water, sewage, and wildlife) and pathways of foodborne pathogens in pre-harvest leafy greens production systems. This paper develops a system model of a hypothetical leafy greens farm in Salinas, California. The model simulates the effect of feral swine and cattle feces as a source of contamination in leafy greens.

6.3 Materials and methods

A dynamic system model representing a lettuce production field in Salinas Valley was developed in MATLAB software (The MathWorks Inc., Natwick, MA, ver. 2015b). The subsystems considered in this system were soil and plant. Inputs to the system model that affect the subsystems are irrigation, cattle and wild pigs in and around the field, rainfall, irrigation, and seasonal effects (**Figure 6.1**). The harvested crop is the output of this system, whereas the contamination in the soil at the time of harvest is the feedback, i.e., it affects the soil conditions for the next crop. Details of various subsystems, data, and equations used in this model are given in the following subsections.

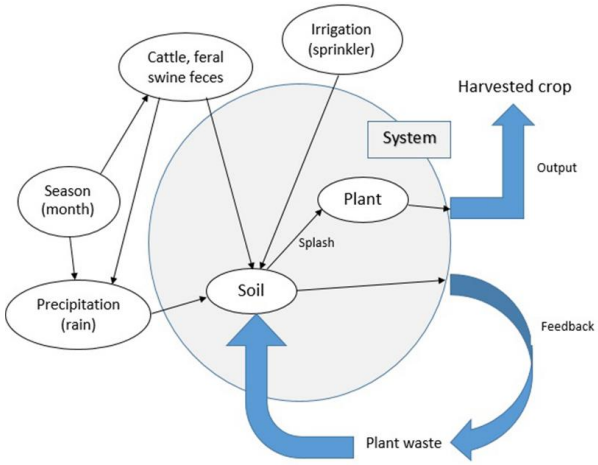

Figure 6.1 Schematic diagram of the system model developed in this study.

6.3.1 Field specifications

A square 1-acre field was assumed for all calculations. Lettuce plants are generally spaced at 30-40 cm (12-16 inches) within the row and 45-76 cm (18-30 inches) between rows (194). Spacing between plants in a row was assumed to be 30 cm (12 inches), and spacing between rows was assumed to be 50 cm (20 inches) in this study. A 1-acre field (63 m × 63 m), would contain 208 plants in a row, and 125 rows in the field, totaling 26,000 lettuce plants in the field. The field was divided into 26,000 subplots (50 cm long and 30 cm wide), each subplot corresponding to the area surrounding a lettuce plant, and any contamination in a subplot was assumed to affect only the plant in the corresponding subplot.

6.3.2 Agricultural timeline

The model assumed two crops of romaine lettuce in a year. Planting was assumed to occur randomly any time between the last week of January and end of April, and between the second week of May and third week of August, respectively (195), as shown in **Figure 6.2**. Time for growth of plants was assumed to be 80-90 days (196). Simulated sprinkler irrigation occurred during the growth period at intervals of 7 days. Simulated irrigation water was assumed to be deep well groundwater (197). Ground preparation takes 3-4 weeks between first and second crop, and the leafy greens fields in Salinas are left uncropped during winter, i.e. between the second crop and next year's first crop (195). Soil was assumed to be properly mixed as a result of plowing, therefore, all the bacteria in different subplots were averaged after the ground preparation operation.

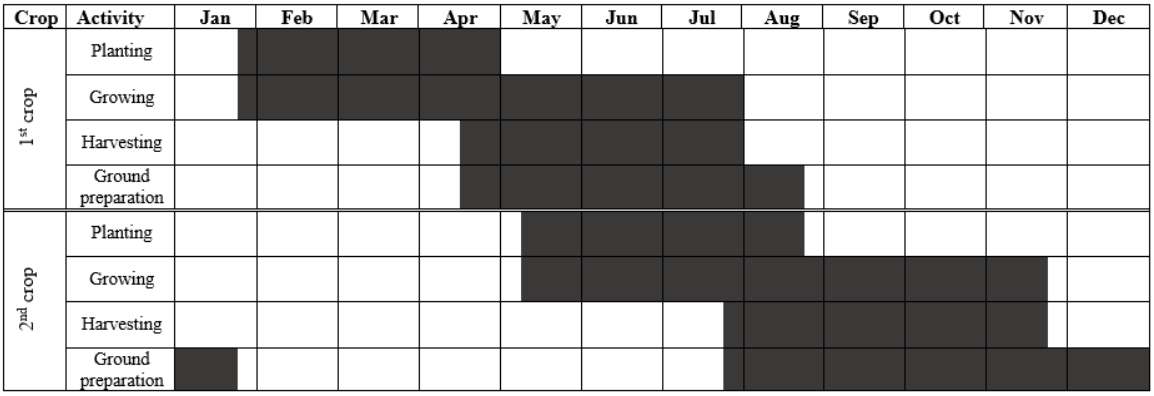

Figure 6.2 Timeline for cropping practices for lettuce in Salinas Valley, California (assuming two crops in a year).

6.3.3 *E. coli* O157:H7 in wild pig and cattle feces

E. coli O157:H7 infection and shedding occurs frequently among cattle and pigs (34, 35, 198, 199). Cooley et al. (200) estimated month-wise prevalence of *E. coli* O157 in samples of cattle and pig feces in the areas surrounding Salinas, including Monterey, San Benito, and San Luis Obispo Counties based on data from 33 farms collected during April 2008-October 2010. These authors estimated their detection limit as 78 CFU of *E. coli* O157:H7 per 10 grams, or 0.89 log CFU/gram. Concentration of *E. coli* O157:H7 in feces (log CFU/g) was assumed to be lognormally distributed. Distribution fitting was performed in @Risk software (Palisade Decision Tools, ver. 6.0) using RiskNormalAlt function, which defines a normal distribution based on two percentiles and their corresponding values. The detection limit (0.89 log CFU/gram) was taken as the lower point for defining the normal distribution. *E. coli* O157:H7 concentration is highly variable in pig and cattle feces, and can range up to 7 log CFU/g in pig feces (198), and 8.4 log CFU/g in cattle feces (35). The maximum values were used as higher point corresponding to the 99.99 percentile in the normal distribution. For example, if the prevalence of *E. coli* O157 in cattle feces is 21.34% in July (78.66% samples were detected negative), the normal distribution for concentration (log CFU/g) of *E. coli* O157:H7 in cattle feces was defined by using the function RiskNormalAlt (78.66%, 0.89, 99.99%, 8.4).

Table 6.1 Month-wise precipitation and normal distribution parameters (mean, standard deviation) for *E. coli* O157:H7 contamination (log CFU/g) in pig and cattle feces

Month	*E. coli* O157:H7 contamination in Pig feces (Mean, Standard deviation) log CFU/g	*E. coli* O157:H7 contamination in Cattle feces (Mean, Standard deviation) log CFU/g	Average precipitation reported (cm)	Normalized precipitation
January	-29.13, 9.72	-10.62, 5.12	5.10	0.86
February	-29.13, 9.72	-7.07, 4.16	5.46	0.92
March	-29.13, 9.72	-25.99, 9.25	5.76	0.97
April	-29.13, 9.72	-3.42, 3.18	2.87	0.48
May	-29.13, 9.72	-6.15, 3.91	0.81	0.14
June	-29.13, 9.72	-36.01, 11.95	0.25	0.04
July	-1.26, 2.22	-1.15, 2.57	0.01	0.00
August	-2.10, 2.44	-3.19, 3.11	0.10	0.01
September	-29.13, 9.72	-3.62, 3.23	0.20	0.03
October	-2.75, 2.62	-1.68, 2.71	1.67	0.28
November	-2.35, 2.51	-6.75, 4.07	2.94	0.50
December	-2.13, 2.46	-8.68, 4.59	5.91	1.00

6.3.4 Modes of contamination of soil

Contamination of soil by cattle and wild pig feces was considered to take place by two ways: direct defecation by wild pigs in the field, and runoff of cattle and

wild pig feces into the field due to rainfall. Since animal feces can harbor human pathogenic microorganisms, pathogen transmission from livestock and wildlife faces to crops and farms from direct deposition, water runoff events or other routes increases risks to human and animal health (201). The strain of *E. coli* O157:H7 linked to the 2006 spinach outbreak was also found in the feces of wild pigs roaming in the Salinas Valley (202). Bacteria can be widely disseminated in soil as a result of water currents and rain runoff carrying contaminated material (203). Runoff from livestock areas has been reported as an important source of microbial contamination of water bodies and agricultural fields (201).

6.3.5 Precipitation

Historical data (January 2005-December 2014) on precipitation for Salinas, California was accessed from National Oceanic and Atmospheric Administration (NOAA, http://www.noaa.gov/). Maximum precipitation occurred during winters; in December, there was maximum average monthly precipitation of 5.91 cm. Summers and early fall months (June-September) are dry, with minimum average monthly precipitation in July (0.01 cm). During 2005-2014, no precipitation was reported in July with the exception of 2005 and 2011, when only small amount of precipitation (< 0.1 cm) was reported, therefore, runoff in July was not assumed in the model. Assuming there would be no runoff in July and maximum runoff in December, precipitation for each month was normalized to scale the range of precipitation in 0 to 1 for further calculations (204).

$$Prec_{norm} = \frac{Prec_m - Prec_{min}}{Prec_{max} - Prec_{min}} \quad \ldots(1)$$

where, $Prec_{norm}$ is the normalized value of precipitation for a month, $Prec_m$ is the observed precipitation for the month, $Prec_{min}$ is minimum average precipitation in any month over the entire dataset (July, 0.01 cm), and $Prec_{max}$ is maximum average precipitation in any month (December, 5.91 cm). The normalized values of precipitation were used to estimate the daily amount of animal feces contaminating the field with the precipitation runoff coming to the field for each month according to Equation 2.

$$PC_d = PC_{dmax} \times Prec_{norm} \quad \ldots(2)$$

Where, PC_d is the amount of animal feces coming to the field with the precipitation runoff in one day, and PC_{dmax} is maximum possible amount of animal feces coming to the field with the precipitation runoff in one day.

6.3.6 Survival models

6.3.6.1 Soil

A log-linear survival model was used to calculate the survival of *E. coli* O157:H7 in the soil. Ma et al. (205) experimentally determined the kinetics of survival of *E. coli* O157:H7 in organic and conventional soils in Salinas by initially contaminating the soil with 5×10^6 log CFU/g. The bacterial population reached the

level of 100 CFU/g in 31.1 days in organic and 28.1 days in conventional soil. This information was used to estimate the log-linear model for survival of *E. coli* O157:H7.

$$\log(N_t) = \log(N_o) - kt \quad \ldots(3)$$

where, N_t is number of survivors per gram at time t (time, in days post contamination), N_o is contamination size per gram, and k is death-rate (log $CFUg^{-1}$ day^{-1}).

6.3.6.2 Lettuce

Erickson et al. (23) conducted experiments to determine the survival of *E. coli* O157:H7 when the bacteria were applied through spray irrigation water to field-grown lettuce. Deactivation data for abaxial spray treatment from Erickson et al. (23) was used to fit the survival model (log-linear) of surface and internalized *E. coli* O157:H7 in lettuce leaves after spray application of contaminated irrigation water using Equation 3.

6.3.7 Transfer of *E. coli* O157:H7 from soil to lettuce

Atwill et al. (43) determined the proportion of *E. coli* O157:H7 transferred from contaminated soil to adjacent heads of lettuce plants because of splash from foliar irrigation. These authors initially contaminated the surrounding soil of each lettuce plant with 5 grams of feces of average spiked load of 1.29×10^8 CFU of *E. coli* O157:H7. After foliar irrigation 61.9% (104/168) samples tested negative for the

pathogen. Of the samples testing positive, 55 samples (32.7%) were positive using low concentration enumeration assays, ranging from 1.3 CFU to 340 CFU per lettuce head, and 9 samples (5.4%) tested positive using high concentration enumeration assay with the maximum concentration of 2.30×10^5 CFU per head. A piecewise linear function was developed in MATLAB to calculate the transfer ratio of *E. coli* O157:H7 from soil to lettuce. For each iteration, a random number (RN) between 0 and 1 was generated, and the Transfer Ratio was calculated using Equations 4 and 5.

$$transfer = \frac{RN-0}{0.619-0} \times 1.3 \qquad \text{for } 0 \leq RN \leq 0.619 \qquad (4a)$$

$$transfer = 1.3 + \frac{RN-0.619}{0.946-0.619} \times (340 - 1.3) \qquad \text{for } 0.619 \leq RN \leq 0.946 \qquad (4b)$$

$$transfer = 340 + \frac{RN-0.946}{1-0.946} \times (230000 - 340) \qquad \text{for } 0.946 \leq RN \leq 1 \qquad (4c)$$

$$Transfer\ Ratio = transfer/(1.29 \times 10^8) \qquad (5)$$

Where, *transfer* is predicted CFU of *E. coli* O157:H7 transferred to lettuce from feces with microbial load of 1.29×10^8 CFU; *Transfer Ratio* is the number of CFU of *E. coli* O157:H7 transferred to lettuce from microbial load of 1 CFU.

6.3.8 Models

Based on the assumptions discussed in the previous sections, five scenarios were modeled to simulate the pathway of *E. coli* O157:H7 in a lettuce field. Each scenario was run for 1,000 iterations, where each iteration represents a field in

Salinas. For each iteration, planting time, harvesting time, and ground preparation durations were randomly selected from the timeline shown in **Figure 6.2**. Level of *E. coli* O157:H7 in animal feces were selected randomly using the lognormal distributions for different months (**Table 6.1**). Within each iteration, separate calculations to calculate the contamination levels were performed for each of the 26,000 subplots. The survival model calculations were performed for each day of the year. This means that for contamination of soil in different subplots, the present model had 1,000×26,000×365 values. During irrigation, the bacteria transferred from each subplot to corresponding plant was calculated and survival model for *E. coli* O157:H7 in lettuce was used for each day before the next irrigation operation. The level of *E. coli* O157:H7 in each plant was calculated at the time of harvest.

6.3.8.1 Baseline model

The baseline model assumed intrusion of an average sized adult pig weighing 56-80 kg (125-175 pounds) into the field every day. Such a pig can defecate 9.4 pounds (4.26 kg) in the field per day (206). The simulated feces from the pig were scattered at 10 random subplots within the field, with each subplot getting equal amount (426 grams). Simulated runoff of cattle and wild pig feces from precipitation in a given month was directly proportional to the normalized value of rainfall for the corresponding month. One kg of feral swine and 10 kg of cattle feces were assumed to contaminate the field with the runoff every day in the month with maximum rainfall (December). The daily runoff in other months was calculated by multiplying

the values of 1 kg of wild pig and 10 kg of cattle feces by the normalized rainfall for the corresponding month (Equation 2). Thus no contamination from runoff occurred in July (the month with the least rainfall). Simulated contamination from runoff equally divided among the 26,000 subplots. The baseline model was assumed for a conventional field, i.e., survival of *E. coli* O157:H7 in the soil was calculated using the Equation 3 for conventional soil.

6.3.8.2 Organic baseline model

Organic baseline model was similar to the baseline model, except that the survival of *E. coli* O157:H7 in soil was predicted using the log-linear survival model of *E. coli* O157:H7 in the organic soil in Salinas (Equation 3).

6.3.8.3 Reduced baseline model

The reduced baseline model used the framework of the baseline model, except contamination was reduced by 90%, i.e., contamination from direct defecation from wild pigs was assumed to be 426 g/day, instead of 4260 g/day. Contamination from runoff was also one-tenth of the baseline model, e.g., 100 g of feral swine feces and 1 kg of cattle feces every day under maximum rainfall.

6.3.8.4 Local defecation model

Local defecation model was developed to understand the effect of direct defecation of wild pigs in the fields on the baseline model. The contamination of the

soil with animal feces because of runoff was not considered in this model, while other assumptions were kept same as the baseline model.

6.3.8.5 Runoff model

Contrary to the local defecation model, the runoff model was developed by eliminating the local defecation parameter from the baseline model. The purpose of this scenario was to understand the contamination of leafy greens field by the runoff carrying animal feces potentially contaminated with *E. coli* O157:H7, and subsequently estimating the concentration of this pathogen in the harvested leafy greens crops.

6.4 Results

6.4.1 *E. coli* O157:H7 population in leafy green field

Simulations were performed for all the five models in order to predict the contamination of lettuce field soil in Salinas throughout the year. **Figure 6.3** show the maximum, mean and minimum values of the average contamination of a subplot over 1,000 iterations. In the baseline model (**Figure 6.3a**), the maximum values of *E. coli* O157:H7 contamination were in the range of 0.5-1.7 log CFU per subplot from January to May. Concentration of *E. coli* O157:H7 in swine feces was very low during this period (**Table 6.1**), thus the contamination is supposed to occur due to the runoff of cattle feces into the field. The maximum, mean, and minimum values declined throughout the month of June and there was a sharp increase in July. The

decline in June could be attributed to the very low prevalence of *E. coli* O157:H7 in cattle and wild pig feces in June. The increase of contamination concentration in July was due to high prevalence of *E. coli* O157:H7 in wild pig feces. Although cattle feces had more prevalence of *E. coli* O157:H7 than wild pig feces, there was no impact of contamination due to cattle feces, because the rainfall was very low (0.006 inches) in the month of July, and hence the normalized value of rainfall for July was 0. The maximum values of average contamination in the field were around the level of 2 log CFU per subplot during July and August, and the high prevalence of *E. coli* O157:H7 in animal feces during these months corroborates this trend. In the month of September, the prevalence of *E. coli* O157:H7 in wild pig feces as well as the amount of runoff were very low, resulting in a decline of more than 1.5 log CFU per subplot of *E. coli* O157:H7 in the field. From October to December, the amount of rainfall gradually increased and the prevalence of *E. coli* O157:H7 in cattle feces decreased (**Table 6.1**). There was an increasing trend in the prevalence of *E. coli* O157:H7 in wild pig feces, but this increase was not very large (8.22, 9.88, and 10.90% samples positive in October, November and December, respectively). The cumulative result of all these factors was that overall the concentration of *E. coli* O157:H7 in the soil did not change considerably from October to December.

The contamination of *E. coli* O157:H7 in soil in the organic baseline model and reduced baseline model followed the similar trend as the baseline model throughout the year, and the contamination level for organic baseline model was about 0-1 log CFU per subplot higher than the concentration levels predicted for the

baseline model. The higher values in the organic baseline model are due to the fact that bacteria survive for a longer period in organic soil. The values predicted for the reduced baseline model were 1-2 log CFU per subplot lower than the values predicted for the baseline model.

For local defecation model (**Figure 6.3d**), the concentration of *E. coli* O157:H7 in the soil declined from January to June. This trend was observed because of very low prevalence of *E. coli* O157:H7 in wild pig feces during these months, thus, it is estimated that there would be very few cases when a large population of *E. coli* O157:H7 contaminated the soil, and the bacterial population during these months represents generally those bacteria that survived during this period. There was a sharp increase in *E. coli* O157:H7 concentration in July, which correlates with high prevalence of *E. coli* O157:H7 in wild pig feces in July. During July-December, there was noticeable change in the bacterial concentration in September, which can be understood by the fact that there was very low prevalence of *E. coli* O157:H7 in feral pig feces in September.

In the runoff model (**Figure 6.3e**), there was an increase in the *E. coli* O157:H7 contamination in the soil in February and April. This trend could be attributed to the increase in *E. coli* O157:H7 prevalence in cattle feces during these months. During June-July, there was a drop in the *E. coli* O157:H7 contamination in soil. During August-December, there was a slow increase in the maximum population of *E. coli* O157:H7 in the soil, which could be attributed to the increasing trend of rainfall during this period.

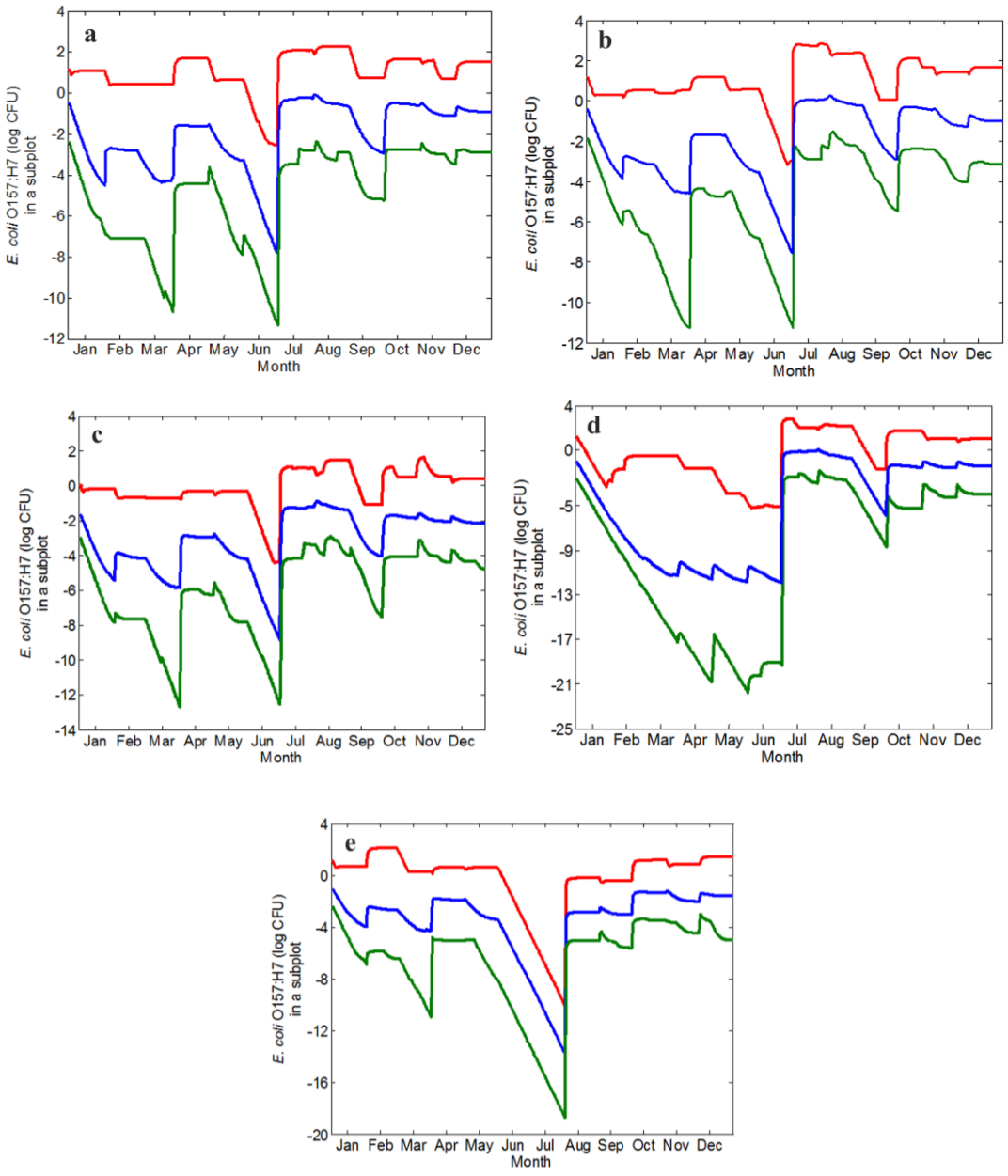

Figure 6.3 Daily predicted population of *E. coli* O157:H7 per subplot (20”×12”) for the (a) Baseline, (b) Organic baseline (c) Reduced baseline, (d) In-field defecation, and (e) Runoff model. Blue line represents mean population, red and green lines represent maximum and minimum populations for 1,000 iterations, respectively.

6.4.2 *E. coli* O157:H7 in the harvested crop

On the basis of the simulated model scenarios, a crop (the whole field with all plants) was considered to be contaminated if it had at least one plant contaminated with more than 1 CFU of *E. coli* O157:H7 at the time of harvest. The results (**Table 6.2**) are in close agreement with the contamination levels shown in **Figure 6.3 (a-e)**. For the baseline model, no contaminated crop was predicted during April-June. The occurrence of contaminated crops was more frequent in the second crops (42 contaminated crops) compared to the first crop (11 contaminated crops). This could be explained by the predictions that maximum levels of *E. coli* O157:H7 contamination in the soil were predicted low (less than 2 log CFU per subplot) from January to June, when more than 70% of the first crops were predicted to be harvested. In July, the maximum level of contamination in the field was 2.08 log CFU per subplot that resulted in an estimation of about 5% (11 out of 221) contaminated crops in July. The maximum level (P_{max}) of *E. coli* O157:H7 in any plant harvested in July was predicted to be 13 CFU (**Table 6.2**). About 8% crops were estimated to be contaminated in August (P_{max} = 43.71 CFU), which was in close agreement with the information that the maximum level of *E. coli* O157:H7 contamination in the field reached the concentration of 2.28 log CFU per subplot during August. In September, only about 1% (P_{max} = 6.03 CFU) crops were estimated to be contaminated, as the maximum level of contamination in the field dropped to the levels of 0.75 log CFU per subplot. The percentage of contaminated crops was 3.6% (P_{max} = 112.65 CFU)

and 5.7% (P_{max} = 62.57 CFU) as the maximum contamination level of *E. coli* O157:H7 in the field crossed the level of 2.0 log CFU per subplot in October and November.

The results of organic baseline, reduced baseline, and local defecation models also depict the same seasonality trends that were observed in the baseline model. There were no contaminated crops predicted in the months of April, May or June. In July, 8.3% (P_{max} = 22.46 CFU), 0.8% (P_{max} = 2.51 CFU), and 4.0% (P_{max} = 6.07 CFU) crops were estimated to be contaminated for organic baseline, reduced baseline, and local defecation model, respectively. In the second crops, 55 crops were predicted as contaminated in the organic baseline model, whereas only 1 crop was predicted as contaminated in the reduced baseline model. For organic baseline and local defecation models, the P_{max} values were computed highest for August (P_{max} = 150.41 and 58.87 CFU for organic baseline and local defecation models, respectively). Total 35 second crops were estimated to be contaminated for the local defecation model. In the runoff model, none of the first and the second crops were predicted as contaminated, but the maximum concentrations of *E. coli* O157:H7 in a plant (P_{max}) were higher in the second crops in comparison to the first crops (**Table 6.2**).

Table 6.2 Results summary of different scenarios of the system models (1,000 iterations for each model)

		Month											
		Jan	Feb	Mar	Apr	May	June	Jul	Jul	Aug	Sep	Oct	Nov	Dec
Baseline Model	Crop number	-	-	-	1	1	1	1	2	2	2	2	2	-
	Contaminated harvests	-	-	-	0	0	0	11	0	21	4	11	6	-
	Total harvests	-	-	-	129	321	329	221	2	253	333	307	105	-
	Maximum *E. coli* O157:H7 observed	-	-	-	0.07	0.19	0.07	13.00	0.10	43.71	6.03	112.65	62.57	-
Organic Baseline Model	Crop number	-	-	-	1	1	1	1	2	2	2	2	2	-
	Contaminated harvests	-	-	-	0	0	0	23	0	24	4	14	13	-
	total harvests	-	-	-	107	297	320	276	3	217	323	331	126	-
	Maximum *E. coli* O157:H7 observed	-	-	-	0.66	0.28	0.01	22.46	0.47	150.41	8.61	50.83	34.24	-
Reduced Baseline Model	Crop number	-	-	-	1	1	1	1	2	2	2	2	2	-
	Contaminated harvests	-	-	-	0	0	0	2	0	0	0	1	0	-
	total harvests	-	-	-	103	338	327	232	1	263	305	329	102	-
	Maximum *E. coli* O157:H7 observed	-	-	-	0.01	0.02	0.06	2.51	0.00	0.86	0.59	12.54	0.41	-

Local Defecation Model	Crop number	-	-	-	1	1	1	1	2	2	2	2	2	-
	Contaminated harvests	-	-	-	0	0	0	10	0	12	2	10	6	-
	total harvests	-	-	-	111	325	316	248	1	250	320	322	107	-
	Maximum *E. coli* O157:H7 observed	-	-	-	0.05	0.02	0.10	6.07	0.01	58.87	6.89	8.98	9.11	-
Runoff Model	Crop number	-	-	-	1	1	1	1	2	2	2	2	2	-
	Contaminated harvests	-	-	-	0	0	0	0	0	0	0	0	0	-
	total harvests	-	-	-	95	326	321	258	1	232	333	313	121	-
	Maximum *E. coli* O157:H7 observed	-	-	-	0.03	0.01	0.00	0.00	0.00	0.01	0.05	0.07	0.16	-

Crop number: First or second crop;

Contaminated harvests: predicted number of harvests with at least one plant contaminated with more than 1 CFU of *E. coli* O157:H7 at the time of harvest;

Total harvests: total number of iterations for which the crop was harvested in the corresponding month;

Maximum *E. coli* O157:H7 observed: maximum population of *E. coli* O157:H7 (CFU) predicted in a plant at the time of harvest;

-: not applicable, no harvesting this month

6.5 Discussion

The pattern or seasonality of contaminated crops in this study was in good agreement with the reported Salinas Valley leafy greens outbreaks. **Table 6.3** shows the list of *E. coli* O157:H7 outbreaks associated with the consumption of leafy greens produced in Salinas Valley region during 1999-2008. During this period, 11 outbreaks were reported, out of which 10 were reported during July-November. The incidence of *E. coli* O157 in feral pigs feces during these months correlated generally with incidence in cattle feces (200). Feral pig activity in and around an implicated field was speculated as a risk in the 2006 *E. coli* O157:H7 outbreak associated with baby spinach, which started in August of that year (188). Animal feces have been reported as an important source of pre-harvest contamination of field, and intrusion by animals either directly into the field or indirectly (through dust or manure) are potential mechanisms of contamination (104). Runoff of animal feces to the agricultural farm may depend upon several topographical factors, such as distance of the field from animal feces, slope of the area, and any hurdles that may prevent the runoff from contaminating the field. Currently, no data of contamination of field with animal feces are available. Intrusion of one wild pig was assumed into the field every day in the baseline model in order to estimate the effect of feral swine feces on contamination in leafy greens at the time of harvest. The results of the baseline model might have over-predicted the contamination of leafy greens, therefore, the reduced

baseline model might be the most representative of actual contamination in leafy greens.

Table 6.3 List of outbreaks attributed to leafy greens produced in Salinas Valley, California

Month	Year	Outbreak state	Number of cases (illness)	Crop	Reference
January	--	--	--	--	--
February	--	--	--	--	--
March	--	--	--	--	--
April	--	--	--	--	--
May	2008	WA	10	Romaine lettuce	(104)
June	--	--	--	--	--
July	2002	WA	29	Romaine lettuce	(189)
August	2006	26 states	208	Spinach	(188)
September	1999	WA	6	Romaine lettuce	(191)
	1999	CA	8	Romaine lettuce	(191)
	2003	CA	57	Romaine, iceberg lettuce	(104)
	2005	MN	11	Romaine lettuce, vegetables	(104)
October	1999	OR	3	Romaine lettuce	(191)
	1999	PA	41	Romaine lettuce	(191)
	2003	CA	16	Spinach	(104)
November	2004	NJ	6	Lettuce	(190)
December	--	--	--	--	--

Irrigation water has the potential to contaminate leafy greens if there is direct contact of water containing human pathogens with edible portions of leafy greens or by means of water-to-soil and soil-to-leafy greens contact (23). Even if the irrigation water is not contaminated, sprinkler irrigation can result in contamination of plants as pathogens present in the soil can be transferred from soil to plant through splashes

created by irrigation water (43). Water with a bigger droplet size will have a higher kinetic energy, and will maximize the erosive forces of irrigation water on contaminated soil or feces (43).

The majority of leafy vegetable production in the Salinas Valley region involves irrigation with well water of high quality. Indeed, well water was reported to be the source of irrigation of leafy vegetables associated with the 2006 outbreak (188). During winter, leafy green production occurs mainly in the Imperial Valley of California and the Yuma region of Arizona, where surface water (lakes, ponds, reservoirs, and watersheds) is primarily used as irrigation water (197). Surface water is much more susceptible than groundwater to contamination with pathogenic microorganisms (207), yet outbreaks associated with produce from these regions have not occurred or have been rare. Indeed, the quality of surface water is important to leafy greens production even when it is not used directly for irrigation, as surface water could be a major source of pathogens affecting aquifer recharging (207), exposure of animals to colonization, and/or transport to produce fields by irrigation, or processes that are yet not identified (104). Because many of the wells in the San Benito county are drilled relatively deep (197), in this study it was assumed that these wells draw most of their water only from deeper groundwater that is of superior quality.

The results of field and experimental studies related with cattle feces highlight the variability of *E. coli* O157 carriage and excretion rates. Field studies have shown that more than 75% fecal samples that are positive for *E. coli* contain less than 2 log

CFU per gram of feces (208). On the other hand, some cattle may excrete *E. coli* O157 at levels of more than 7 log CFU per gram of feces (34, 35). Such a variation in levels of *E. coli* O157 excretion cannot be explained by a single distribution that represents one homogeneous population (208, 209). The maximum contamination level of *E. coli* O157:H7 in cattle feces (8.4 log CFU/g) used in the present system model was taken from a large systematic study (35) that did not account for animal age or super-shedding, thus, it was considered to be a part of the representative range in the general cattle population (199).

This study is the first attempt towards developing a mathematical system model to understand the pathway of *E. coli* O157:H7 in leafy greens production. The present model included two key subsystems in a leafy greens farm: soil and plants. There are several other subsystems and inputs that can be included in the future studies, including contaminated manure, application of contaminated irrigation water, impact of birds and insects, human handling, and climatic variation on survival of pathogen. Including all these parameters in the future system models will be challenging in terms of computational efforts, as well as availability of data.

6.6 Conclusions

Leafy greens are implicated with large number of outbreaks associated with *E. coli* O157:H7 in the U.S. Salinas Valley in California is a major leafy greens producing region in the country. Most of the outbreaks listed in the available literature occurred during July-November. Results of the presented system model

indicate that the seasonality of *E. coli* O157:H7 associated outbreaks was in good agreement with the prevalence of this pathogen in cattle and wild pig feces. The current system model also suggested that probability of presence of *E. coli* O157:H7 in the harvested crop was higher during July-November. On the basis of comparison between the results of different scenarios, it can be recommended that concentration of *E. coli* O157:H7 in leafy greens can be reduced significantly if contamination of soil with wild pig and cattle feces is mitigated. Among the scenarios simulated in this study, the scenario assumed in the reduced baseline model might be the most representative of real contamination of leafy greens farms in the Salinas Valley.

Chapter 7. Summary and future studies

7.1 Summary

Leafy green vegetables have been associated with an increasing number of foodborne illness outbreaks the United States. This project systematically estimated the growth and death of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in leafy greens stored at different temperatures. Furthermore, models related to the deterioration of different sensory quality parameters were also used in order to optimize the storage temperature of leafy greens while minimizing the storage cost and maintaining the desired standards of sensory quality and microbial safety. Finally, a system model was developed to evaluate the impact of several pre-harvest factors on the probability of presence of *E. coli* O157:H7 in leafy greens.

Chapter 2 focused on the literature related to available data on contamination sources and pathogen ecology, predictive microbial models at various steps, and overall quantitative risk assessment models for different pathogens in leafy greens in the farm-to-table continuum. The review of literature also emphasizes that microbial contamination in leafy greens mostly originates from the pre-harvest environment. Growth of pathogens in leafy greens can effectively be controlled by storing these at appropriate temperature and time, and by the application of intervention steps such as washing and irradiation.

In the published literature, there are large number of growth and death data available for *Salmonella* and *L. monocytogenes* in leafy greens. However, there is a need to compile these data in order to develop a generic growth-death model and to address the variability in the growth rates. Chapter 3 focused on developing a growth-death model for *Salmonella* and *L. monocytogenes* in leafy greens during non-isothermal conditions. These growth-death models for *Salmonella* and *L. monocytogenes* were validated using several dynamic time-temperature profiles during the production and supply chain of leafy greens. Furthermore, these models for *Salmonella* and *L. monocytogenes* and a similar growth-death model for *E. coli* O157:H7 were used to predict the growth of these enteric pathogens in leafy greens without temperature control (Chapter 4).

Chapter 5 utilized nonlinear programming approach to optimize the storage and transportation temperature of leafy greens during supply chain, while minimizing the refrigeration cost and controlling the bacterial growth and deterioration of sensory qualities to certain levels. The types of leafy greens considered were fresh-cut iceberg and romaine lettuce, and fresh-cut chicory. The studied sensory quality parameters were fresh appearance, browning, wilting, and off-odor.

In Chapter 6, a system model consisting of subsystems and inputs (soil, irrigation, cattle, wild pig, and rainfall) to the system simulating a hypothetical farm in Salinas (California) was developed. This model assumed two crops of lettuce in a year, with the first and the second crops being planted any time between January and

April, and between May and August, respectively. The model was simulated assuming the events of plantation, irrigation, harvesting, ground preparation for the new crop, contamination of soil and plants, and survival of *E. coli* O157:H7. The model predicted results suggested that probability of presence of *E. coli* O157:H7 in the harvested crop was higher during July-November. On the basis of comparison among results from different scenarios, it could be recommended that concentration of *E. coli* O157:H7 in leafy greens can be reduced considerably if contamination of soil with wild pig and cattle feces is mitigated.

7.2 Future studies

This dissertation represents use and development of predictive models for enteric pathogens in leafy greens at pre- and post-harvest levels. Some possible areas of future research pertaining to enteric pathogens in leafy greens are proposed as follows.

(1) More studies related to decline of *Salmonella* and *Listeria monocytogenes* in leafy greens at refrigerated temperature are needed. Studies currently available for decline of *Salmonella* have reported the decline of this pathogen at 4 and 5°C. This information is not sufficient to model the decline of *Salmonella* as a function of temperature. Also, available information regarding decline of *L. monocytogenes* in leafy greens at lower temperature is not sufficient to model any decline of this pathogen at temperatures below 3°C.

(2) There was considerable variability in the growth data of *Salmonella* in cut and uncut leafy greens. Bacterial growth in cut leafy greens is higher than the growth in uncut leafy greens. More studies are needed to model the growth behavior of pathogens in cut and uncut leafy greens separately.

(3) Experimental studies are needed in the future for the growth of pathogens in leafy green without temperature control. This could provide more insight into whether leafy greens undergo a new lag-phase when these are taken out of refrigeration or pathogens start to grow as soon as leafy greens are exposed to the ambient temperatures.

(4) Information about several other varieties of leafy green vegetables such as spinach, kale, and cabbage can be added to the tool developed in Chapter 5. In addition, changes in the sensory properties of uncut leafy greens may also be included in the future studies.

(5) A user-friendly web-tool may be developed for the study conducted in Chapter 5, which could have a larger outreach to different stakeholders, such as people from industry, academia, and regulatory agencies.

(6) There are several gaps in the available information regarding agricultural practices in the Salinas Valley region, which is currently a hurdle in the development of a complete system model with several subsystems and inputs that were not included in Chapter 6. These data gaps include:

surveys pertaining to the microbial quality of manure used, sources of irrigation water and irrigation methods, seasonality of birds, insects, and wildlife, and experimental studies for modeling survival of *E. coli* O157:H7 in soil at different temperatures. More studies could be conducted to fill these data gaps.

(7) When more information about different components of the system is available, a system model consisting of several other subsystems and inputs such as contaminated irrigation water, birds and insects, contaminated manure, effect of climatic factors (temperature, wind speed, and wind direction) could be developed. The developed system model could help develop guidelines to mitigate the occurrence of outbreaks in the future.

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