QUANTITATIVE MICROBIAL RISK ASSESSMENT FOR *LISTERIA MONOCYTOGENES* ON FRESH BABY SPINACH LEAVES

A Thesis

by

BASRI OMAC

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Chair of Committee,	Elena Castell-Perez
Committee Members,	Rosana G. Moreira
	Alejandro Castillo
Head of Department,	Stephen W. Searcy

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ABSTRACT

The increase in foodborne illness outbreaks associated with leafy green vegetables can be attributed to the minimal processing required of this group of fresh produce. Neglecting effective microbial elimination steps result in leafy green vegetables that naturally contain microorganisms such as pathogens microorganisms. This quantitative microbial risk assessment describe the change of *L. monocytogenes* concentration in fresh baby spinach from farm to table. This model also consists of several scenarios to evaluate decontamination treatments, cross-contamination, and temperature abuse.

Cross-contamination was the biggest issue when ensuring safety of leafy green vegetables. Temperature abuse also increased the risk of listeriosis. On the other hand, irradiation treatment with Modified Atmosphere Packaging reduced the number of annual cases of listeriosis by 99.99%.

In addition, the validation of *L. innocua* used as a surrogate for *L. monocytogenes* in fresh baby spinach was evaluated based on reductions by washing (water and chlorine) treatments and growth at temperatures between 5 and 36^{o}C. According to the results of the washing treatments, the concentration of *L. innocua* was reduced by almost 0.5 log CFU/g. This is more than that of *L. monocytogenes.*

Furthermore, the effect of natural background microflora on the growth of *L.* monocytogenes and *L. innocua* in fresh baby spinach was determined. The results showed that the growth of *L. innocua* was affected more than that of *L. monocytogenes*

at temperatures 10> T >30ºC. Although *L. innocua* and *L. monoctogenes* are genetically similar, their behavior changes under stress conditions.

Ultimately, the dynamic growth models for *L. monocytogenes* in fresh baby spinach was presented. These models can be used to estimate the growth of *L. monocytogenes* in fresh baby spinach during distribution, storage or market, and potential growth at a consumer level.

DEDICATION

To my parents, Mehmet and Medine Omac

To my siblings, Zeki, Hayriye, Ramazan, Fatma, Emine, and Yasin Omac

For their unconditional love. Thank you.

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CHAPTER I

INTRODUCTION

The number of foodborne diseases linked to consumption of fresh produce in the United States has risen during the last twenty years. According to the Food and Drug Administration (FDA), there have been 72 foodborne illness outbreaks linked to fresh produce from 1996 to 2006 with a quarter of those outbreaks linked to fresh-cut produce (Adavi, 2011).

Surveillance of vegetables and outbreaks associated with green leafy vegetables has demonstrated that these foods can be contaminated with miscellaneous bacterial pathogens, including *Escherichia coli O157:H7*, *Salmonella*, and *Listeria monocytogenes* (Warriner et al., 2009; Franz et al., 2010). Green leafy vegetables, such as spinach and lettuce, are the commodity group of most concern regarding microbiological hazards of fresh produce because they have been connected to multiple outbreaks of foodborne disease (FAO/WHO, 2008). For instance, 502 foodborne outbreaks caused by various pathogens linked to leafy greens were reported in the U.S. from 1973 to 2006, of which 18,242 cases were foodborne illness and 15 cases resulted in death (FAO/WHO, 2008).

Listeria monocytogenes is a pathogen of concern because it causes approximately 1591 cases of foodborne illness annually. Of these cases, there are 1455 hospitalizations and 255 deaths in the United States (Scallan et al., 2011). Furthermore, studies on the presence of *L. monocytogenes* in vegetables have shown that it should be a concern

because although *L. monocytogenes* is rare in fresh produce, the lethality ratio of this pathogen is higher than the other food borne pathogens (Lianou and Sofos, 2007; Koseki et al., 2011; Sant`Ana et al., 2012a). In addition, lower doses of *L. monocytogenes* can still cause infection in immunocompromised populations (Lianou et al., 2007) and grow under refrigeration temperatures. For instance, after shredded romaine lettuce was stored at 5^{o}C for 10 days, the populations of *E. coli O157:H7* and *Salmonella* were reduced by 1 $\log_{10}$ CFU/g whereas *L. monocytogenes* populations approximately grew 1 $\log_{10}$ CFU/g (Tian et al., 2012). Depending on commodities, the temperature for storing fresh and fresh-cut vegetables should be controlled for the safety of consumer. As a result, all of this information shows that quantitative assessment for potential contamination of *L. monocytogenes* in fresh baby spinach or other green leafy vegetables is crucial to predict outcomes before the actual implementation of prevention or decontamination steps.

Listeria innocua strains (M1, DSM 20649, LM 105 (ATCC 33090), Seeliger 1983 strain CIP 80.12) have been used as a surrogate for *L. monocytogenes* in food systems to evaluate the effect of thermal processing (Murphy, 2000), sanitizer effectiveness (Omary et al., 1993; Houtsma et al., 1994), and super-atmospheric oxygen and carbon dioxide (Geysen et al., 2005), on the growth of *L. monocytogenes*, respectively. However, Duh and Schaffner (1993) determined that the growth of *L. innocua PFEI* and *L. monocytogenes Scott A PFEI* in brain heart infusion changed differently depending on the temperature. O`Bryan et al. (2006) determined that *L. innocua M1* cannot be an appropriate surrogate to show the heat-resistance of *L. monocytogenes* in meat because many factors such as the age of the culture, growth

conditions, and the stress response of both microorganisms may not be same. More recently, Friedly et al. (2008) showed that the thermal survivability characteristics of *L. innocua M1* are not similar to those of *L. monocytogenes* in hamburger patties. These results indicate that the validation of *L. innocua* for use as a surrogate for studies on green leafy vegetables should be demonstrated because the stress response of *L. innocua* and *L. monocytogenes* may not be similar when the products are treated with water and chlorine. In addition, the growth of these two microorganisms in green leafy vegetables may not be similar under different temperatures.

There are several reasons for survival and growth of *L. monocytogenes* and other pathogens in spinach and other green leafy vegetables. First, biofilms on fresh produce develop as groups of bacterial cells combine in exopolysaccharide materials. These materials serve to prevent harm coming from environmental stressors as well as containing desiccation and bactericidal agents (Morris and Monier, 2003). Second, the phyllosphere parts of the plant protect the pathogen against washing or surface sanitization methods such as water washing and chlorine (Whipps et al., 2008). The internalization of pathogens into the plant vascular system is also an important concern because decontaminants such as chlorine are commonly inadequate in eliminating pathogens during washing procedures (Hora et al., 2005; Gomes et al., 2009).

Conventional decontamination techniques used in the produce industry such as washing with chlorine, chlorine dioxide, use of organic acids, and ozone do not eliminate pathogens in fresh and fresh-cut produces; they just reduce microbial

populations (Zhang and Farber, 1996; FAO/WHO, 2008). In addition, several studies determined incidence and transmission of pathogen microorganisms, methods for pathogen control, and how to decrease the risk of pathogen contamination in fresh and fresh-cut produce (Harris et al., 2003; Lianou and Sofos, 2007; Raybaudi-Massilia et al., 2009; Park et al., 2012). Additionally, other than disinfection treatments, there is no additional control point to eliminate or prevent contamination with pathogenic microorganisms before the produces are consumed. Thus, processors need new approaches to eliminate pathogenic microorganisms in spinach and other fresh produces and want to know how long their products can be safe for consumers. Furthermore, fresh produce processors do not want to increase their cost due to recall products.

Naturally existing microbiota on the surface of fresh and fresh-cut produces is believed to play a considerable role in maintaining safety and quality of fresh and fresh-cut fruits and vegetables. According to Babic et al. (1997), *L. monocytogenes*-inoculated at 4.0 log CFU/g did not grow on fresh-cut spinach leaves stored in air at 10 and 5ºC, while mesophilic aerobic microorganisms and especially *Pseudomonas* spp. increased acutely. Carlin and Morris (1996) found that when endive leaves were treated with 10 % hydrogen peroxide, the number of native microorganisms on the leaves decreased; however, *L. monocytogenes* grew very quickly on endive leaves. Similarly, Francis and O`Beirne, (1998) reported that background microflora linked to lettuce restrained the growth of *Listeria spp*. on the surface of shredded lettuce.

Risk assessment tools and predictive microbiology concepts are crucial to estimate growth of pathogens in fresh baby spinach and other fresh and fresh-cut produce, because they can present insight to evaluate the safety of features of the production process and allow the manufacturer to estimate results before actual implementation (Puerta-Gomez et al., 2013b). Furthermore, quantitative risk assessment methods can be helpful to inform policy decisions, which depend on the problem, the time frame, and the specific risk management questions to be addressed. These models also provide information to predict the microbiological shelf-life of products.

Quantitative microbiological risk assessment (QMRA) and predictive modeling have become popular in the food microbiology and food safety engineering fields in the last two decades (Mafart, 2005; Marks, 2008; Vercammen et al., 2013). These quantitative tools enhance food safety by evaluating the effects of intervention measures in food production processes. QMRA can be an important tool to figure out the impact of different alleviation strategies (washing, chlorination, and cross contamination) on the number of pathogens present in fresh baby spinach and other leafy vegetables. Because the estimation of microbial risk naturally consists of variability and uncertainty, simulation methods such as Monte Carlo are generally used in QMRA (Danyluk and Schaffner, 2011).

Predictive modeling is necessary to estimate growth of pathogens in QMRA because it provides information on the growth of pathogens in foods during processing and storage (Perez-Rodriguez and Valero, 2013). Likewise, a dynamic model can be

used to estimate the population dynamics of pathogens in food systems at time-varying temperature profiles (non-isothermal conditions) (Puerta-Gomez et al., 2013a).

The main goal of this study was to conduct a quantitative risk assessment analysis for human health risk involving *Listeria monocytogenes* and fresh baby spinach leaves. The main goal was achieved by carrying out these specific objectives:

(1) Validation of the feasibility of using *L. innocua* as a surrogate of *L. monocytogenes* to predict growth of the pathogen on fresh baby spinach leaves under storage (at constant temperature) and establish means to reduce microbial counts on the produce during washing (water and chlorine) treatments.

(2) Evaluating of the effect of the initial level of *L. innocua, L. monocytogenes,* and natural microbiota load on the surface of baby spinach leaves, and their interaction, on the growth curves of *L. innocua* and *L. monocytogenes* at different storage temperatures (5, 10, 20, 30, and 36^{o}C).

(3) Prediction of the growth patterns of *L. innocua* and *L. monocytogenes* on fresh baby spinach containing different natural microbiota levels stored at different temperatures (5, 10, 20, 30, and 36^{o}C).

(4) Development of predictive growth equations to estimate the growth of *L. innocua* and *L. monocytogenes* in fresh baby spinach leaves.

(5) Development of a quantitative risk assessment model to determine prevalence, concentration, and cross contamination of *L. monocytogenes* in fresh baby

spinach leaves by determining dose-response assessment based on the quantity of *L. monocytogenes* consumed and magnitude and frequency of adverse health.

CHAPTER II

LITERATURE REVIEW

2.1 Consumption of Spinach in the U.S.

Spinach (*Spinacia oleracea*) is a functional food valued for its antioxidants, and anti-cancer constituents and because it is also a good source of nutrients including high levels of vitamin C, lutein, iron, folic acid, and magnesium (Neal, 2009). The U.S. Department of Agriculture (USDA), the Centers for Disease Control and Prevention (CDC), the National Cancer Institute (NCI) as well as the healthcare community and numerous other organizations recommend consumption of spinach (Neal, 2009). Spinach was consumed by 5.51% of the U.S. population who consumed 40% of spinach in fresh as fresh baby spinach at restaurants and at home (Hoelzer et al., 2012a). Based on Hoelzer et al. (2012a), the odds of consuming fresh leafy greens were significantly lower for men than women, whereas adults (15 to 49 years of age) were significantly more likely to consume leafy greens than older and elderly adults (i.e., adults older than 49 years old).

Although the harvesting area and the production capacity of spinach decreased between 2010 and 2012, the production of spinach in 2012 was still over 240,000 ton and occurred mainly in California (60%), Arizona (25%), New Jersey (5%), and Texas (3.1%) (USDA/NASS, 2013). The average spinach consumption per person in the U.S. was reported 1.9 pounds (0.86 kg) and this amount decreased 32% between 2007and 2009 (Lucier and Glaser, 2010). Fahs et al. (2009) reported that this reduction in consumption of spinach could be related to the *E. coli 0157:H7* outbreak associated with

contaminated baby spinach in 2006. Therefore, the fresh produce industry has had to address this issue while increasing the shelf life of the leafy green.

2.2 Foodborne Disease Outbreaks Associated with Fresh Produce and Leafy Green Vegetables

The number of foodborne disease outbreaks linked to fresh produce have increased in the past three decades (Sivapalasingam et al., 2004; Lynch et al., 2009; Critzer and Doyle, 2010; Kozak et al., 2013). Over 13,352 foodborne disease outbreaks were reported in the United States throughout 1998-2008 (Painter et al., 2013), which caused 271,974 illnesses. According to some studies, these outbreaks are caused by changes in dietary habits, the increasing amount of fresh produce consumed, and cutting and coring of the fresh produce (Burnett and Beuchat, 2001; Brandl, 2006; Lynch et al., 2009). With the independent nutrition and health authorities in the U.S. encouraging people to eat more fresh produce, the risk of exposure to fresh produce contaminated with pathogenic microorganisms has increased (De Roever, 1998; Sivapalasingam et al, 2004; Beuchat, 2006). Although the consumption of leafy greens in the U.S. rose 17.2%, the number of foodborne illnesses outbreaks linked to leafy greens rose 59.6% throughout the years between 1996 and 2005 (Neal, 2009). Likewise, the consumption of leafy greens increased 9.0% and foodborne illnesses outbreaks linked to leafy greens rose 38.6% during those years between 1996 to 2005 (Neal, 2009). Hence, the amount of consumption of leafy greens is one reason for the increased number of foodborne disease associated with leafy greens (Painter et al., 2013). In addition, contamination can occur from the “farm to table” chain because of handling-by workers, intensification and

centralization of production and more sources of produce (De Roever, 1998; Burnett and Beuchat, 2001; Olaimat and Holley, 2012).

Tauxe et al. (1997) reported that rises in the size of *at risk* population, increased epidemiological surveillance, and improved methods of identifying and tracking pathogens have added to the increase in fresh produce associated outbreaks. Centralized processing from multiple farms also create a longer food chain, which increases the growth of pathogens and their distribution may put more people at risk of foodborne disease (De Roever, 1998; Painter et al., 2013). Furthermore, improvements in healthcare have added to a raising proportion of elderly as well as immunocompromised people (Carrasco et al., 2010). Burnett and Beuchat (2001) reported that several fresh produce, including raspberries, basil, lettuce, alfalfa sprouts, radish sprouts, carrots, salad vegetables, cabbage, tomatoes, watermelon, cantaloupe, green onions, parsley, and spinach, have been recently linked to foodborne illnesses.

During 1998-2008, 22 % of all foodborne illnesses in the United States were attributed to leafy green vegetables. In addition, illnesses linked to leafy green vegetables were the second most frequent cause of hospitalization and the fifth most frequent cause of death (Painter et al., 2013).

Preharvest and postharvest processing steps of leafy green vegetables contribute to the increased incidence of foodborne illness outbreaks associated with leafy greens (Prazak et al., 2002; Lianou and Sofos, 2007; Olaimat and Holley, 2012).

Bacteria, viruses, and protozoan cause foodborne disease outbreaks in fresh produce epidemiologically; however, bacterial origin foodborne disease outbreaks linked

to leafy green vegetables were commonly reported (Olaimat and Holley, 2012). Listeriosis outbreaks associated with leafy green vegetables are very important because *L. monocytogenes* causing listeriosis can survive and grow at low temperature and has a high mortality rate (Lianou and Sofos, 2007). *L. monocytogenes* is a big concern for processors of leafy green vegetables such as spinach as well as ready-to-eat foods, meat products, and dairy products (Francis et al., 1999; Szabo et al., 2000; Harris et al., 2003; WHO&FAO, 2008). The first listeriosis outbreak was determined in 1981 when the consumption of coleslaw contaminated by *L. monocytogenes* caused at least 41 cases of illness and 18 deaths (Hoelzer et al., 2012b). Likewise, cantaloupes grown in Colorado were involved in a multistate outbreak that sickened 139 people and caused 29 deaths in 2011 (CDC, 2012). Moreover, celery contaminated by *L. monocytogenes* from Sangar Fresh Cut Produce in Texas included 10 cases of illness and 5 deaths in 2010 (FDA, 2010). Some other listeriosis outbreaks are presented in Table 2.1 (Hoelzer et al., 2012). Recalls of produce due to contamination by *L. monocytogenes* are another issue because they result in increased cost of products. Several recalls of various fresh produce between 2012 and 2013 are shown in Table 2.2. These two tables clearly illustrate that most outbreaks have been linked to fresh and fresh-cut products. Therefore, new strategies must be developed to ensure their safety and avoid the potential of future outbreaks due to consumption of fresh and fresh-cut produce.

Table 2.1. Outbreaks of listeriosis associated with produce (Adapted from Hoelzer et al., 2012b).

Year	Source	No. of cases	No. of hospitalizations	No. of fatalities	Location
1979	Tuna fish and chicken salads with celery, lettuce, and tomatoes, cheese	20	20	5	U.S.
1981	Coleslaw	41	40	18	Canada
1986	Vegetables or unpasteurized milk	28	28	5	Australia
1988	Vegetable rennet	1	Mother-child pair (miscarriage)		United Kingdom
1989	Salad containing lettuce (hypothesized)	1			United Kingdom
1989	Salted mushrooms	1			Finland
	Frozen broccoli, cauliflower	7			U.S.
1993	Rice salad (cheese, pickled vegetables, frozen vegetables, hard-boiled eggs)	18	4	0	Italy
1997	Corn and tuna salad	1566	292	0	Italy
1998-1999	Fruit salad	6	6	5	Australia
	Melon and watermelon	Case-control of Sporadic cases			U.S.
2001	Potato salad	56	1	0	U.S.
2006	Taco/nacho salad	2	0	0	U.S.
2010	Alfalfa sprouts	20	16	0	U.S.
2010	Celery	10	10	5	U.S.
2011	Cantaloupe melon	146	142	30	U.S.

Table 2.2. U.S. recall cases associated with produce in 2012 and 2013 (between January and September) due to the presence of *Listeria monocytogenes* (Adapted from FDA, 2013).

Year	**Source**
2013	Kale
2013	Salads
2013	Fresh potato salad
2013	Cantaloupes
2013	Cantaloupes
2013	Thai peppers
2013	Fruit and grain salad
2013	Sprouted seeds
2012	Romaine lettuce
2012	Apple slices
2012	Bagged salad
2012	Romaine hearts
2012	Romaine hearts
2012	Spinach
2012	Spouts
2012	Bagged salad
2012	Fruits, vegetables
2012	Cantaloupes
2012	Onions
2012	Bagged salad
2012	Bagged salad
2012	Alfalfa Sprouts
2012	Bagged salad
2012	Soybean sprouts

2.3 Contamination of Leafy Greens

2.3.1 Preharvest Contamination

During preharvest processing, spinach can be contaminated with pathogens at any point. Potential risky preharvest points can be irrigation water, water used to apply insecticides and fungicides, manure, feces, soil, wild, and domestic animals such as birds, reptiles, and insects (Beuchat, 1996a; De Roever, 1998; Burnett and Beuchat, 2001; Park et al., 2012). Water quality is crucial during irrigation because the large amount of surface area of leafy green vegetables such as spinach commonly obtains the greatest amount of contamination (WHO/FAO, 2008). The methods of irrigation can also cause contamination with pathogens such as *L. monocytogenes* (Park et al., 2012). For example, drip and flood irrigation is better than spraying irrigation (De Roever, 1998). In addition, fields can be contaminated by feces either from the use of improperly composted manure or from free roaming wild or domestic animals (Park et al., 2012). Furthermore, leafy green vegetables such as spinach grow close to the soil making them vulnerable to contamination (WHO/FAO, 2008). Lastly, poor workers hygiene and the lack of proper sanitary facilities in the field can be a source of pathogenic microorganisms (De Roever, 1998; Park et al., 2012).

2.3.2 Postharvest Contamination

During postharvest processing, leafy green vegetables such as spinach can be contaminated by using water or ice, handling by workers, and equipment and transporting vehicles (Prazak et al., 2002; FAO&WHO, 2008). In addition to postharvest processing, these leafy green vegetables may be vulnerable to cross-

contamination during distribution in retail stores, food service facilities and at home when temperature abuse throughout storage, packaging, and displaying can occur (Prazak et al., 2002; Lianou and Sofos, 2007).

During processing of spinach or other leafy green vegetables, there are several critical issues: (1) the process can cause damage such as cuts or bruises to the leafy green, increasing the availability of nutrients for microorganisms to grow; (2) the lack of a lethality step minimizing microbial growth; and (3) these products may be transported to multiple locations which raises the risk for contamination (Prazak et al., 2002). Fresh produce can be contaminated during the *farm to table* process in spite of using proper hygiene, Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs) (Anciso and Gregory, 2010). U.S. Food and Drug Administration (FDA) and the Center for Food Safety and Applied Nutrition (CFSAN) published some guidelines to improve safety of fresh fruits and vegetables (FDA, 2009). The last version of guidance published in 2009, covering leafy greens, highlights the importance of employing prevention of microbiological hazards instead of elimination strategies of them because once pathogens contamination has occurred at fresh produce, removing or killing the microbial pathogens is very difficult despite maintaining the fresh attribute of the produce (FDA, 2009).

Several studies have helped to define critical points in preharvest conditions as well as post harvesting and production (Beuchat, 1996a; Prazak et al., 2002; Gagliardi et al., 2003; Anciso and Gregory, 2010; Gomez-Lopez et al., 2013). GAPs contain water, worker health and hygiene, sanitary facilities, the use of municipal bio-solids and

manure, field sanitation, and transportation. Critical points linked to GMPs include the level of interest of the personal working at a facility, the building facilities, equipment, production, process controls, and policies and procedures. It has been that *L. monocytogenes, E.coli 0157:H7* and *Salmonella* can be found on fresh produce from the field through the packing process (Johnston et al., 2005a).

2.4 *Listeria monocytogenes*

Although *L. monocytogenes* was discovered in 1924 by E.G.D. Murray and others (Friedly, 2007), it did not get attention from the food industry until thirty years ago when it was connected in a series of foodborne diseases in the U.S. (Carpentier and Cerf, 2011). *L. monocytogenes* is a Gram-positive, small, rod-shaped bacterium, facultative anaerobic, non-sporulate, and oxidase negative, catalase positive (Farber and Peterkin, 1991). The genus of *Listeria* includes seven species, *Listeria monocytogenes*, *Listeria innocua, Listeria ivanonovii, Listeria welshimeri, Listeria grayi, Listeria murrayi*, and *Listeria seeligeri*) and the pathogenic genus of *Listeria* for human is only *L. monocytogenes* (Monsalve, 2008).

Listeria innocua has been used as a surrogate organism to design and develop an understanding of the behavior of *L. monocytogenes* in foods and food processing environments because of similarities between them (Rodriguez et al., 2006; Milillo et al., 2012). *L. innocua* has been also recommended as an indicator of the presence of *L. monocytogenes* (Milillo et al., 2012). This microorganisms has very close genetic relationship with *L. monocytogenes* (Glaser et al., 2001). Therefore, it may be a suitable option as a surrogate for *L. monocytogenes*.

Several studies on thermal processing, use of aerosol, gaseous, ozone, antimicrobial organic acids, and other food additives, used *L. innocua* as a surrogate for *L. monocytogenes*, to design ways to control the growth, eliminate the presence, or decrease the transmission of this pathogen in foods and a variety of systems (Fan et al., 2007; Milly et al., 2008; Millillo et al, 2012). Kamat and Nair (1996) used *L. innocua F5646* and *F5643* strains to analyze the response of four *L. monocytogenes* strains under gamma radiation, heat, lactic acid, and sodium nitrite treatments. According to this study, the survival of all six strains was similar under all treatments. Similarly, several studies for the dairy industry showed that *L. innocua* can be used as a suitable biological indicator for the evaluation of pasteurization process lethality (Foegeding and Stanley 1990; Fairchild and Foegeding, 1993).

However, O`Bryan et al. (2006) determined that *L. innocua* surrogate strains cannot precisely mirror the behavior of *L. monocytogenes* in all test conditions or foods, containing thermal and other stresses. Friedly et al. (2008) also found that the survival abilities of *L. innocua* and *L. monocytogenes* are different during thermal treatments. In addition, Tompkin (2002) stated that *L innocua* is not a good indicator for the presence of *L. monocytogenes* because that can lead to unnecessary cleaning and sanitizing procedures and increase using resources and taking valuable time from production. All these results highlight that although *L. innocua* and *L. monocytogenes* are very similar genetically, they can behave differently under the same conditions.

2.5 Survival and Growth of *Listeria monocytogenes* on Leafy Green Vegetables

L. monocytogenes is a psychrotrophic bacteria and can grow under low temperatures (Warriner and Namvar, 2009). Koseki and Isobe (2005) determined the minimum growth temperature of *L. monocytogenes* on lettuce is -4.26^{o}C. Several studies determined that *L. monocytogenes* found on and internalized in fresh and fresh-cut vegetables may survive during long periods of time and it may even grow under appropriate conditions (WHO&FAO, 2008; Hoelzer et al., 2012b). A study on different leafy greens stored at 4^{o}C for 9 days showed that *L. monocytogenes* concentration remained constant (Farber et al., 1998). Similarly, *L. monocytogenes* on lettuce stored at 5^{o}C and 10^{o}C grew after 14 days of storage at both temperatures (Beuchat, 1996b).

L. monocytogenes attaches to fresh and fresh-cut produce which provides some degree of protection to the pathogen. Factors such as cell`s surface charge, hydrophobic effects, bacterial structures, excreted extracellular polysaccharides, and the type of commodity affect the attachment of bacteria such that they survive and grow on leafy green vegetables such as spinach (Neal, 2009). Babic et al. (1996) determined that spinach leaves were colonized mainly in areas where the waxy cuticle was broken during storage conditions. Besides, hydrophobic pockets and folds in the leaf`s surface provides the protection for microorganisms such as *L. monocytogenes* during disinfection treatments (Zhang and Farber, 1996).

Another means of protection for microorganisms on fresh produce is the formation of a biofilm an extracellular polysaccharide matrix which ties multiple cells together and attaches them to the surface of the plant (Sapers, 2001). Basically, biofilms

create physical and chemical barriers for microorganisms because there are composed of multiple layers of microbial cells (Morris et al., 1997). Biofilms are formed by pathogenic microorganisms such as *L. monocytogenes*, *Salmonella,* and *E. coli 0157:H7* (Sapers, 2001). Ronner and Wong (1993) demonstrated that *L. monocytogenes* biofilm cells and extracellular matrices would remain on sanitized surfaces. Morris et al. (1997) showed biofilms formation inherently happening on the surface of spinach, lettuce, and parsley.

Furthermore, pathogenic microorganisms penetrate the interior of cut leaf edges or become internalized within leafy green vegetables` tissue (Takeuchi et al., 2000). In addition, negative temperature differences in fresh produce through immersion can cause internalization of *E. coli 0157:H7* and *Salmonella* (Zhuang et al., 1995; Buchanan et al., 1999). Thus, during the disinfection treatment, produce should be placed in water which is colder than produce.

As a means to minimize the growth of *L. monocytogenes* on leafy greens, several factors must be considered such as temperature, water activity, pH, and microbial competition. *L. monocytogenes* can be survive at freezing temperatures (-6^{o}C) (Hitchins et al., 2001). Therefore, this pathogen can grow under the refrigerator temperatures (usually from 0 to 8^{o}C) (Pouillot et al., 2010). In addition. *L. monocytogenes* can grow between pH 4.1 to 9.6, with an optimum range of 6.0-8.0 (Monsalve, 2008). Various factors such as incubation temperature, available nutrients, moisture content, and product composition affected this pH range for *L. monocytogenes* growth. Furthermore, water activity affects the growth of this pathogens as well as temperature and pH. Petran and

Zottola (1989) determined the growth of *L.monocytogenes* in a 39.4% sucrose solution with water activity of 0.92. Thus, the combination of low aw with low temperature and pH can be considered to reduce this food safety hazard.

Natural microflora of food products can impact the growth of *L. monocytogenes* because of microbial competition (Francis and O`Beirne, 1998; Amezquite and Brashears, 2002; Cornu et al., 2011; Al-Zeyara et al., 2011).The natural microflora of spinach between, 10^4-10^7 CFU/g, includes mesophilic aerobic bacteria, psychrotrophic bacteria, *Pseudomonadeceae*, *Enterobacteriaceae*, *Micrococcaceae*, *Lactic acid bacteria*, and yeast (Babic et al., 1996). Babic et al. (1997) showed that the growth of *L. monocytogenes* in tryptic soy broth (TSA) could be reduced by native mesophilic aerobic microorganisms on fresh-cut spinach at 30 and 10^{o}C. *Enterobacter* spp. and *Lactic acid bacteria* also reduced the growth of *Listeria innocua* on minimally processed lettuce (Francis and O`Beirne, 1998). Bennik et al. (1998) reported that bacteriocinogenic strains of *Pediococcus* and *Enterococcus* could be used to control the growth of *L. monocytogenes* on mung-bean sprouts. Johnston et al. (2009) determined antagonistic activity against *E. coli 0157:H7* due to production of antimicrobial peptides and acid by native microflora on fresh-cut lettuce and spinach. Hwang et al. (2011) stated that the native microflora in cooked ham at 4-8^{o}C affected the growth characteristics of *L. monocytogenes* and reduced the maximum population density (MPD) of *L. monocytogenes* in cooked ham. Besides, lactic acid flora in pork products reduced *L. monocytogenes* concentration at 8^{o}C (Cornu et al., 2011).

2.6 Decontamination Methods for Fresh Produce

Decontamination treatment steps are crucial in processing of fresh and fresh-cut products because during this step, pathogenic microorganisms can be removed or reduced (Olaimat and Holley, 2012). Since pathogens cannot be completely eliminated because of the issue of attachment, the effectiveness of decontamination treatments must be verified (Brackett, 1999) and new intervention strategies such as irradiation are worth their evaluation (Gomes et al., 2011). Decontamination methods currently being used can reduce 1-2 log units of microbial population on produce (WHO&FAO, 2008). There are many sanitizing agents such as chlorine, chlorine dioxide, organic acids, ozone, and peracetic acid to eliminate pathogens such as *L. monocytogenes*; however, the effectiveness of these agents depend on many factors such as the temperature of water, pH, water hardness, contact time, and presence of organic matter (Olaimat and Holley, 2012).

2.6.1 Chlorine

Chlorine is commonly used as a chemical disinfectant in the fruits and vegetables industry (Beuchat, 1996a; Zhang and Farber, 1996; Keskinen et al., 2009). Generally, 50-200ppm of chlorine solutions is usually used in wash, spray, and flume waters in fruits and vegetables industry (WHO, 2008). The efficiency of chlorine for reducing microbial populations in fresh fruits and vegetables depends on the amount of free available chlorine in water because chlorine reacts with organic matter (Zhang and Farber, 1996). Furthermore, a result of the reaction between chlorine and organic matter has been related to the production of carcinogenic compounds (Olmez and Kretzschmar,

2009). Zhang and Farber (1996) determined that the population of *L. monocytogenes* artificially inoculated onto shredded lettuce and cabbage was reduced by 1.7 and 1.2 log CFU/g respectively when treated with 200mg/L of chlorine solution for 10 minutes. Francis and O`Beirne (2002) reported that *L. innocua* concentration on shredded lettuce was reduced 1-1.5 log when agitated in a water-chlorine solution (10 ppm) for 10 minutes Burnett et al. (2004) reported that *L. monocytogenes* inoculated iceberg lettuce treated with water or with 200mg/L chlorine had reductions in population of 0.60 and 1.76 log CFU per lettuce piece, respectively.

2.6.2 Irradiation

Irradiation exposes the food to a suitable level of ionizing radiation in the form of electron beams, gamma rays or X-rays to control or eliminate bacteria, viruses or insects that might be present. In the U.S., irradiation has been legalized for poultry, spice, dry ingredients in August, 2008, the U.S. FDA approved the irradiation of spinach and iceberg lettuce at a maximum absorbed dose of 4.0 kGy for microbial inactivation (US FDA, 2008).

Most surface decontamination methods fail to assure produce safety whereas application of irradiation has been reported as very effective (Niemira, 2007; Gomes et al, 2008). Neal (2009) reported that low dose e-beam irradiation may be a feasible tool for decreasing microbial populations or removing *E. coli 0157:H7* and *Salmonella* from spinach with minimal product damage. Similarly, Gomes et al. (2011) pointed out that a treatment by e-beam irradiation of 0.7 kGy on baby spinach leaves under 100% oxygen at room temperature would assure a 5-log reduction of either *Salmonella* spp. and

Listeria spp. without detrimental effects on product quality. Furthermore, compared to other decontamination methods, irradiation is a promising decontamination method for leafy greens for other reasons besides its high efficacy. First, irradiated spinach is free of chemical residues. Second, irradiation treatment may be conducted either before or after packaging. Lastly, irradiation only causes minimal environmental pollution. In addition, it has been determined that irradiation treatment with a dose up to 1 kGy will maintain the overall quality of spinach (Gomes et al., 2008). Nevertheless, market acceptance of the application of irradiation technology on spinach is still debatable and the high cost of irradiation-associated facilities and equipment make it unaffordable to small manufacturers (Wu, 2010).

2.7 Predictive Microbiology

The main concern of the food industry is protecting food products from microbial contamination. Microbial growth and survival in foods are affected by several conditions such as temperature, pH, water activity, antimicrobials, sodium nitrite, and sodium chloride (McKellar, 1997; Juneja, 2003; Moller et al., 2013).

Predictive microbiology in foods is a wide broad scientific field containing insight for evaluating the effects of processing operations on microbial growth, and the resulting shelf life and safety of food products. It brings together mathematical, statistical, and microbiological principles to predict quantitatively the behavior of microbial populations in foods. Predictive models are useful tools, capable of studying the effects of various variables on growth and reduction of pathogens in foods (McMeekin et al., 2013; Perez-Rodriguez and Valero, 2013).

Predictive models provide a description of a real system by using mathematical equations. They could be very useful tools to support decision making such as justification for determining critical limits in HACCP plans. These models can also be very helpful to processors of as meat and poultry, milk, and fruit and vegetables and regulatory agencies to minimize the risk of *L. monocytogenes* in the food products (McMeekin et al., 1993; McKellar, 1997; Coroller et al., 2006).

Several growth model software programs are available for the food industry right now (Perez-Rodriguez and Valero, 2013). The Pathogen Modelling Program (PMP), a predictive software program, developed by the USDA Agriculture Research Service (USDA, 2013) provides growth models for *L. monocytogenes, Escherichia coli 0157:H7, Bacillus cereus, Staphlylococcus aureus, Clostridium perfringens, Salmonella* spp, and *Shigella flexneria* under various environmental conditions. Another software program to model bacterial growth is DMFit (IFR, 2013) an excel add-in program, developed based on the work of Baranyi and Roberts (1994).

The development of a predictive model (also called the primary model) starts by establishing microbial growth or death under constant conditions of temperature, pH, and/or water content. Then, it determine the effect of the environmental variables on the parameters of the primary model (this is called a secondary model). Afterward, primary and secondary models are integrated to develop a dynamic model for predicting the growth of bacteria under different environmental conditions (Perez-Rodriguez and Valero, 2013).

2.7.1 Primary Model

The purpose of the primary model is to define the kinetics parameters of a process such as maximum growth rate, lag phase, and inactivation rate as a function of treatment time. An appropriate primary model should explain the kinetics of microbial growth with the fewest of variables. Growth curves are used to explain observation in processing operations, and help in assessing methods for enhancing the overall process effectiveness and risk assessment (Marks, 2008).

Bacterial growth curves are identified with the change in the number of microorganisms with time as affected by a number of intrinsic and extrinsic factors which are used for predicting growth, survival, and control of desirable and undesirable microorganisms in food systems. Monsalve (2008) characterized microbial growth curve by four main phases as follows: (1) the lag phase or the adaptation period described as an adjustment period throughout which bacterial cells adapt themselves to get advantage of the new environment and initiate exponential growth; (2) the exponential or logarithmic phase defined as the grow of microorganisms in their environment until they reach a maximum population level; (3) the stationary phase defined as the time when the growth rate of microorganisms equals the death rate of microorganisms; (4) the death phase stated as the period when the microbial population starts to decrease because of reduced concentration of nutrients or physiological sate of cells.

Some examples of primary growth models (Monsalve, 2008; Perez-Rodriguez and Valero, 2013) are:

2.7.1.1 The Logistic Model

Fujikawa et al. (2004) reported that although bacterial growth curves are generally sigmoid on a semi-logarithmic plot, the logistic model generates a convex curve consisting of a monotonously increasing portion and stabilizing one, without a lag phase at the initial period. Therefore, for fitting the bacterial growth data, the equation of logistic model was modified as follows (Chowdhury et al., 2007):

$$y(t) = C + \left(A/(1 + \exp\left(-B(t - M)\right))\right) \quad (2.1)$$

herein, *C* is the initial level of inoculation (log CFU/g); *A* represents the difference between the maximum and minimum growth values (log CFU/g); *M* is the time (hours) at which the slope of the sigmoidal growth reaches a maximum value; and *B* represents the maximum growth rate relative to the amount of growth at time *M*.

2.7.1.3 Gompertz Model

The Gompertz growth model is given as follows (Gibson et al., 1988):

$$N(t) = C + A * \exp(-\exp\left(-B(t - M)\right)) \quad (2.2)$$

herein, *C* is the value of the lower asymptote (log CFU/g); *A* is the asymptotic term ($\log_{10}$), *M* is the time at which the slope of the sigmoidal growth reaches a maximum value and the *B* is the maximum growth rate relative to the amount of growth at time.

The Gompertz model was used in several studies (Juneja et al., 2009; Kreyenschmidt et al., 2009; Slongo et al., 2009; Huang, 2011; Zimmermann et al., 2011; Li et al., 2013). The most of them mentioned that the growth rate and lag time estimated from the Gompertz model were a little higher than other models.

2.7.1.2 Baranyi Model

Baranyi proposed the following differential equation (Baranyi and Roberts, 1994):

$$\frac{dx}{dt} = \alpha(t) * \mu_{max} * u(x) * x \qquad (0 \leq t < \infty;\ 0 < x) \qquad (2.3)$$

herein, $\alpha(t)$ is a process of adjustment function (CFU/g); $u(x)$ indicates the inhibition function because it explains the transition of the growth curve to the stationary phase (CFU/g); μ_{max} represents the maximum growth rate (h^{-1}).

The logarithm of the solution of Eq. (2.3), y(t)=ln(x(t), can be expressed as:

$$y(t) = y_o + \mu_{max} * F(t) - \ln\left(1 + \frac{e^{\mu_{max}F(t)} - 1}{e^{(y_{max} - y_o)}}\right) \qquad (2.4)$$

$$F(t) = t + \frac{1}{v} ln(e^{-vt} + e^{-h_o} - e^{(-vt - h_o)}) \qquad (2.5)$$

herein, $y(t)$ is the natural logarithm of the population at time t (ln CFU/g); y_o represents the initial population number (ln CFU/g); y_{max} represents the maximum population (ln CFU/g); h_o represents $\mu_{max} * t_{lag}$, where t_{lag} is the lag time (hours); μ_{max} represents the maximum specific growth rate (1/hours); v is the rate of increase of the critical substrate. Assuming that after the inoculation, the critical substrate grows at the same specific rate as the cells in the exponential phase, $v = \mu_{max}$.

Based on the physiological state of the cell, the model of Baranyi and Roberts (1994) predicts microbial growth very well. In several studies, this dynamic model was successfully implemented for a variety of growth conditions such as temperature, pH, and water activity (Koseki and Isobe, 2005a; Ding et al., 2012; Puerta-Gomez et al., 2013a). Koseki and Isobe (2005b) used the Baranyi model to predict the growth of *E.*

coli 0157:H7, Salmonella, and *L. monocytogenes* on lettuce at fluctuating temperatures from the farm to retail market. In that study, the growth kinetic parameters were presented at isothermal temperatures (5, 10, 15, 20, and 25ºC). Then, these model parameters were used to anticipate pathogen growth under fluctuating temperatures in the Baranyi primary model together with the secondary model of Ratkowsky (Koseki and Isobe, 2005b). According to this study, the Baranyi model can be used to accurately predict the growth of pathogens under fluctuating temperatures in fresh produce after harvest. Ding et al. (2012) verified that the performance of these predictive models is good for the growth of *E. coli 0157:H7* on cabbage. In addition, Puerta-Gomez et al. (2013a) reported that the Baranyi model is adequate to determine the growth of *E. coli* spp. and *Salmonella Typhimurium* LT2 in baby spinach leaves under slow cooling. In comparison to the Baranyi model, Pathogen modeling program (PMP) model and the modified Gompertz model overestimated the growth rate of pathogens in produce because PMP-derived media, so microorganisms are not exposed to factors such as the structure of foods and the presence of background of microflora among other issues and the modified Gompertz model has a different structure (Koseki and Isobe, 2005b; Huang, 2011; Sant`Ana et al., 2012b).

2.7.2 Secondary Models for the Maximum Growth Rate

Secondary models define the effects of environmental conditions such as temperature, pH, water activity, oxygen availability, added preservatives and modified atmospheres on the values of the parameters of a primary model (Monsalve, 2008).

These models predict the changes in the parameters of primary models such as the maximum specific growth rate and lag time.

Most commonly used secondary models (Monsalve, 2008) are:

2.7.2.1 Square-Root Models

At the beginning, these secondary models were suggested by Ratkowsky et al. (1982), who determined a linear relationship between the square root of the maximum growth rate and temperature.

$$\sqrt{\mu_{max}} = b * (T - T_{min}) \quad (2.13)$$

herein, *b* represents a regression coefficient ($^oC^{-1}*h^{-1/2}$); *T* represents the intercept of the predicted function and the temperature axis (^oC); T_{min} represent the notional minimum temperature below which maximum growth rate is equal to 0 (^oC).

Then, this model was developed to cover the whole temperature growth range (Perez-Rodriguez and Valero, 2013):

$$\sqrt{\mu_{max}} = b * (T - T_{min})(1 - e^{c(T-T_{\max})}) \quad (2.14)$$

herein, *c* is a parameter (^oC); T_{max} is the theoretical maximum temperature at which growth can be observed (^oC).

2.7.2.2 Gamma Concept

The gamma-concept-based model was used to determine the μ_{max} of an experiment, containing growth and/or recovery, can be stated as a function of an environmental factor, if all other environmental factors are kept constant as (Giffel and Zwietering, 1999; Leroy and Vuyst, 2005):

$$\gamma = \frac{\mu_{max}}{(\mu_{max})opt} = \gamma(T) * \gamma(pH) * \gamma(aw) \quad (2.15)$$

herein, γ is a self-inhibition factor; μ_{max} is the growth rate at actual environmental conditions (h^{-1}); $(\mu_{max})opt$ is the growth rate at optimal environmental conditions and the absence of inhibitory substances (h^{-1}). The inhibition function γ determines the initial inhibition because of sub optimal temperature ($\gamma(T)$), water activity ($\gamma(aw)$), and ($\gamma(pH)$) conditions. Furthermore, inhibition because of the initial presence of inhibitory compounds, such as lactic acid (γ_{La}), sodium chloride (γ_{NaCl}), and sodium nitrite (γ_{NaNO2}) has be taken into account.

2.7.2.3 Cardinal Parameters Models (CPMS)

Cardinal parameters models are used to determine the effect of temperature on microbiological growth rates. In CPMs, explicit models parameters T_{min} and T_{max} (^oC) are contained (Rosso et al., 1995):

$$\mu_{max} = \mu_{opt}\left(\frac{(T-T_{max})(T-T_{min})^2}{(T_{opt}-T_{min})((T_{opt}-T_{min})-(T_{opt}-T_{max})(T_{opt}+T_{min}-2T))}\right) \quad (2.16)$$

$$\mu_{max} = 0, \quad \text{for} \quad T_{min}<T<T_{max}$$

$$\text{for} \quad T \leq T_{min}$$

$$\text{for} \quad T \geq T_{max}$$

herein, T_{max} is the maximum temperature at which no growth occurs (^oC); T_{min} is the minimum temperature at which no growth occurs (^oC); T_{opt} is the temperature at which μ_{max} is equal to its optimal value (^oC); μ_{max} is the specific growth rate (h^{-1}) ; and μ_{opt} is the optimum specific growth rate (h^{-1}).

2.7.3 Secondary Models for Lag Time

Lag times are affected by various environmental factors. For instance when the media is changed the growth rate will be influenced resulting in changes in the lag time.

Table 2.3. Secondary lag time models (Adapted from Monsalve, 2008).

Model	[1]Equation	Equation number
Hyperbola model	$t_{lag} = e^{\left(\frac{p}{(T-q)}\right)}$	(2.17)
Extended hyperbola model	$t_{lag} = \left(\frac{p}{T-q}\right)^{m}$	(2.18)
Davey model (linear Arrhenius)	$t_{lag} = e^{-C_o-\left(\frac{C_1}{T}\right)-\left(\frac{C_2}{T^2}\right)}$	(2.19)
Inverse simple Ratkowsky-type model	$t_{lag} = \frac{1}{(b(T-T_{min}))^2}$	(2.20)

[1]The model parameters are described as follows:

p is the rate of change of lag time as a function of temperature (hours)

q is the temperature at which the lag time is infinite (no growth) (^{o}C)

m is the exponent devoid of biological meaning

t_{lag} is the lag time (hours)

T is the absolute temperature (^{o}C)

C_o, C_1, C_2 and *b* are coefficients of Equations (2.19) and (2.20)

T is the theoretical minimum temperature beyond which growth is not possible (^{o}C)

Some secondary models that are used to predict the effect of temperature on the lag time of microorganisms are presented in Table 2.3 (Monsalve, 2008).

2.8 Quantitative Microbial Risk Assessment (QMRA)

Quantitative microbial risk assessment (QMRA) has been developed at the national and international levels as a systematic and objective approach for appraising information identified with microbial hazards in foods and linked risks, pointing out both likelihood and impact of disease (Lammerding and Paoli, 1997; Romero-Barrios et al., 2013). QMRA is as a predictive and decision-making tool and aims to determine the data gaps in the database and requirement of additional information (Montville and Schaffner, 2005). The U.S. Department of Agriculture (USDA) and Environmental Protection Agency (EPA) published a microbial risk assessment guideline for microbial contamination of food and water (USDA/EPA, 2012). The QMRA approach includes four components: (1) hazard identification, (2) exposure assessment, (3) hazard characterization (Dose-response assessment), and (4) risk characterization (Cassin et al., 1998). These four components are described in detail in Chapter IV.

QMRA generally includes different mathematical models describing the growth and survival of bacteria, consumption of the bacteria in food, and subsequent illness, with probability distributions and Monte Carlo simulation. A probability distribution is a mathematical representation of the relative possibility of a spontaneous parameters which choosing on a specific value. Uncertainty linked to the model and the level of the human illness are estimated by using Monte Carlo simulation. Disinfection and

decontamination strategies may be computed in the same way and cost/benefit may be managed for prevention of illness.

So far, available QMRA models for the risks linked to leafy greens vegetables throughout the supply chain are very limited. In the U.S., the preliminary QMRA framework for risk linked to leafy greens from farm to consumption contributed with initial risk estimates for *E. coli 0157:H7* in leafy greens (Danyluk and Schaffner, 2011). Carrasco et al. (2010) determined the risk of *L. monocytogenes* in ready-to-eat lettuce salads from farm to table in Spain. In this study, the estimated number of listeriosis cases was 10^2 and 10^5 for low and high risk subpopulations respectively. Besides, according to this study, MAP was a very effective method to decrease the number of cases. Tromp et al. (2010) assessed the risk of *E. coli 0157:H7, Salmonella*, and *L. monocytogenes* in leafy green vegetables consumed at salad bars, based on modeling supply chain logistics in the Netherlands. This study showed that the risk of listeriosis-induced fetal mortality in the perinatal population raised from $1.24*10^{-4}$ (fixed storage time) to $1.66*10^{-4}$ (supply chain logistic). Franz et al. (2010) also assessed the risk of *E. coli 0157, Salmonella*, and *L. monocytogenes* in leafy green vegetables consumed at salad bars in Netherlands. They estimated the average number of cases per year linked to the consumption of leafy greens at salad bars were 166, 187, and 0.3 for *E.coli 0157:H7, Salmonella,* and *L. monocytogenes* respectively. Ding et al. (2013) determined risk of *L. monocytogenes* on lettuce from farm to table in Korea. This study found that the final contamination levels of *L. monocytogenes* at restaurant and home were -1.50 log CFU/g and -0.146 log CFU/g respectively. They also estimated the average number of annual

listeriosis cases varied from 559 to 817, depend on the different *r*-values employed in the exponential dose-response model (this is explained in Chapter IV), which means the incidence of listeriosis ranged from 11.9 to 17.4 cases per million person. Puerta-Gomez et al. (2013b) assessed the risk of contamination of ready-to-eat spinach with *Salmonella* in the U.S. they and found that irradiation was the most effective means to reduce the number of tainted samples from 84% to 0.1% for highly cross-contaminated lots (3 log CFU/g).

CHAPTER III

MODELING GROWTH OF *LISTERIA MONOCYTOGENES* AND *LISTERIA INNOCUA* ON FRESH BABY SPINACH LEAVES

3.1 Overview

Predictive models are valuable tools for manufacturers to develop process controls to reduce the risk of pathogen contamination in fresh and fresh-cut produce. These predictive models can also provide an estimate of the produce shelf life based on "microbial safety".

The objectives of this study were to: (1) validate the use of *L. innocua* as a surrogate for *L. monocytogenes* to simulate the growth of the pathogenic strain on fresh baby spinach leaves and, reduction of bacterial counts on the produce during washing; (2) develop and validate a dynamic model for prediction of growth of *L. monocytogenes* and *L. innocua* under different storage temperature; and (3) evaluate the effect of temperature and natural microflora on the growth of both microorganisms on fresh baby spinach leaves.

Results showed that the total reduction of *L. monocytogenes* and *L. innocua* inoculated in fresh baby spinach leaves after washing was significantly different ($P<0.05$) for both microorganisms. For water and chlorine washing, the observed average $\log_{10}$ reduction of *L. monocytogenes* and *L innocua* was 0.96 and 1.60 at room temperature (~22^{o}C), respectively.

Growth data for *L. monocytogenes* and *L. innocua* on fresh baby spinach leaves were collected at 5, 10, 20, 30, and 36ºC. Three replications were performed for each temperature. Growth curves were fitted to the primary model (Baranyi) to estimate the initial cell concentration, (y_o), lag time, (t_{lag}), maximum population density, (y_{max}), and maximum growth rate, (μ_{max}). With the exception of R^2=65 for *L. innocua* (the initial concentration of total mesophilic bacteria was 5.51 log CFU/g at 10ºC), the model showed a good correlation coefficient at all temperatures and concentration of natural microflora (R^2>0.70) for both microorganisms. Root mean squared error (RMSE) ranging from 0.09 to 0.47 log CFU/g for *L. monocytogenes* and from 0.08 to 0.42 log CFU/g for *L. innocua* was also used to evaluate the model`s performance. In addition, the natural background microflora affected the growth of *L. innocua* more than *L. monocytogenes* on fresh baby spinach leaves especially at temperatures 10>T>30.

For each microorganism, a dynamic model was developed and validated for y_{max}, t_{lag}, and μ_{max}. The accuracy factor, (A_f), bias factor, (B_f), and the standard error of prediction, (%SEP), were also calculated and evaluated between observed data and predicted values. The results pointed out that the developed secondary models displayed a good agreement between the observed and predicted values, with the exception for the lag time of *L. innocua*.

These results suggest that, before *L. innocua* is used as a surrogate for *Listeria monocytogenes*, it should be tested for each particular produce. These models for *L. monocytogenes* can be suitable to estimate and control the growth of *L.monocytogenes* growth on fresh baby spinach.

3.2 Introduction

The consumption of fresh and fresh-cut vegetables has increased in recent years worldwide (Maatta et al., 2013). Codex Alimentarius Commission (CAC) wanted that the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to contribute scientific advice to develop commodity-specific annexes for the Codex Alimentarius "Codex of Hygienic Practice for Fresh Fruits and Vegetables". The reason behind this request was that the committee emphasized the need to determine connections with the control of specific hazards of concern, especially fresh fruit and vegetable products, and support terms of reference as guidance to the type of scientific advice needed (CAC, 2003).

In the United States, there are nearly 2500 cases of listeriosis annually and these cases result in about 500 deaths (Morrissey et al., 2010). Due to these high infection rates and especially high number of fatalities, approximately 20-30% of all foodborne fatalities, listeriosis is an important concern (Milillo et al., 2012). *L. monocytogenes* is a common pathogenic microorganism in nature and has been found on numerous domesticated animals (Park et al., 2012). *L. monocytogenes* and *L. innocua* are the two most widespread *Listeria* spp. isolated in human feces, sewage, and soil (Beuchat, 1996b). Therefore, food products can be contaminated with *L. monocytogenes* during the pre-harvesting process. *L. monocytogenes* can grow in a wide pH range (from 4.1 to 9.6) and at refrigerator temperature and it has high resistance against high levels of salt (up to 10%) (Cole et al., 1990). Furthermore, Kalmokoff et al. (2001) showed that *L. monocytogenes* has displayed the ability to form biofilms adhering to food products.

Biofilm formation allows necessary chemical transfer processes to happen between cells and the external environment whereas protecting bacteria against potentially harmful environment al conditions such as disinfectants (Nilsson et al., 2011).

L. monocytogenes is feasible for laboratory experiments, but because of the risk of exposing vulnerable individuals it cannot be safely studied in such environments if they are not Biosafety level 2 labs. Therefore, the use of a surrogate organism is crucial to understand the behavior of *L. monocytogenes* in foods and food processing environments. *Listeria innocua* is usually used as a surrogate for *L. monocytogenes* due to their close genetic relationship (Guo et al., 2013). However, O`Bryan et al. (2006) determined that *L. innocua* surrogate strains cannot exactly mirror the behavior of *L. monocytogenes* in all test conditions/foods, containing thermal and other stresses. Friedly et al. (2008) also demonstrated that the survival ability of *L. innocua* strains in thermal treatments is different from *L. monocytogenes*. Therefore, the validation of *L. innocua* as a surrogate for *L. monocytogenes* should be proved for each food matrix and decontamination treatments such as water and chlorine washing, especially in the case of leafy greens.

Several bacterial growth models have been developed. Although most predictive models have been constructed in an artificial nutrient broth environment, many of them have been developed in real foods (Farber et al., 1995; Murphy et al., 1996; Koseki and Isobe, 2005a). Several bacterial growth models have been built in for fresh produce (Rodriguez et al., 2000; Koseki and Isobe, 2005b; Puerta-Gomez et al., 2013a). Nevertheless, factors such as temperature, the food product, and the indigenous

microflora of fresh produces significantly influence the growth and death of pathogenic microorganisms. Duh and Schaffner (1993) determined that *L. innocua* grows faster than *L. monocytogenes* at temperatures below 42°C. This study also showed that the lag time of *L. monocytogenes* is longer than that of *L. innocua* at temperatures below 8°C. Thus, if *L. innocua* is used as a surrogate for *L. monocytogenes* at these conditions ($8 \geq T \leq 42$°C), the result collected will include errors. Therefore, the validation of *L. innocua* as a surrogate for *L. monocytogenes* under the same conditions should be tested.

The objectives of this study were to (1) validate the feasibility of using *L. innocua* as a surrogate of *L. monocytogenes* to predict growth of *L. monocytogenes* on fresh baby spinach leaves under storage (constant temperature) and reduction of the pathogen on the produce during washing (water and chlorine) treatments; (2) determine the effect of initial level of *L. innocua* and *L. monocytogenes,* and natural microbiota load on the surface of baby spinach leaves, and their interaction on the growth curves of *L. innocua* and *L. monocytogenes* at different storage temperatures (5, 10, 20, 30, and 36°C); (3) predict the growth patterns of *L. innocua* and *L. monocytogenes* when fresh baby spinach contains different natural microbiota levels at different storage temperatures (5, 10, 20, 30, and 36°C); and (4) develop predictive growth equations to estimate the growth of *L. innocua* and *L. monocytogenes* on fresh baby spinach leaves.

3.3 Materials and Methods

3.3.1 Food Material

Bags of fresh baby spinach leaves (*Spinacea oleracea*) were obtained from a local grocery store. All bags with same expiration date were selected to ensure

uniformity of the produce. The product in package was stored at 5°C for no more than 24 hours prior to the experiments. All leaves showing signs of wilt and decay were discarded and then 5-g was weighted and dispensed into a sterile stomacher bags (18 oz Whirl Pak® bag) before inoculation.

3.3.2 Initial Natural Microbiota Enumeration

Fresh baby spinach leaves were analyzed at the beginning of every experiment following a plate count enumeration technique. Aerobic mesophilic bacteria were enumerated by spread plating on Tryptic Soy Agar (TSA) incubated at 36°C for no more than 48 hours. Yeasts and molds were quantified by spread plating on Sabouraud Dextrose Agar (pH 5.6, adjusted with 0.1% citric acid) after 5 days of incubation at 20°C.

3.3.3 Inoculation and Preparation of Spinach Samples

Five-gram portions of spinach leaves were dispensed into sterile stomacher bags (18 oz Whirl Pak® bag) and inoculated with 0.5 ml of the 10^2 CFU/ml (*L. innocua and L. monocytogenes* spp.) inoculum. The bags were gently shaken for 30 times to evenly spread the inoculum over the sample. Four bags were prepared for each sampling time and the experiment was performed in triplicate. The inoculated samples were then placed in an incubator (VWR International, Model 1510E, IL, USA) maintained at constant temperature (5, 10, 20, 30, and 36^{o}C).

3.3.4 Bacterial Cultures

L. monocytogenes (ATCC 15313, ATCC 33090, Scott A, Strain A) and rifampicin-resistant (80 μg/ml) culture of *Listeria innocua* (NRCC B33076) were acquired from Dr. Carmen Gomes' stock laboratory (Department of Biological and Agricultural Engineering, Texas A&M University) stored at -80°C. Before being used, an inoculum was removed from the frozen culture with a loop, streaked onto Tryptose Phosphate Broth (TPB; Difco, Detroit, MI), and incubated for 24 hours at 36°C. Next, single colony isolates were obtained by streaking on Oxford Listeria-selective agar supplemented with 80 μg/ml of rifampicin (OLR) and incubated at 36 °C for 24 h through two successive transfers on Oxford Listeria plus rifampicin (OLR). Colonies were stored on TSA slant at 5°C as working cultures and used within 90 days.

3.3.5 Inoculum Preparation

A loop inoculum was transferred from the working culture at 5°C to TPB test tubes and incubated at 36°C for 18 hours. After incubation, each test tube was centrifuged and washed for three consecutive times (3000 X *g* for 15 min) with Difco buffered peptone water. Subsequently, each pellet was resuspended in 0.1% peptone water (PW). The Optical density at 600nm (OD_{600}) of the cell suspensions was adjusted to 0.5 of absorbance for bacterial preparation. An approximate initial concentration of 10^7 CFU/ml was confirmed by making serial dilutions of the inoculum suspension in 9-ml test tubes of PW. The suspension was plated on OLR and incubated at 36°C until visible black colonies could be counted. To prepare the inoculum, serial dilutions of

initial population in PW were carried out to reach a target level of 10^2 CFU/ml of *Listeria innocua* and *L. monocytogenes* strains.

3.3.6 Washing and Sanitation (Chlorine) Treatments

Four bags of fresh baby spinach samples (5-g per sample in 18 oz Whirl Pak® bag) for each bacteria with different initial inoculum loads (10^3, 10^4, and 10^5 CFU/ml) of *L. innocua* and *L. monocytogenes* were washed with tap water for 10-minutes at room temperature. During the treatment, the washing solution was occasionally stirred. Next, four different bags of produce samples (5-g per sample in18 oz Whirl Pak® bag) with same initial loads as for the water washing treatment were treated with 200 ppm of chlorinated water at pH 7.0 (reduced with 0.1 N of HCI) for 10-minutes at room temperature. The solution was occasionally stirred during the treatment. After the treatment, each sample (5-g) was placed in an 18-oz. stomacher bag and kept at 5^{o}C for 2 hours. Then, the number of microorganisms remaining on the surface of the fresh baby spinach was determined by using microbial enumeration methods.

3.3.7 Microbial Enumeration

Each 5-g sample of fresh baby spinach inoculated with *L. monocytogenes* and *L. innocua* were hand pummeled with 45 ml of Difco buffered peptone water (BPW; Difco, Detroit, MI) in an 18 oz Whirl Pak® bag until samples were reduced to small pieces, allowing the internal leaf structure to be exposed. Samples of 1 ml from original Whirl Pak® bag and 0.1 ml from serial dilution in 0.1 % of PW were plated in duplicate (0.1ml) on Oxford *Listeria*-selective agar supplemented with Oxford *Listeria*-selective

supplement, which was incubated 24 h at 37ºC for *Listeria monocytogenes* enumeration. For *Listeria innocua* enumeration, the dilutions were plated on Oxford Listeria-selective agar with 80 µg/ml of rifampicin, which was incubated 24 h at 37ºC. After incubation, visible colonies were enumerated with the use of a magnifier counter (detection limit was 10 CFU/g of sample).

3.3.8 Isothermal Growth Data

Growth data of *Listeria monocytogenes* and *Listeria innocua* at 5, 10, 20, 30 and 36 °C were collected to obtain the bio-kinetic growth temperatures at different levels of initial inoculums and natural microbiota for both microorganisms. The inoculated samples (stomacher bags) were placed in an incubator (VWR International, Model 1510E, IL, USA) maintained at constant temperature. Three independent replications for each temperature were performed. Four samples (18 oz stomacher bags) were prepared for each sampling time. About 10 min were allowed for temperature stabilization, and 4 samples were removed to estimate initial inoculum using microbial enumeration methods.

3.3.9 Growth Models

3.3.9.1 Primary Model

Growth curves were fitted using the DMFit Excel Add-In software (kindly provided by J. Baranyi, Institute of Food Research, Norwich, UK) to fit the Baranyi and Roberts (1994) model in order to estimate the main growth parameters, i.e. specific

growth rate and lag time. The four- parameter Baranyi functions are described in Chapter II (Equations 2.4 and 2.5).

The ability of the primary model to predict the growth pattern of *L. innocua* and *Listeria monocytogenes* in fresh baby spinach leaves was evaluated by the root mean square error (RMSE) and coefficient of determination (R^2). Both RMSE and R^2 were calculated as follows;

$$R^2 = 1 - (SSR/SST) \tag{3.1}$$

where, *SSR* is the sum of squares of residuals and *SST* is the total sum of squares. The root mean squared error (*RMSE*) was also used to evaluate the model's performance:

$$RMSE = \sqrt{\frac{SSE}{N-p}} \tag{3.2}$$

where, *N* is the number of observations, and *p* is the number of model parameters. The above two statistical quantities were calculated for each temperature after fitting the growth data into Eq. (2.4, Chapter II).

3.3.9.2 Secondary (Dynamic) Models

The modified Ratkowsky equation was used to describe the effect of temperature on growth rate (from Eq. 2.13, Chapter II):

$$\mu_{max} = (b * (T - T_{min}))^2 \tag{3.3}$$

where, the parameter $T_{\min}$ represents the theoretical minimum temperature beyond which growth of the organism is impossible. The parameter *b* is a regression coefficient.

Equation (3.3) represents the growth rate up to the optimum growth temperature for any temperature change (dynamic model).

An inverse simple Ratkowsky-type model was also used to predict lag time, (t_{lag}), (from Eq. 2.20, Chapter II):

$$t_{lag} = (c * (T - T_{\min}))^{-2} \quad (3.4)$$

where c is a regression coefficients.

In addition, a second-order polynomial was used to estimate the values of y_{max} as a function of temperature (Puerta-Gomez et al., 2013a):

$$y_{max} = A_1 * T^2 + A_2 * T + A_3 \quad (3.5)$$

where, y_{max} refers to maximum cell concentration (log CFU/g), and A_1, A_2, and A_3 are regression coefficients.

3.3.9.3 Validation of Dynamic Models

Before using the dynamic models for quantitative microbial risk assessment (QMRA), the efficiency of the dynamic models should be validated (Ding et al., 2012). Therefore, the performance of the dynamic model for each microorganism was evaluated using three performance indices: Bias Factor (B_f), Accuracy Factor (A_f), and the standard error of prediction expressed as a percentage (%SEP).

The bias factor is used to measure the model prediction bias that computes the mean difference between the actual and predicted values (Ding et al., 2012),

$$B_f = 10^{(\Sigma_{i=1}^{n} \log(\frac{O}{P})/n)} \quad (3.6)$$

The accuracy factor is a measurement of accuracy calculating the proximity of predicted values to the actual values (Ding et al., 2012),

$$A_f = 10^{(\Sigma_{i=1}^{n} |\log(\frac{P}{O})|/n)} \quad (3.7)$$

The Standard Error of prediction is a relatively typical deviation of the mean prediction values. Hervas et al. (2001) reported that %SEP is more accurate to evaluate the fitting and prediction accuracy of the models because it is a dimensionless criterion,

$$\%SEP = \frac{100}{Average(O)} \sqrt{\frac{\sum(O-P)^2}{n}} \quad (3.8)$$

where, *O* is the observed value; *P* is the predicted value; and *n* is the number of observations and predictions.

3.3.10 Statistical Analysis

Data analysis for washing treatments was performed using SPSS software (version 20.0 for Windows, 2011). Statistical differences between variables were analyzed for significance by one-way ANOVA using Tukey`s multiple range tests. Statistical significance were determined at the $P<0.05$ levels.

3.4 Results and Discussion

3.4.1 Water and Chlorine Washing

The effect of water and chlorine washing treatments on the reduction of *L. monocytogenes* and *L. innocua* inoculated on fresh baby spinach leaves is presented in Table 3.1. As expected, a particular level of initial population load and washing with chlorine was more effective ($P<0.05$) than water washing for both microorganisms. Furthermore, treatment of fresh baby spinach inoculated with different initial population load of *L. monocytogenes* and *L. innocua* using water and chlorine water had no significant ($P>0.05$) effect in reducing bacterial numbers. However, when the initial population load was decreased to 10^3 CFU/g for *L. innocua*, a significantly ($P<0.05$) higher log reduction of bacterial population was observed for chlorine washing (Table 3.1). This result suggests that washing fresh baby spinach leaves inoculated with low levels of *L. innocua* with water-chlorine was significantly more effective ($P<0.05$) in removing *L. innocua*. This may be due to the fact that low microbial population are not able to attach as well as higher microbial population. Singh et al. (2002) found that when the initial population load of *E.coli 0157:H7* on lettuce was further reduced, a significantly ($P<0.05$) higher log reduction of bacterial population was observed as compared to bacterial log reduction from higher initial populations.

The levels of reduction of microbial populations due to water washing were significantly different ($P<0.05$) for *L. monocytogenes* and *L. innocua* (Table 3.1). Therefore, the total reduction of both microorganisms was significantly different

Table 3.1. Effect of washing treatment and initial population loads on the log-reductions of *Listeria monocytogenes* and *Listeria innocua* inoculated in fresh baby spinach leaves.

		Reductions (log CFU/g)	
Microorganism	**Initial population load (CFU/g)**	**Water washing**	**Chlorine washing**
Listeria monocytogenes	10^3	0.58^{a} [1](0.17)	0.97^{a} (0.34)
	10^4	0.59^{a} (0.16)	$1.05^{a,c}$ (0.31)
	10^5	0.46^{a} (0.05)	0.87^{a} (0.22)
Listeria innocua	10^3	1.11^{b} (0.26)	1.92^{b} (0.34)
	10^4	1.10^{b} (0.14)	1.48^{c} (0.30)
	10^5	1.01^{b} (0.22)	1.41^{c} (0.19)

[1]: Standard Deviation

[a,b,c] : Means within a column, which are not followed by a common superscript letter are significantly different ($P<0.05$).

($P<0.05$) with *L. monocytogenes* being more difficult to remove from the leafy green. Ijabadeniyi et al. (2011) found similar results for total (water and chlorine) reductions of *L. monocytogenes* on spinach. These authors reported that *L. monocytogenes* was able to attach to the surface of spinach rapidly. In a previous study, Ells and Hansen (2006) also determined that *L. monocytogenes* could attach to whole and cut cabbage within 5 minutes of exposure. Furthermore, several studies found the same time range of attachment of *L. monocytogenes* to lettuce, cantaloupe, and *Arabidopsis thuliana* (Li et al., 2002; Ukuku and Fett, 2002; Milillo et al., 2008). Besides, Warning and Datta (2013) reported that each type of microorganisms has its own method of attachment and can vary even within different strains of the same bacteria. Milillo et al. (2012) reported that *L. monocytogenes EGD-e* has 19 genes which is not found in *L. innocua* that putatively encode proteins containing leucine-rich repeats (*LRR*), implicated in *L. monocytogenes* attachment to and invasion of host cells, and Leu-Pro-X-Thr-Gly (*LPXTG*) motifs. These authors also determined that the motility of *L. monocytogenes* is different from that of *L. innocua*.

Van Houdt and Michiels (2010) determined that the major bacterial factors affecting attachment and strength of attachment are the bacterial growth conditions, hydrophobicity, surface charge, attachment surface, and surface appendages. These authors also reported that *L. monocytogenes* biofilms were more resistant to cleaning agents and disinfectants such as trisodium phosphate, chlorine, ozone, hydrogen peroxide, peracetic acid (PAA), and quaternary ammonium compounds. According to Szomolay et al. (2005), biofilm resistance to cleaning agents is because of biocide into

the biofilm. They determined the mechanism of biocide resistance is based on that some of the biofilm cells are able to sense the biocide challenge and actively respond to it by deploying protective stress responses more effectively than planktonic cells. As a result, the different levels of microbial reductions of water-chlorine washing for *L. monocytogenes* and *L. innocua* can be explained by the fact that *L. innocua* does not attach as well as *L. monocytogenes* to the surface of fresh baby spinach leaves.

3.4.2 The Effect of the Presence of Background Microbiota on the Growth of *Listeria monocytogenes* and *Listeria innocua*

Both *L. monocytogenes* and *L. innocua* grew in fresh baby spinach very well at the different temperatures tested in this study. Growth data for each bacteria and temperature were fitted into the Baranyi model (Eq.2.4) and the fitted curves produced are presented in Figures A.1 to A.10 (Appendix). Next, the obtained growth parameters (y_o, y_{max}, t_{lag}, and μ_{max}) for each temperature and the initial concentration of total mesophilic bacteria, (M_o), for *L. monocytogenes* and *L. innocua* were determined (Tables 3.2 and 3.3, respectively). The RMSE values varied from 0.10 to 0.47 log CFU/g and from 0.08 to 0.42 log CFU/g for *L. monocytogenes* and *L. innocua*, respectively.

At 5^{o}C, when the initial concentration of total mesophilic bacteria, (M_o), increased by 27.86% and 17.87%, t_{lag} of *L. monocytogenes* decreased by 32.45% (Table 3.2) and that of *L. innocua* increased 244.22% (Table 3.3), respectively. In addition, although, μ_{max} of *L. monocytogenes* on fresh baby spinach leaves decreased by 28.50% and 20.41 % when the M_o on fresh baby spinach leaves increased by 27.86% and

Table 3.2. Effect of natural microflora on estimated maximum population density, maximum growth rate, and lag time, of *Listeria monocytogenes* inoculated in fresh baby spinach leaves determined using Baranyi model (Eq. 2.4).

Temperature (°C)	**[a]M_o (log CFU/)**	**[b]y_o (log CFU/)**	**[c]y_{max} (log CFU/)**	**[d]μ_{max} (log CFU/g/hour)**	**[e]t_{lag} (Hours)**	**R^2**	**[f]RMSE (log CFU/g)**
5	4.94	2.26	3.97	0.0421	158.45	0.91	0.10
5	5.53	1.92	4.30	0.0350	109.91	0.95	0.12
5	6.31	2.07	4.14	0.0301	107.03	0.96	0.26
10	4.94	2.58	5.41	0.0500	45.60	0.93	0.39
10	5.53	2.42	5.66	0.0490	39.98	0.95	0.47
10	6.31	2.64	5.76	0.0499	43.06	0.95	0.37
20	5.43	2.44	5.88	0.2128	6.03	0.97	0.39
20	5.98	2.30	5.73	0.2747	3.07	0.90	0.11
20	6.31	1.92	5.55	0.5894	4.12	0.76	0.32
30	5.22	2.38	7.42	0.7411	3.47	0.94	0.29
30	5.78	1.92	7.67	0.8507	1.29	0.90	0.12
30	6.52	2.11	5.60	0.7539	0.29	0.78	0.19
36	4.94	2.26	7.43	1.2503	2.403	0.87	0.23
36	5.53	1.90	7.73	1.1100	0.808	0.87	0.20
36	6.31	1.99	7.43	0.995	0.995	0.89	0.16

[a] (M_o) :Initial concentration of total mesophilic bacteria (log CFU/g); [b]y_o: Initial population density (Eq. 2.3) (log CFU/g); [c]y_{max}: Maximum population density (Eq. 2.3) (log CFU/g); [d]μ_{max}: Maximum growth rate (Eq. 2.3) (log CFU/g/h); [e]t_{lag}: Lag time (Eq. 2.3) (hours); [f]RMSE: Root mean square root (Eq. 3.2) (log CFU/g)

Table 3.3. Effect natural microflora on estimated maximum population density, maximum growth rate, and lag time of *Listeria innocua* inoculated in fresh baby spinach leaves determined using Baranyi model (Eq. 2.4).

Temperature (°C)	**[a]M_o (log CFU/g)**	**[b]y_o (log CFU/g)**	**[c]y_{max} (log CFU/g)**	**[d]μ_{max} (log CFU/g/ hours)**	**[e]t_{lag} (Hour)**	**R^2**	**[f]RMSE (log CFU/g)**
5	5.51	2.52	4.93	0.0280	70.36	0.96	0.24
5	5.46	1.57	3.88	0.0515	169.76	0.89	0.08
5	6.50	1.75	3.25	0.0331	242.19	0.79	0.29
10	5.51	2.49	6.15	0.0976	17.32	0.65	0.36
10	5.29	2.19	5.71	0.0751	48.09	0.91	0.29
10	6.50	1.86	5.04	0.0595	30.34	0.84	0.42
20	5.51	2.52	6.84	0.4586	2.32	0.75	0.24
20	5.30	2.41	7.09	0.3554	3.89	0.91	0.37
20	7.08	2.43	4.24	0.2962	4.16	0.72	0.27
30	5.81	2.70	7.60	0.8114	0.13	0.82	0.23
30	5.12	1.97	6.26	0.9141	0.26	0.73	0.31
30	6.03	1.93	7.17	0.9561	0.69	0.80	0.33
36	7.02	3.36	7.59	0.9898	2.10	0.87	0.16
36	7.19	2.14	7.05	1.1881	2.05	0.86	0.23
36	7.15	1.45	5.73	1.8580	2.89	0.76	0.26

[a] M_o: Initial concentration of total mesophilic bacteria (log CFU/g); [b]y_o: Initial population density (Eq. 2.3) (log CFU/g); [c]y_{max}: Maximum population density (Eq. 2.3) (log CFU/g); [d]μ_{max}: Maximum growth rate (Eq. 2.3) (log CFU/g); [e]t_{lag}: Lag time (Eq. 2.3) (hours); [f]RMSE: Root mean square root (Eq. 3.2) (log CFU/g)

27.83% at 5 and 36ºC (Table 3.2), respectively. Furthermore, when the M_o increased by 17.87%, 28.52%, 3.90% and 1.70%, μ_{max} of *L. innocua* decreased by 39.02% and 35.41% at 10 and 20ºC while those values increased by17.84% and 87.71% at 30 and 36ºC (Table 3.3), respectively.

In addition, these differences on behavior caused by total mesophilic bacteria affected the maximum population density, y_{max}, of both microorganisms. At 5ºC, when the initial concentration of total mesophilic bacteria, M_o, increased, although the y_{max} of *L. monocytogenes* increased by 4.49%, that of *L.innocua* decreased by 34.09%. Furthermore, when the M_o increased by 27.83% and 1.70%, the y_{max} of *L. innocua* decreased by 24.46% (Table 3.3) whereas that of *L. monocytogenes* did not change at 36ºC, respectively. These results show the total mesophilic bacteria at 5ºC and 36ºC affected the growth of *L. innocua* more than *L. monocytogenes*. Francis and O`Beirne (1998) reported similar results for the effects of the indigenous microflora of minimally processed lettuce on the survival and growth of *L. innocua*. Likewise, several studies reported similar results verifying that the initial background bacteria did not affect the growth of *L. monocytogenes* (Beuchat and Brackett, 1990; Gleeson, 2005; Carrasco et al., 2008). However, Koseki and Isobe (2005a) determined that the background flora of iceberg lettuce significantly decreased the y_{max} of *L. monocytogenes* on iceberg lettuce when temperatures were below 25ºC. This result may be due to fact that competitive subpopulations of the microflora such as lactic acid bacteria (LAB) and *Enterobacter* spp. in food matrices is different or reductions of these competive subpopulations in

commodities is different after disinfection treatments (Johnston et al., 2005b; Carrasco et al., 2008).

The different growth behaviors of these two microorganisms under the presence of natural microbiota may be due to different mechanisms responsible for bacterial inhibition by biocontrol agents, containing production of inhibitory compounds, competition for nutrients, space or even colonization sites on produce affected each microorganism differently (Olaimat and Holley, 2012). Several microorganisms including lactic acid bacteria (LAB) on fresh baby spinach produce bacteriocins, cationic antimicrobial peptides (Trias et al., 2010). Alegra et al. (2013) reported that *Pseudomonas* spp. showed antagonistic capacity to inhibit the growth of *E. coli 0157:H7*, *Salmonella*, and *L. innocua* on fresh-cut apple and peach. In another study, Alegra et al. (2010) determined that *Enterobacteriaceae* spp. effectively inhibited the growth of, or reduced, in some cases to below the limit of detection, *E. coli 0157:H7, Salmonella* and *L. innocua* on fresh-cut apples and peaches.

In addition to these mechanisms, the different growth behaviors of these two microorganisms may be due to the different stress responses of *L. innocua* and *L. monocytogenes*. Stress response is crucial for microorganisms because their habitats are subjected to continual changes in temperature, osmotic pressure, toxic compounds, and nutrients availability (Capozzi et al., 2009). Nufer et al, (2007) determined that some *L. monocytogenes* strains possessed better cold tolerance than other *Listeria* spp., containing *L. innocua* in the brain heart infusion broth at4 ^{o}C. Milillo et al. (2012) reported that the heat resistance of *L. innocua M1* is higher than that of *L.*

monocytogenes. However, Patil et al. (2010) reported that heat resistance of *L. monocytogenes* was dependent on the type of strain. For example, Cava-Roda et al. (2012) reported that a sub-population of strain Scott A, containing a mutation in *ctsR* (class III heat-shock regulator), was more thermo-resistant than the wild type strain. In addition, *L. monocytogenes* can be more resistant against acid than *L .innocua* because Lactic acid bacteria (LAB) on fresh baby spinach can produce acid. Skandamis et al. (2008) showed that acid resistance of *L. monocytogenes* are also strain dependant as heat resistance. For instance, Milillo et al. (2012) reported that the protein products of *gad* genes contribute to the acid resistance of *L. monocytogenes* and the *gadD1/T1* homolog is not found in *L. innocua CLIP11262*.

In conclusion, the combined treatments (temperature and the native bacterial flora of fresh baby spinach leaves) had a different effect on the growth inhibition of *L. innocua* and *L. monocytogenes* due to genomic difference and bacterial stress response. Thus, *L. monocytogenes* and *L. innocua* respond differently under the presence of natural background microflora on fresh baby spinach.

3.4.3 Growth Models of *L. monocytogenes* and *L. innocua* in Fresh Baby Spinach

In general, the maximum growth rate of the *L. monocytogenes* was between 0.0023 and 0.2264 log CFU/g/h less than that of *L. innocua* when temperatures changed between 5 and 36ºC (Tables 3.4 and 3.5). The RMSE values varied from 0.09 to 0.41 log CFU/g and from 0.20 to 0.31 for *L. monocytogenes* and *L. innocua*, respectively (Tables 3.4 and 3.5). The lower RMSE values indicate a better fit of the model. The R^2 values varied from 0.85 to0.94 and from 0.79 to 0.92 for *L. monocytogenes* strains and *L.*

innocua, respectively. Overall, the Baranyi model provided a good fit to the data from all temperatures at which growth was observed (Figures A.11 to A.15, Appendix).

Table 3.4 shows the maximum population density (y_{max}) of *L. monocytogenes* growth on fresh baby spinach leaves varied from 4.15 to 7.53 log CFU/g and when temperature raised from 5 to 36°C, respectively. These values for *L. innocua* ranged from 4.02 to 7.01 log CFU/g (Table 3.5). The difference in results between the two microorganisms may be because of the different effect of the background microflora on the growth of *L. monocytogenes* and *L. innocua*. Koseki and Isobe (2005a) and Carrasco et al (2008) determined similar y_{max} values for *L. monocytogenes* on iceberg lettuce for temperatures between 5 and 25°C. However, Fang et al. (2013) reported y_{max} for *L. monocytogenes* in fresh-cantaloupe of 8.0 log CFU/g, with a standard deviation of 0.5 log CFU/g, at temperatures between 10 and 40°C. The discrepancy with our results may be because of the different type of produce which has different pH (5.5-6.3) (Babic and Watada, 1996), free water activity (92.1%) (Bhattacharjee et al., 1999), and composition concentration of background microflora (Babic et al., 1996). Hoelzer et al. (2012b) reported that food chemistry affected by produce variety, growing season, storage conditions, and processing and actual values may differ considerably from the proxies, so that may also cause the different results .

The maximum growth rate (μ_{max}) for *L. monocytogenes* and *L. innocua* on fresh baby spinach leaves increased when temperature increased. It varied from 0.036 to 1.119

Table 3.4. Estimated maximum population density, maximum growth rate, and lag time of *Listeria monocytogenes* inoculated in fresh baby spinach leaves by using Baranyi model (Eq. 2.4).

Temperature (°C)	**[b]y_o (log CFU/g)**	**[c]y_{max} (log CFU/g)**	**[d]μ_{max} (log CFU/g/hr)**	**[e]t_{lag} (Hours)**	**R^2**	**[f]RMSE (log CFU/g)**
5	2.08 ±0.17	4.15 ±0.17	0.0357 ±0.01	125.13 ±28.89	0.94	0.09
10	2.55 ±0.12	5.61 ±0.18	0.0496 ±0.00	42.88 ±2.81	0.94	0.41
20	2.22 ±0.27	5.72 ±0.16	0.3590 ±0.20	4.41 ±1.50	0.85	0.33
30	2.14 ±0.23	6.90 ±1.13	0.7819 ±0.06	1.68 ±1.62	0.89	0.19
36	2.05 ±0.18	7.53 ±0.17	1.1186 ±0.13	1.40 ±0.87	0.88	0.2

[b]y_o: Initial population density (Eq. 2.3) (log CFU/g)

[c]y_{max}: Maximum population density (Eq. 2.3) (log CFU/g)

[d]μ_{max}: Maximum growth rate (Eq. 2.3) (log CFU/g/h)

[e]t_{lag}: Lag time (Eq. 2.3) (hours)

[f]*RMSE*: Root mean square root (Eq. 3.2) (log CFU/g)

Table 3.5. Estimated maximum population density, maximum growth rate, and lag time of *Listeria innocua* inoculated in fresh baby spinach leaves by using Baranyi model (Eq. 2.4).

Temperature (°C)	**[b]y_o (log CFU/g)**	**[c]y_{max} (log CFU/g)**	**[d]μ_{max} (log CFU/g /hr)**	**[e]t_{lag} (Hours)**	**R^2**	**[f]RMSE (log CFU /g)**
5	1.95 ±0.51	4.02 ±0.85	0.038 ±0.01	160.77 ±86.27	0.92	0.2
10	2.18 ±0.31	5.63 ±0.56	0.077 ±0.02	31.92 ±15.45	0.85	0.31
20	2.45 ±0.06	6.06 ±1.58	0.370 ±0.08	3.46 ±0.99	0.79	0.26
30	2.20 ±0.43	7.01 ±0.69	0.894 ±0.07	0.36 ±0.29	0.79	0.24
36	2.32 ±0.97	6.79 ±0.96	1.345 ±0.45	2.35 ±0.47	0.81	0.2

[b]y_o: Initial population density (Eq. 2.3) (log CFU/g)

[c]y_{max}: Maximum population density (Eq. 2.3) (log CFU/g)

[d]μ_{max}: Maximum growth rate (Eq. 2.3) (log CFU/g/h)

[e]t_{lag}: Lag time (Eq. 2.3) (hours)

[f]*RMSE*: Root mean square root (Eq. 3.2) (log CFU/g)

log CFU/g/h for *L. monocytogenes* and from 0.038 to 1.345 log CFU/g/h for *L. innocua* at 5-36 ^{o}C (Tables 3.4 and 3.5, respectively). Similarly, Duh and Schaffner (1993) reported that μ_{max} of *L. innocua* was higher than *L. monocytgenes* in brain heart infusion broth when temperature was below 42^{o}C. In addition, Hoelzer et al. (2012b) reported that several studies had similar results for *L. monocytogenes* on produce at 10^{o}C. On the other hand, Wang et al., (2013) stated that μ_{max} values for *L. monocytogenes* growth on cabbage were between 0.008 and 0.320 log CFU/g/h at 4-30 ^{o}C. Other studies reported that μ_{max} is changed by changing commodity, processing form, atmosphere composition and storage conditions (Beuchat and Brackett, 1990; Gonzalez-Fandos et al., 2001; Corbo et al., 2005; Hoelzer et al., 2012b).

As shown in Tables 3.4 and 3.5, the estimated lag time (t_{lag}) for both *L. monocytogenes* and *L. innocua* on fresh baby spinach leaves decreased when temperature increased, with the exception for *L. innocua* at 36^{o}C. For *L. monocytogenes*, t_{lag} ranged from 125.13 to 1.40 hours as temperature varied from 5 to 36^{o}C, respectively. For *L. innocua*, those values ranged from 160.77 to 0.36 hours (at 30^{o}C), 2.34 hours (at 36^{o}C) as temperatures varied from 5 to 36^{o}C. However, Duh and Schaffner (1993) determined that at temperatures below 8^{o}C, t_{lag} of *L. monocytogenes* was longer than that of *L. innocua* and when temperatures were greater than 8^{o}C, t_{lag} of *L. monocytogenes* was slightly shorter than that of *L. innocua* in brain heart infusion broth. The discrepancy with our results may be explained by the fact that although, under stress conditions such as low and high temperature, *L. innocua* has better tolerance than *L.*

monocytogenes, the effect of natural background microflora of fresh baby spinach leaves affected the growth of *L innocua* more than that of *L. monocytogenes*.

3.4.4 Secondary (Dynamic) Growth Models *Listeria monocytogenes* and *Listeria innocua*

Figures A.16 to A.21 (Appendix) illustrate the effect of temperature on maximum growth rate (μ_{max}) (Eq. 3.3), lag time (t_{lag}) (Eq. 3.4), and maximum population density (y_{max}) (Eq. 3.5), of *L. monocytogenes* and *L. innocua* on fresh baby spinach leaves. The coefficients of Eq. (3.3) for *L. monocytogenes* and *L. innocua* are presented in Table 3.6. In addition, Coefficients of Eq. (3.4) and Eq. (3.5) are presented in Tables 3.7 and 3.8, respectively.

Equations determining the relationship of growth parameters and temperature for *L. monocytogenes* and *L. innocua* grown in fresh baby spinach leaves from 5 to 36°C are shown in Equations 3.9, 3.10, and 3.11 and Equations 3.12, 3.13, and 3.14, respectively:

$$\mu_{max} = (0.026 * (T + 4.26))^2 \quad (3.9)$$

$$t_{lag} = (0.0099 * (T + 4.26))^{-2} \quad (3.10)$$

$$y_{max} = (-0.0009) * T^2 + 0.259 * T + 9.0512 \quad (3.11)$$

$$\mu_{max} = (0.028 * (T + 4.26))^2 \quad (3.12)$$

$$t_{lag} = (0.0090 * (T + 4.26))^{-2} \quad (3.13)$$

$$y_{max} = (-0.0086) * T^2 + 0.5431 * T + 7.3206 \quad (3.14)$$

Table 3.6. The coefficient of [a]Eq. (3.3) used to predict the values of maximum growth rate as a function of temperature for each microorganisms inoculated in fresh baby spinach leaves.

Microorganisms	**[b]b (log CFU/g/hr/^{o}C)**	**[c]T_{min} (^{o}C)**	**R^2**
L. monocytogenes	0.026	-4.26	0.99
L.innocua	0.028	-4.26	0.98

[a]Eq. (3.3): $\mu_{max} = (b * (T - T_{min}))^2$

[b]b : Coefficient of [a]Eq. (3.3)

[c]T_{min} : Minimum growth temperature of *L. monocytogenes* (^{o}C) (Koseki and Isobe, 2005a)

Table 3.7. Coefficients of [a]Eq. (3.4) used to predict the values of lag time as a function of temperature for each microorganisms inoculated in fresh baby spinach.

Microorganisms	**[b]c (1/hours*[o]C)**	**[c]T_{min} ([o]C)**	**R^2**
L. monocytogenes	0.0099	-4.26	0.97
L.innocua	0.0090	-4.26	0.92

[a]Eq. (3.4): $t_{lag} = (c * (T - T_{\min}))^{-2}$

[b]c : Coefficients of [a]Eq. (3.4)

[c]T_{min} : Minimum growth temperature of *L. monocytogenes* ([o]C) (Koseki and Isobe, 2005a)

Table 3.8. Coefficients of [a]Eq. (3.5) used to predict the values of maximum population density as a function of temperature for each microorganism inoculated in fresh baby spinach leaves.

Microorganisms	**[b]A_1 (log CFU/g/$^oC^2$)**	**[c]A_2 (log CFU/g/^oC)**	**[d]A_3 (log CFU/g)**	**R^2**
L. monocytogenes	-0.0009	0.2592	9.0512	0.92
L.innocua	-0.009	0.54	7.32	0.93

[a]Eq. (3.5): $y_{max} = A_1 * T^2 + A_2 * T + A_3$

[b]A_1, [c]A_2, [d]A_3: Coefficients of [a]Eq. (3.5)

The overall performance of the obtained secondary models was estimated with R^2 values. For *L. monocytogenes*, R^2 values of the predictive secondary model for μ_{max}, t_{lag}, and y_{max} were 0.99, 0.97, and 0.92, respectively. For *L. innocua*, R^2 values of predictive secondary model for μ_{max}, t_{lag}, and y_{max} were 0.98, 0.92, and 0.93, respectively. These results indicate that the secondary models provided good estimates of the growth parameters for both microorganisms.

3.4.4.1 Model Validation

The maximum growth rate, lag time, and maximum population density values of *L. monocytogenes* and *L. innocua* in fresh baby spinach leaves calculated from observed data were compared to predictions from the dynamic model to figure out performance indices of the model.

The Bias factor (B_f) values of the predictive models for μ_{max}, t_{lag}, and y_{max} were 0.70, 0.40, and 1.00 for *L. monocytogenes* and 0.72, 0.25, and 1.01 for *L. innocua*, respectively (Tables 3.9 and 3.10). Valero et al. (2007) and Ross et al. (1996) reported that the B_f value is a suitable measure for performance of predictive models and observed that B_f values ranging from 0.9 to 1.15 proved the model was good for determining the growth parameters. Ding et al. (2012) determined that the B_f values in range of 0.7 to 1.15 were considered as acceptable. The obtained B_f values of the present study were in the acceptable range for both microorganisms and proved that the developed dynamic models for μ_{max} and y_{max} showed a good agreement between the observed and predicted values for both microorganisms. However, B_f values of the lag

time for both microorganisms were not in the acceptable range because the inverse simple Ratkowsky –type model (Eq. 3.4) did not fit the data very well.

As shown in Tables 3.9 and 3.10, the A_f values of the predictive models for μ_{max}, t_{lag}, and y_{max} were 1.43, 2.53, and 1.00 for *L. monocytogenes* and 1.37, 3.99, and 1.00 for *L. innocua*, respectively. A_f values should be close to 1.00 to provide acceptable descriptions of the data. However, the acceptable range of A_f value depends on the number of variables in the predictive model (Ding et al., 2012). Hence, the produced A_f values were all within acceptable range, with the exception for the lag time of both microorganisms. The A_f value is a measurement of preciseness to determine how close the estimated values are to the real values. For *L. monocytogenes* and *L. innocua*, the results indicate that on average, about 43% and 37% of the estimated values were different from the observed μ_{max} values, while 113% and 299% for t_{lag} and 0% for y_{max}, respectively. Thus, the predictive model was more precise for μ_{max} and y_{max} than for t_{lag}. This is because the inverse simple Ratkowsky –type model (Eq. 3.4) did not fit the data very well. Duh and Schaffner (1993) found similar results when using this model *on L. monocytogenes* and *L. innocua* in brain heart infusion broth.

As shown in Tables 3.9 and 3.10, the standard error of prediction expressed in percentage, %SEP values of the secondary models for μ_{max}, t_{lag}, and y_{max} were 11.22%, 23.18%, and 5.61% for *L. monocytogenes* and 12.39%, 44.24%, and 4.87% for *L. innocua*, respectively. This showed that the newly developed models could be considered to provide a good fit between the experimental and predicted data.

Table 3.9. Validation indices of developed models for maximum population density, maximum growth rate, and lag time of *Listeria monocytogenes* in fresh baby spinach leaves.

	[c] y_{max} **(log CFU/g)**	[d] μ_{max} **(log CFU/g/h)**	[e] t_{lag} **(hours)**
Bf (Eq. 3.6)	1.00	0.70	0.40
Af (Eq. 3.7)	1.00	1.43	2.53
%SEP (Eq. 3.8)	5.61	11.22	23.18

[c] y_{max}: Maximum population density (log CFU/g)

[d] μ_{max}: Maximum growth rate (log CFU/g/h)

[e] t_{lag}: Lag time (hours)

Table 3.10. Validation indices of developed models for maximum population density, maximum growth rate, and lag time of *Listeria innocua* in fresh baby spinach leaves.

	[c] y_{max} **(log CFU/g)**	[d] μ_{max} **(log CFU/g/h)**	[e] t_{lag} **(hours)**
Bf (Eq. 3.6)	1.00	0.72	0.25
Af (Eq. 3.7)	1.00	1.37	3.99
%SEP (Eq. 3.8)	4.87	12.39	44.24

[c] y_{max}: Maximum population density (log CFU/g)

[d] μ_{max}: Maximum growth rate (log CFU/g/h)

[e] t_{lag}: Lag time (hours)

3.5 Conclusions

1. Experiments with water and chlorine (200 ppm) washing treatments on the total reduction of populations of *L. monocytogenes* and *L. innocua* (a surrogate) inoculated in fresh baby spinach leaves demonstrated that both microorganisms had different responses to the type of washing treatment.
2. The levels of microbial population reduction due to water washing were significantly different ($P<0.05$) for *L. monocytogenes* and *L. innocua* and the pathogen was more difficult to remove than the surrogate.
3. Natural microflora of fresh baby spinach leaves affects the growth behavior of *L. innocua* more significantly than the growth of *L. monocytogenes*.
4. *L. monocytogenes* grows faster than *L. innocua* on fresh baby spinach leaves at temperatures $10>T>30^{o}C$. These results support the recommendation that multiple commodities should be tested for the validation of the *L. innocua* for use as a surrogate for *L. monocytogenes*.
5. Predictive models were developed to investigate the effect of simulated storage temperature on the growth patterns of L. *monocytogenes* and *L. innocua.* The validation results show that these models could provide reliable estimates for growth of *L. monocytogenes* and *L. innocua* as a function of temperature.
6. The dynamic models can be used by manufacturers to estimate the growth of *L. monocytogenes* in fresh baby spinach during distribution, storage, or retail, and potential growth at consumer levels.

7. These models can also estimate potential growth of *L. monocytogenes* in fresh baby spinach under constantly varying (dynamic) condition. This is crucial because temperature can drastically vary from 'farm to table'.

CHAPTER IV

QUANTITATIVE MICROBIAL RISK ASSESSMENT FOR *LISTERIA MONOCYTOGENES* ON FRESH BABY SPINACH LEAVES

4.1 Overview

The risk of listeriosis because of the consumption of fresh baby spinach contaminated by *Listeria monocytogenes*, a foodborne pathogen with high fatality rate, has become a concern with serious public health and economic consequences. The purpose of this study was to conduct a quantitative microbial risk assessment (QMRA) for *L. monocytogenes* infection from consumption of fresh baby spinach leaves and provide some recommendations regarding the effectiveness of intervention strategies such as washing, chlorine, and irradiation.

The whole food chain of fresh baby spinach from farm to table containing the following information initial contamination at the farm, growth, and cross-contamination throughout transportation, storage conditions at retail and at home, was simulated employing @Risk software. Model output showed mean final contamination levels of -3.277 log CFU/g and 3.9035 log CFU/g based on different scenarios. The quantitative risk assessment model estimated an average number of annual listeriosis cases in the U.S. that ranged between $1.3*10^{-3}$ and $2.0*10^{5}$ for high number of servings and between $1.9*10^{-6}$ and 280 for low number of servings.

Based on the different scenarios evaluated in this study, cross-contamination seems the most probable scenario for prevalence of contamination a whole lot of daily production. In addition, when temperature abuse was occurred after the pathogens

concentration increased, the number of annual cases of listeriosis increased by 200%. However, when the irradiation treatment was used, the average number of annual listeriosis cases in the U.S. could be reduced by 99%. Furthermore, the practice of using Modified Atmosphere Packaging (MAP) with irradiation reduced the number of annual cases of listeriosis by 99.99%.

This study determined that fresh baby spinach processors could ensure a highly safe product-in a scenario where cross-contamination is possible-if the product is exposed to irradiation treatment with a dose of 1 kGy and kept under the cold chain.

4.2 Introduction

Food safety is a crucial issue for consumers, authorities, and producers. Food producers are primarily concerned with ensuring food safety and minimizing any risk linked to consumption of a given food item. This is done by using a systematic approach to determine hazards linked to a given product and process, by implement suitable control measures, and by applying general Good Manufacturing Practices (GMP). Risk assessment, or the determination of quantitative or qualitative value of risk related to a concrete situation and transparent manner, is the scientific basis for risk management and safety criteria for foods. The objective of risk assessment is to determine the characteristics of the nature and graduation of the risk to human health linked to hazards, and to make clear the degree of scientific certainty of the assumptions used to develop the estimates (Mufty, 2012).

The purpose of a microbiological risk assessment for a specific pathogen or group of pathogens is to predict the level of disease that can be anticipated in a target population from product or group of products. The risk assessment process covers the description of a food production system and the identification of possible points of failure that could raise the risk for a specific hazard. A microbiological risk assessment provides information that will assist the producers to figure out whether a pathogen is, or could be, significant hazard in their production system and how best to prevent the hazard being accomplished.

In recent years, *L. monocytogenes* has been of great interest to researchers because of the increased susceptibility to contamination by this pathogen (RTE products) (Garrido et al., 2010). This microorganism has been isolated from a wide range of raw and RTE (ready-to-eat) meats, poultry, dairy products, and vegetables as well as various food processing environments (Harris et al., 2003; Sant`Ana et al., 2012a; Pradhan et al., 2010; Gombas et al., 2003;Prazak et al., 2002; Lianou and Sofos, 2007; Park et al., 2012). *L. monocytogenes* has a great resistance in different environments. Thus, its eradication is very difficult. Despite the zero tolerance policy in the U.S., the assessment of the risk posed by the pathogen is of high relevance due to the high mortality rate of the illness (20 to 40%) and how wide spread the pathogen is in foods and the environment (Carrasco et al., 2010).

In 2003, quantitative microbial risk assessment (QMRA) of listeriosis for 23 categories of RTE products containing vegetables had been carried out by Food and Drug Administration (FDA) and Center for Food Safety and Applied Nutrition

(CFSAN). In this study, vegetables were categorized as relatively low risk groups (<1 case/year). However, these authors suggested that additional investigations for the subdivision of the vegetables category into several different groups are needed because of high uncertainty caused by the diversity of the products. Therefore, several studies have been recently published QMRA for *E. coli 0157:H7*, *Salmonella* and *L. monocytogenes* in leafy green vegetables (Franz et al., 2010; Tromp et al., 2010; Carrasco et al., 2010; Danyluk and Schaffner, 2011; Ding et al., 2013; Puerta-Gomez et al., 2013b).

Microbial risk assessment with scenario analysis can be considered a powerful tool to evaluate the safety features of the production process and allow the producers to predict consequences before actual implementation. In the present study, the unit operations of whole farm to table pathway and related pathogen events containing initial contamination on the farm, growth, and cross-contamination before packaging were considered and described. The contamination levels of *L. monocytogenes* on fresh baby spinach at time of consumption were used to predict the likelihood of infection resulting from a single exposure.

The objective of this study was to develop a quantitative risk assessment model to determine prevalence, concentration, and cross-contamination of *L. monocytogenes* in fresh baby spinach leaves by determining dose-response assessment based on the quantity of *L. monocytogenes* consumed and magnitude and frequency of adverse health effects.

4.3 Risk Assessment Methodology and Data Sources

4.3.1 Hazard Identification

Hazard identification is defined by Codex Alimentarius (1999) as "the identification and biological, chemical, and physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods."

L. monocytogenes, causing listeriosis in humans with diverse symptoms including mild diarrhea, meningitis, and septicemia, is a widely seen foodborne pathogen in several foods, such as milk, vegetables, and meat (Painter et al., 2013). Foodborne illness caused by *L. monocytoogenes* has become relevant nationally since 2000 (CDC, 2013). This pathogen can grow up to notable number at refrigeration temperature with adequate time. Although the occurrence of listeriosis is less than other pathogens such as *E. coli O157:H7,* it is also considered as an important illness because of its high fatality rate, particularly in immunocompromised individuals (Carrasco et al., 2010; Ding et al., 2013). According to records, the occurrence of listeriosis in the United States was 2.9 cases per million populations throughout 2008 (CDC, 2010). In 2011, *L. monocytogenes* also contaminated cantaloupes which caused 28 deaths and 133 total confirmed cases (Ding et al., 2013). Beside this, the consumption of RTE vegetables has caused several listeriosis outbreaks worldwide (Table 2.1, Chapter II). This fact indicates that *L. monocytogenes* cannot be completely eliminated by commercial decontamination methods used by producers if it is present in raw vegetables. Therefore, it is crucial to control the methods at all stages in the food chain to prevent or reduce contamination in foods with *L. monocytogenes* to avoid series foodborne illnesses.

4.3.2 Hazard Characterization

Hazard characterization is defined by Codex Alimentarius (1999) as "the qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with the hazard. For the purpose of microbiological risk assessment, the concerns relate to microorganisms and/or their toxins."

Hazard characterization is also described as the evaluation of adverse health effects connected to *L. monocytogenes* and it was estimated as the likelihood that a single cell of *L. monocytogenes* would cause a disease (Ding et al., 2013). In this study, an exponential dose –response non-threshold model was used to estimate the risk of listeriosis linked to fresh baby spinach consumption. Dose-response relationships have not been determined for humans yet. A main assumption in dose-response modeling for microbial risk assessment is that a single cell has a finite probability of causing illness (Tromp et al., 2010), which is described by the following equation:

$$P(D) = 1 - e^{-r*D} \tag{4.1}$$

where, *P (D)* is the probability of illness for individual exposed to a certain dose (D cells); *D* is the number of *L. monocytogenes* consumed (CFU/serving) and *r* refers to the probability that a single cell will cause invasive listeriosis. The r-values used for evaluating risks of consumed number of *L. monocytogenes* were reported by Franz et al. (2010) and Tromp et al. (2010) (r = 1.91*10-10). Because of "zero tolerance" in the U.S., 10^{-5} of the probability illness was used as tolerance level in this study. Besides,

different subpopulations with different levels of vulnerability to illness following exposure was not considered in the present study.

In the present study, serving size of fresh baby spinach data was based on collected from the study published by Hoelzer et al. (2012a). Serving size was defined by cumulative probability distributions. Thus, the final output of the exposure model will be determined as the probability distribution of the ingested dose in colony-forming units (CFU) in serving size of fresh baby spinach, with which the risk of infection was predicted by the dose-response model.

Two total numbers of annual servings of fresh baby spinach were used to estimate cases of listeriosis in the U.S. annually. First, the total number of annual servings (high number of servings) of fresh baby spinach was assumed equal to $8.5*10^{10}$ (FDA/CFSAN, 2003). Second, Hoelzer et al. (2012a) reported that 40% of fresh baby spinach consumed by 3.17% of the U.S. population is consumed as fresh, annually. In addition, per capita consumption of spinach is reported as 870.9 g by Lucier and Glaser (2010) and the population of the U.S. was reported 308,745,538 in 2010 by Howden and Meyer (2011). The total number of annual servings (low number of servings) of fresh baby spinach was then calculated as:

$$C = P * 0.0317 * 0.40 * 870.9 \quad (4.2)$$

$$S_N = \frac{C}{S_S} \quad (4.3)$$

Herein, P is the population of the U.S.; S_N is the number of servings annually; C is the amount of fresh baby spinach consumed in the U.S.; and S_S is serving size (g).

Next, the estimated cases of listeriosis for population caused by fresh baby spinach consumption per year were calculated as (Danyluk and Schaffner, 2011):

$$ECL = P(D) * S_N \tag{4.4}$$

herein, *ECL* is the estimated number of cases of listeriosis.

4.3.3 Exposure Assessment

Codex Alimentarius (1999) determines exposure assessment as "the qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food, as well as exposures from other sources if relevant."

The scope of the exposure assessment for *L. monocytogenes* on fresh baby spinach leaves within the food chain is shown in Figure 4.1. Fresh baby spinach leaves were assumed to be contaminated with *L. monocytogenes* during harvest by an unknown source. The process and packing line has several unit operations consisting of harvesting, washing treatments (water and water- chlorine), packaging, irradiation, storage, and transportation, and display for sale at markets before consumption at home. The model inputs in terms of initial contamination level after harvest, transportation, and cross contamination levels were based on available literature data. Otherwise, the growth of *L. monocytogenes* was determined by laboratory experiments and growth models developed for this microorganism in Chapter III.

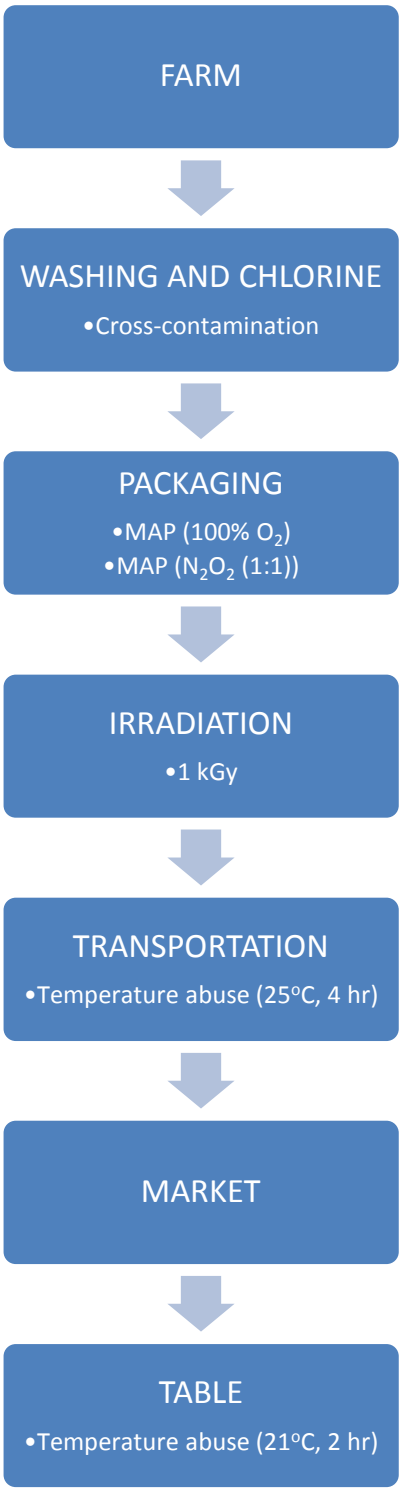

Figure 4.1 Flow chart of fresh baby spinach process and distribution.

4.3.3.1 Description of the Supply Chain of Fresh Baby Spinach

Fresh baby spinach is a crucial crop in Texas, mostly in Uvalde County and the Rio Grande Valley. The climate of these areas have been determined as continental, semi-arid, and subtropical-sub humid. The range of temperatures in these areas is between 3-17^{o}C in January and 22-37^{o}C in July (Puerta-Gomez et al., 2013b). Fecal contamination is more probable to take place in Uvalde County than in Rio Grande Valley due to wild animals such as deer, pigeon, and hogs. In the field, baby spinaches are harvested by machine. The field crop is analyzed to control pathogen contamination after harvesting. Then, fresh baby spinach is sent to washing (water and water-chlorine) line. There are two methods for washing. First, the amount of water which is purged is replaced with fresh water (together with chlorine and citric acid as needed). Second, the water in the first tank is thrown away and then the water from the second tank transferred to the first tank (CDHS and USFDA, 2007). This process continues for four hours (CDHS and USFDA, 2007). After that, the produce exits over a de-watering belt and moves to the packaging line.

After packing, almost 7 hours later, the fresh baby spinach is transported to markets. It is assumed that there should be no contamination at this point (CDHS and USFDA, 2007). However, during transportation, temperature (4-15^{o}C) is adequate for growth of *L. monocytogenes* and sometimes cannot be controlled due to accidental circumstances (Ding et al., 2013).

4.3.3.2 Prevalence and Initial Level of *Listeria monocytogenes*

The status of *L. monocytogenes* in fresh baby spinach is defined by the prevalence and concentration of the pathogen in the fresh baby spinach at the time of consumption. There are no microbial surveys regarding prevalence of *L. monocytogenes* in fresh baby spinach. However, Beuchat, (1996) stated that vegetables are likely contaminated with *L.monocytogenes* because this pathogen can survive in decaying vegetation, animal faces, soil, surface, river, and canal waters or sewage treatment operations. Prazak (2002) detected *L. monocytogenes* in samples acquired from cabbage farms in Texas, so fresh baby spinach can be contaminated with *L. monocytogenes* during harvesting. According to this study, two positive samples of *L. monocytogenes* were identified within 130 samples. Gombas et al. (2003) also collected 2,966 bagged salad samples and *L. monocytogenes* was detected in 22 of them. Prevalence and initial contamination level of *L. monocytogenes* on fresh baby spinach are the initial inputs of the quantitative microbial risk assessment (QMRA) model developed in this study. Therefore, the distribution for *L. monocytogenes* on fresh baby spinach was estimated based on the combined data derived from literature (Table 4.1) in order to make the QMRA more reliable and rational. The different initial population of the pathogen was not used in the present study because it is well known that increasing the initial population of the pathogen increases the risk of listeriosis.

The initial contamination level refers to the concentration of *L. monocytogenes* in the fresh baby spinach leaves after harvest. There is no survey regarding concentration of the pathogen in fresh baby spinach. Therefore, concentration of the pathogen was

assumed equal to the concentration found by Carrasco et al. (2010) for bagged salads (Table 4.2). In the present study, both prevalence and concentration of *L. monocytogenes* in fresh baby spinach were described separately by the cumulative probability distribution. In addititon, the relative growth was calculated as:

$$RG = \left((y_0 - y_t) * 100\right)/y_0 \quad (4.5)$$

Herein, RG is the relative growth (%); y_o is initial concentration density (log CFU/g); y_t is concentration of the pathogens at time t (log CFU/g).

4.3.3.3 Washing and Sanitizing (Chlorine)

Fresh and fresh –cut products are not exposed to any thermal treatments to decrease microbial load. Therefore, cleaning washing and sanitizing are important steps in the processing of these products. Microorganisms on fresh and fresh-cut products are little affected from washing by tap water. Garg et al. (1990) determined that total counts and *Enterobacteriaceae* on spinach leaves only decreased between 0.2 to 0.5 log cycles by washing by tap water. Use of a sanitizer such as chlorine is the logical next step because chlorine (200-ppm, 10 min) reduced population of *L. monocytogenes* on shredded lettuce and cabbage by 1.7 and 1.2 log CFU/g, respectively (Zhang and Farber, 1996). In addition, it is recommended that the temperature of the processing water should be kept up at least 10^{o}C above than that of the produce to decrease the probability of microbial infiltration caused by a temperature-generated pressure differential (Gil and Selma, 2009). Nevertheless, during the washing step, pathogen microorganisms such as *L. monocytogenes* can likely occur due to reuse of washing water and washing tanks.

Table 4.1. Prevalence of *Listeria monocytogenes* in vegetables.

Source	Food	Number of Samples	Prevalence (%)	F(x)	Number of positive samples
Gombas et al., 2003	Bagged salads	2966	0.74	0.067	22
Heisick et al., 1989b	Cabbage	92	1.1	0.133	1
Prazak et al., 2002	Cabbage	130	1.5	0.200	2
Lin et al., 1996	Vegetables salads	63	1.6	0.267	1
Velani and Roberts, 1991	Salad vegetables	108	1.8	0.333	2
Carrasco et al., 2010	Salad vegetables	263	2.3	0.400	6
Carrasco et al., 2010	Lettuce	28	3.6	0.533	1
FDA/CFSAN, 2003	Vegetables	9223	3.6		332
Legnani et al., 2004	Raw vegetables	43	6.9	0.600	3

Table 4.1. Prevalence of *Listeria monocytogenes* in vegetables (continued).

Source	Food	Number of Samples	Prevalence (%)	F(x)	Number of positive samples
De Simon et al., 1992	Vegetables salads	103	7.8	0.667	8
Harvey and Gilmour, 1993	Raw vegetables	66	10.6	0.733	7
Olaimat and Holley, 2012	Radish	132	14.4	0.800	19
Arumugaswamy et al., 1994	Leafy vegetables	22	22.7	0.867	5
Heisick et al., 1989b	Radish	68	36.8	0.933	25

Table 4.2. Concentration assumed for *Listeria monocytogenes* in vegetables (Adapted from Carrasco et al., 2010).

Concentration (Log CFU/25 g	**Number of positive Samples**	**f(x)**	**F(x)**
0-0.4	17	0.77	0.77
0.4-1.4	1	0.04	0.82
1.4-2.4	1	0.04	0.86
2.4-3.4	2	0.09	0.95
3.4-4.4	1	0.04	1

As a result, pathogens, if present in raw vegetables, cannot be destroyed by tap water and chlorine washing. Hence, probability distributions were fitted to the experimental data using @RISK (Palisade Corp. NewField, NY). Thus, the normal distribution was used to express the log reduction of water and chlorine washing (Carrasco et al., 2010; Danyluk and Schaffner, 2011). The change of concentration of *L. monocytogenes* by decontamination treatments from farm to table for low (Scenario #2) and high initial concentration (Scenario #4) are presented in Figure A. 21 and A. 22 (Appendix).

4.3.3.4 Packaging

After the washing and sanitizing steps, the produce enters the packing line. In the packaging process, there are several surfaces such as conveyors, handling equipment, sorting tables, and packing containers (Johnston et al., 2006a). Poor cleaning on these surfaces will not protect the produce against contamination by microorganisms. Even pathogens such as *L. monocytogenes* cause cross-contamination (FAO and WHO, 2008). Johnston et al., (2006a) determined that the contamination with *E. coli* on cabbages rose from 0.7 log CFU/g to 0.86 log CFU/g during their removal from the conveyor belt to the final box. Likewise, Prazak et al., (2002) pointed out that *E. coli* and *L. monocytogenes* was present on transport bins, conveyor belts, and cooler surfaces used for processing cabbages in the packaging process. As a result, these studies emphasize the importance of hygiene and equipment sanitation to reduce the possibility of cross-contamination.

4.3.3.5 Cross-contamination

Cross-contamination occurs when bacteria are passed from one food item to another by handling mistakes, water washing, and packing equipment (Ding et al., 2013). A recent study showed that the contamination loads of pathogens on various kinds of surfaces ranged from 2.12 to 7.43 log CFU/g (Chen et al., 2001). Leafy green vegetables such as spinach are kept under low temperature from farm to table, so the growth of pathogenic microorganisms such as *E. coli 0157:H7* and *Salmonella* is limited. However, *L. monocytogenes* can grow during this period because the theoretical minimum temperature for the growth of *L. monocytogenes* is -4.26ºC (Koseki and Isobe, 2005a; Tromp et al., 2010). In this study, the cross-contamination scenario (Scenario #3, #4, #5, and #6) was modeled by using a uniform distribution.

4.3.3.6 Irradiation

The reduction of *L. monocytogenes* in fresh baby spinach was determined by the irradiation death model from experimental data (Gomes et al., 2011). According to this study, the D_{10}-value of *Listeria* spp. was 0.213 kGy. Therefore, the survival of *L. monocytogenes* was calculated as:

$$S_{irr} = \frac{N}{N_o} = e^{-D/D_o} = e^{-2.303D/0.213} = e^{-10.8122D} \quad (4.6)$$

herein, N_o refers to the initial number of microorganisms (CFU/g), N refers to the number of remaining microorganisms (CFU/g) after exposure to dose D (in kGy), D_o is the mean lethal dose (in kGy), or the dose required to reduce the survival fraction S to

1/e (i.e., 37%) and D_{10} is the radiation D-value or required dose for 90% reduction of the microbial population.

In the present study, the radiation dose was assumed as 1 kGy at the room temperature (~21^{o}C)) because this dose level is approved by the FDA and maintains fresh baby spinach`s quality (Gomes et al., 2011).

4.3.3.7 Radiosensitization Strategies

When considering irradiation of fresh produce, producers need approaches to reduce the required dose because the actual radiation dose required to reduce pathogen can be very high (Puerta-Gomez et al., 2013b). One alternative is the combination of Modified Atmosphere Packaging (MAP) with different atmospheres (e.g., 100% 0_2 and 50% N_2) and ionizing radiation (Gomes et al., 2011). This approach showed that although the doses required for the inactivation of pathogens (*Salmonella* spp. *E. coli*, and *Listeria* spp.) in fresh baby spinach leaves decreased, the level of reduction of pathogens increased. Gomes et al. (2013) also proved that 100% O_2 atmospheres did not cause any quality changes on fresh baby spinach leaves.

Irradiation treatment at low temperature is less effective to reduce microorganisms in produce. Gomes et al. (2011) found a linear relationship between radiation sensitivity and temperature, with a slope of $-2.74*10^{-3}$ ($\pm 3.79*10^{-4}$), D_{10}-values/^{o}C. This relationship was included in the irradiation treatment module of a risk assessment model (Puerta-Gomez et al., 2013b). The reduction in *Listeria* spp. population was then calculated as:

$$S_{irr} = S_{water} = \frac{D_T}{D_{MAP}} \quad (4.7)$$

herein, S_{irr} refers to the microbial population after the irradiation treatment (log CFU/g), S_{water} refers to the population after the washing treatment (log CFU/g), D_T refers to the target dose (kGy), and D_{MAP} refers to the calculated D_{10}-value (kGy) at the specific temperature and atmospheric conditions inside the package (MAP). Furthermore, Figure A.1 (Appendix) shows the microbial distribution in the field and reduction at the intervention steps, for two different initial loads of contamination.

4.3.3.8 Transport

Fresh baby spinaches should be transferred to market for sale and home for consumption after harvesting. Temperature and time throughout transportation and distribution are very important factors for the growth of *L. monocytogenes*. In this study, it was assumed that the fresh baby spinach was distributed to chain markets and then it was consumed at home. The temperature of transportation usually ranges from 4 to 15^{o}C, whereas it sometimes increases up to 25^{o}C (Ding et al., 2013). The time of transportation of baby spinaches from the Rio Grande Valley to the closest city (San Antonio, TX) and furthest big city (El Paso, TX) is approximately between 4 and 10 hours (Google Earth 7, Mountain View, CA). Therefore, the time throughout the transportation process was determined by using a uniform distribution.

4.3.3.9 Growth of *Listeria monocytogenes*

The growth of *L .monocytogenes* in fresh baby spinach leaves from transportation to the table was estimated using the Baranyi model (Eqs. 2.3 and 2.4,

Chapter II). The parameters of the Baranyi model (μ_{max}, t_{lag} and y_{max}) were also calculated from Equations 3.9, 3.10, and 3.11 in Chapter III, respectively.

Time and temperature data used in this study was reported by Danyluk and Schaffner (2011). These authors reported that the extreme value distribution was the best fitting distribution of retail temperatures. They stated that leafy green vegetables is kept between 4 and 7 days in the retail storage. The next step, home storage, represents the expected change in pathogen level throughout storage in the consumer`s home. In the present study, these data were modified by using several distributions (Table A.1, Appendix).

4.4 Scenario Analysis

Table 4.3 presents all the different scenarios evaluated in this study. Each scenario was simulated separately. ***Scenario #1*** is based on the baseline of the supply chain (from transportation to table) of fresh baby spinach. ***Scenario #2*** is based on a combination of baseline and the irradiation treatment (1 kGy at room temperature). Cross-contamination was considered in ***Scenario #3***, without irradiation. ***Scenarios #4, #5, and #6*** includes not only cross-contamination but also irradiation and Modified Atmosphere Packaging (MAP), (O_2 (100%) and N_2:O_2 (1:1). Lastly, ***Scenarios #7 and #8*** simulated the case of temperature abuse at the table and at the transportation step, respectively. Table A.1 (Appendix) summarizes all the cells in the Excel spreadsheet used for subsequent risk calculation. Assumption made for this model including the following:

- Cross-contamination did not occur without cross contamination **Scenarios** (#3, #4, #5, #6).
- Temperature abuse was not occurred without temperature abuse **Scenarios** (#7 and #8).
- The initial contamination of *L. monocytogenes* in fresh baby spinach was assumed to occur before the water washing treatments.
- Time before the transportation step and after table was neglected.
- All fresh baby spinach was consumed at home and any decontamination methods would not be used without the decontamination treatments in the processing line.

4.5 Risk Characterization

Codex Alimentarius (1999) defines as "the process of determining the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization, and exposure assessment."

The contamination levels of *L. monocytogenes* in fresh baby spinach at the point of consumption were acquired from the exposure assessment and then fed into the exponential dose-response model to administer the risk characterization portion of risk assessment. According to the scenario of quantitative risk assessment model determined in Table 4.3, the quantitative microbial risk assessment (QMRA) was built in an Excel (Microsoft, Redmond, WA) spreadsheet, and then simulated by using Monte Carlo techniques.

Table 4.3 Scenarios used in the present study.

Scenarios	**Temperature (°C)**	**Duration (hours)**	**What if scenario**
#1		From farm to table	Baseline
#2		From farm to table	Baseline + irradiation treatment
#3		From farm to table	Cross contamination
#4		From farm to table	Cross-contamination + irradiation treatment
#5		From farm to table	Cross-contamination + irradiation treatment + MAP (O_2 (100%))
#6		From farm to table	Cross-contamination + MAP (N_2:O_2 (1:1)) + irradiation treatment
#7	21	2	Temperature abuse at home
#8	25	4	Temperature abuse at the transportation step

4.6 Monte Carlo Simulation

The Monte Carlo simulation, an alternative to analytic techniques, expresses a powerful and accurate method for including both the stochastic and epistemic uncertainty of a problem (Hald et al., 2004). A single point is spontaneously chosen from each of the likelihood distributions assigned to each input parameter including epistemic uncertainty in Monte Carlo simulation. These spontaneously chosen single points are then used to compute a mathematical solution, as described by the risk assessment model. This outcome is kept and this series iterate many thousand times. Throughout each iteration, a unlike set of points for the inputs is chosen with points that are more probably to happen, as described by the likelihood distribution, chosen more repeatedly. The end outcome is a likelihood distribution for the output of interest, represented by the mixture of ranges of repetitions of the input parameters. In this study, each risk assessment model was simulated three times using the @RISK software (Palisade Corp. New Field, NY) with 10,000 iterations.

The @Risk software is an important tool to conduct a probabilistic quantitative microbiological risk assessment (QMRA). It provides an opportunity to simulate the QMRA model built in an Excel spreadsheet.

4.7 Results and Discussion

The mean of prevalence (Figure A.23, Appendix) and initial concentration (Figure A.24, Appendix) of *L. monocytogenes* on fresh baby spinach were calculated as 9.34% with a standard deviation of 0.121 and -0.669 log CFU/g with standard deviation of 1.11 from several surveys (Table 4.1) and Carrasco et al. (2010) (Table 4.2),

respectively. The population of *L. monocytogenes* in fresh baby spinach leaves increased by between -389.8 and 683.5% from transportation to consumption, based on the different scenarios (Table 4.3). Higher relative growth (683.5%) was observed in the cross-contamination Scenario (#3).

When evaluating the effectiveness of the decontamination treatments, application of irradiation combined with modified atmosphere packaging MAP (N_2:O_2 (1:1)) was more effective than other combinations because the relative growth of *L. monocytogenes* was 66.8% (Table 4.4) even though cross-contamination occured. Carrasco et al. (2010) also stated that the use of MAP (usually 5 to 10% CO_2, 0.5 to 3% O_2, and the balance of N_2 for RTE lettuce salads) postpones the growth of *L. monocytogenes*. In addition, although the fresh baby spinach was contaminated by *L. monocytogenes* at harvesting time, the population level of the pathogen at the table was reduced by the irradiation treatment (Scenario #2) (Table 4.4). Thus, the irradiation treatment is a feasible alternative to ensure the safety of fresh leafy greens without reducing their quality attributes (low dose treatment at 1kGy).

Temperature abuse is an important issue for fresh produce. According to Scenario #7, although cross contamination did not occur, the relative growth of *L. monocytogenes* on fresh baby spinach leaves increased 296.7%. Furthermore, temperature abuse at the transportation step (Scenario #8) increased 248.0% of the relative growth of *L. monocytogenes* (Table 4.4). In addition, the point of temperature abuse is important because if temperature abuse occurs before the retail step, the annual number of cases of listeriosis will be 66% less than that those occurring at home (Table

Table 4.4 Calculated prevalence, initial and final population of *Listeria monocytogenes* on fresh baby spinach leaves at time of consumption and relative growth.

Scenarios	Prevalence (%)	Initial population (log CFU/g)	Final population (log CFU/g)	Relative growth (Eq. 4.5) (%)
#1	9.34 (0.121)	-0.669 (1.1083)	0.9778 (2.5450)	246.158
#2			-3.277 (3.342)	-389.8356
#3			3.9035 (1.1988)	683.483
#4			0.996 (2.776)	248.879
#5			0.00258 (3.058)	100.386
#6			-0.222 (3.082)	66.8161
#7			1.316 (2.4524)	296.71
#8			0.99 (2.5174)	247.98

#1: Baseline

#2: Baseline + Irradiation

#3: Baseline + Cross contamination

#4: Baseline + Cross Contamination + Irradiation

#5: Baseline + Cross contamination + MAP (O2 (100%)) + Irradiation

#6: Baseline + Cross contamination + MAP (N_2O_2 (1:1)) + Irradiation

#7: Baseline +Temperature abuse (21^{o}C, 2 hr) at the table

#8: Baseline + Temperature abuse (25^{o}C, 4 hr) the transportation step

A.2, Appendix). These results support the recommendation that fresh baby spinach leaves should be kept under the cold chain.

The cumulative frequencies for the overall probability of infection for the eight scenarios are presented in Figure 4.2. With the exception of Scenario #2, no cross-contamination, all the other scenarios yielded over 10% of the lot (Table 4.5). The Scenario #2 yielded 5.8% of the lot as contaminated samples (Table 4.5). In addition, the cross-contamination scenario (Scenario #3) yielded a percentage of tainted samples of 20.8%. Although the condition of Scenario #8 (25^{o}C, 4 hours) represents higher temperature abuse than that of Scenario #7 (21^{o}C, 2 hours), Scenario #7 yielded 2 % of tainted samples more than Scenario #8 (Figure 4.3). Thus, temperature abuse increases the percentage of tainted samples due to an increase in pathogen population.

The effect of using Modified Atmosphere Packaging (MAP) strategies on the cumulative frequency, in the sense of probability of infection is shown in Figure 4.4. These results were simulated from same input and irradiation dose of 1 kGy. Scenarios #5 and #6, (100% O_2) and (N_2:O_2 (1:1)) yielded 10.3 and 10.1% of tainted product, respectively. Therefore, the safety of fresh leafy greens, without considering the effect on their quality attributes, can be ensured by the combination of the irradiation treatment with MAP.

Table 4.5. Model parameters and calculated values for eight scenarios of the probability of infective dose of *Listeria monocytogenes* in fresh baby spinach leaves.

	Scenarios #							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Initial concentration of *L. monocytogenes* (log CFU/g)	-0.67 (1.11)	-0.67 (1.11)	-0.67 (1.11)	-0.67 (1.11)	-0.67 (1.11)	-0.67 (1.11)	-0.67 (1.11)	-0.67 (1.11)
Water washing (log CFU/g)	0.54	0.54	0.54	0.54	0.54	0.54	0.54	0.54
Chlorine washing (log CFU/g)	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Cross-contamination levels (log CFU/g)	0	0	4.77 (1.53)	4.77 (1.53)	4.77 (1.53)	4.77 (1.53)	4.77 (1.53)	4.77 (1.53)
Irradiation dose (kGy)	0	1	0	1	1	1	1	1
Temperature abuse (°C&hours)	0	0	0	0	0	0	21°C, 2 h	25°C, 4h
Temperature (°C)	5.33 (3.1)	5.33 (3.1)	5.33 (3.1)	5.33 (3.1)	5.33 (3.1)	5.33 (3.1)	5.33 (3.1)	5.33 (3.1)
Time (hours)	197.9 (54.4)	197.9 (54.4)	197.9 (54.4)	197.9 (54.4)	197.9 (54.4)	197.9 (54.4)	197.9 (54.4)	197.9 (54.4)
L. monocytogenes concentration at consumption (log CFU/g)	0.97 (2.54)	-3.27 (3.34)	3.90 (1.20)	0.996 (2.78)	0.002 (3.06)	-0.22 (3.08)	1.31 (2.45)	0.999 (2.52)
Median log probability of positive leaves being infectious	-9.10	-13.2	-5.34	-8.94	-10.1	-10.3	-8.72	-9.15
% of samples over the safety limit (Pif>10^{-5})	12.3	5.8	20.8	12.6	10.3	10.1	14.7	12.8

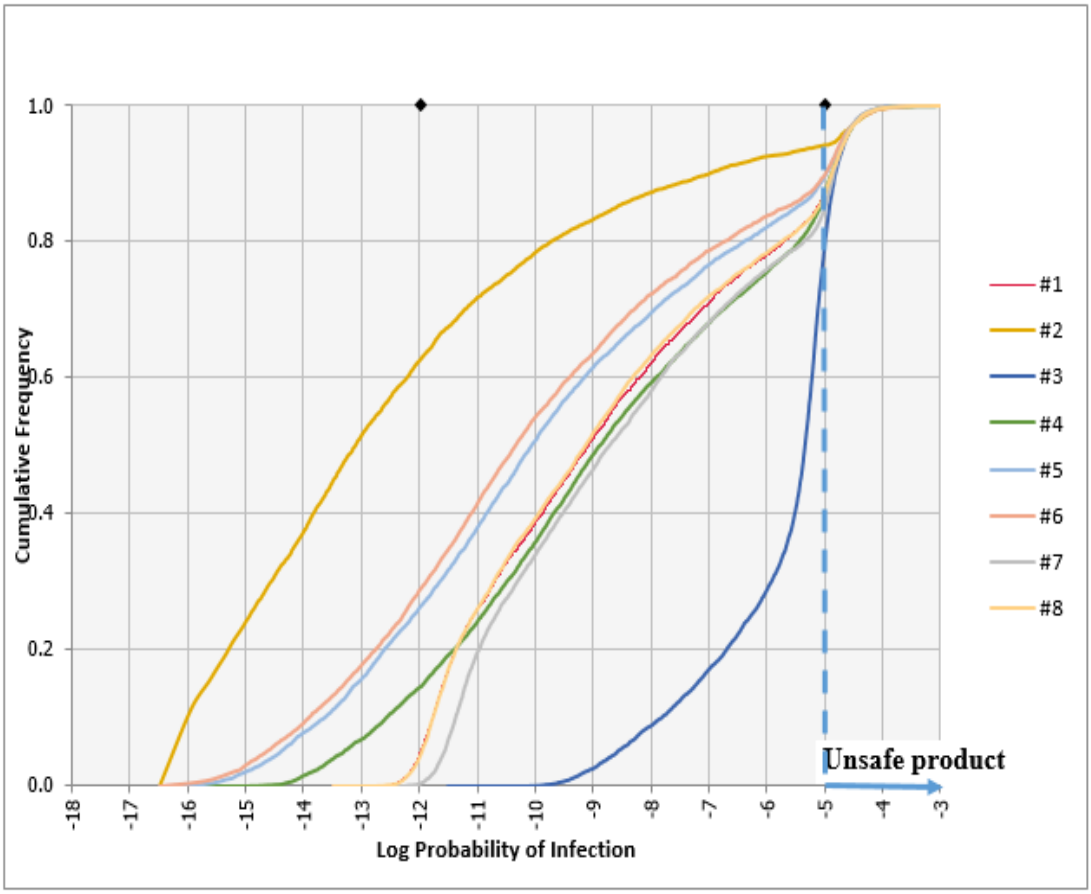

Figure 4.2 Probability of illness occurrence for Scenarios #1- #8.

#1: Baseline

#2: Baseline + Irradiation

#3: Baseline + Cross contamination

#4: Baseline + Cross Contamination + Irradiation

#5: Baseline + Cross contamination + MAP (O2 (100%)) + Irradiation

#6: Baseline + Cross contamination + MAP (N_2O_2 (1:1)) + Irradiation

#7: Baseline +Temperature abuse (21^{o}C, 2 hr) at the table

#8: Baseline + Temperature abuse (25^{o}C, 4 hr) the transportation step

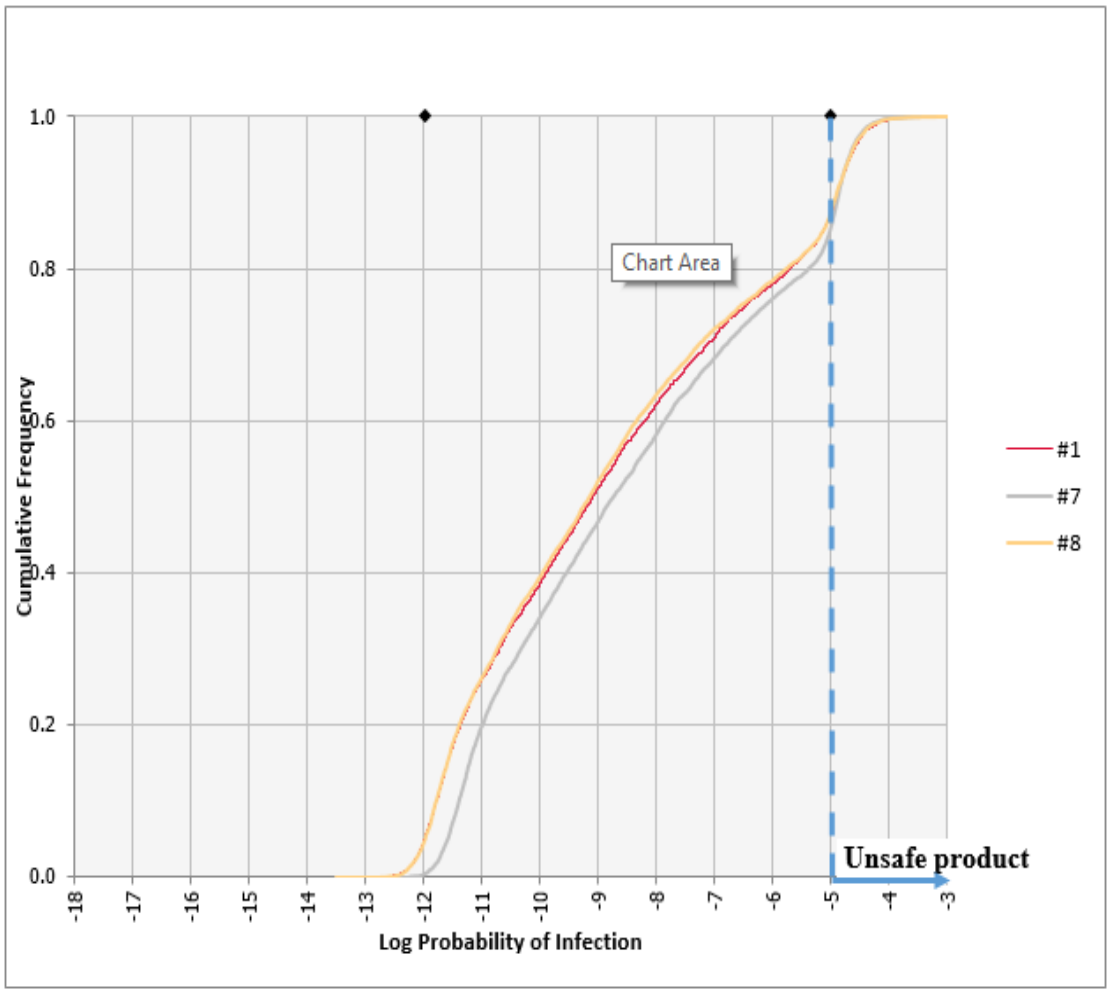

Figure 4.3 Probability of illness occurrence in fresh baby spinach leaves base on scenarios of temperature abuse.

#1: Baseline

#7: Baseline +Temperature abuse (21^{o}C, 2 hr) at the table

#8: Baseline + Temperature abuse (25^{o}C, 4 hr) the transportation step

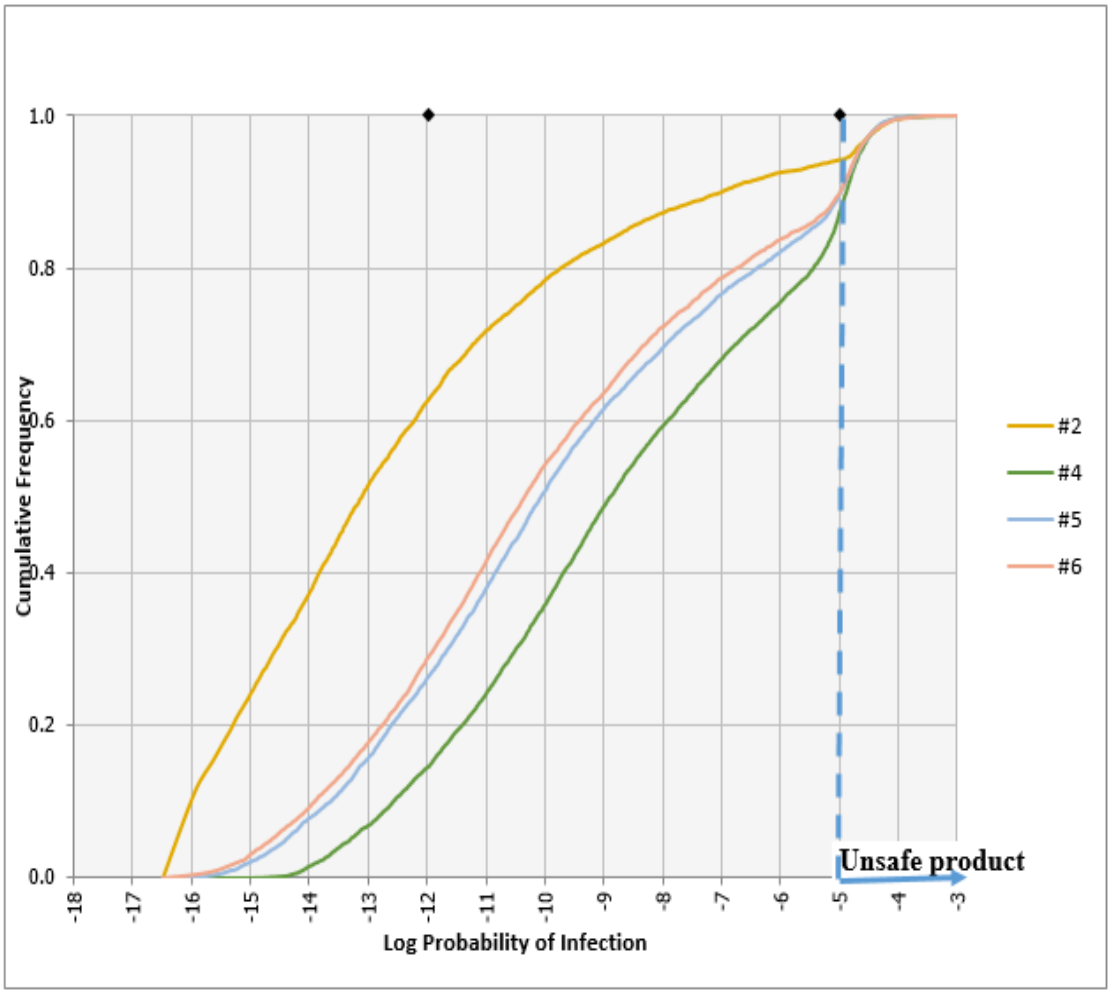

Figure 4.4 Probability of infection occurrence in fresh baby spinach leaves irradiated at 1 kGy under different atmospheres at 21°C.

#2: Baseline + Irradiation

#4: Baseline + Cross Contamination + Irradiation

#5: Baseline + Cross contamination + MAP (O2 (100%)) + Irradiation

#6: Baseline + Cross contamination + MAP (N_2O_2 (1:1)) + Irradiation

The mean of serving size was 29-g (Figure A.25) and the annual number of servings was calculated as 1.70×10^{8} (Eq. 4.3). Similar serving size (28-g) for vegetables was reported by Food and Drug Administration and Center for Food Safety and Applied Nutrition (FDA/CFSN) (2003). The estimated cases of listeriosis for the population caused by fresh baby spinach consumption per year, ranged from 1.3×10^{-3} to 2.0×10^{5} for high number of servings and ranged from 1.9×10^{-6} to 280 for low number of servings. Similarly, Lynch et al. (2009) reported that the outbreaks related to fresh produce increased because the number of servings increased. FDA/CFSAN (2003) reported that predicted median cases of listeriosis for total U.S. population consuming vegetables on per annum are 0.2. This result confirmed that the estimated cases of listeriosis for low number of servings is more realistic than that for high number of servings.

The log value of exposure and cases are shown in Figure 4.5. There is always a risk to increase the cases of illness with exposure in the home. This exposure was postponed in the home storage and consumption stages. In addition, if the irradiation treatment were applied, the estimated cases of listeriosis would be reduced by 99.95%. Furthermore, the combination of irradiation and MAP could reduce the annual cases of listeriosis by 99.99%. This result highlights the importance of introducing an irradiation step to reduce the number of annual cases of listeriosis associated with leafy greens.

Up to now, the outbreaks of listeriosis associated with fresh baby spinach have not been reported in the U.S. According to current policies in the U.S., a policy of "zero-tolerance" of *L. monocytogenes* in ready-to-eat foods (i.e., products that may be consumed without any further cooking or heating) is maintained by the FDA (Trinetta et

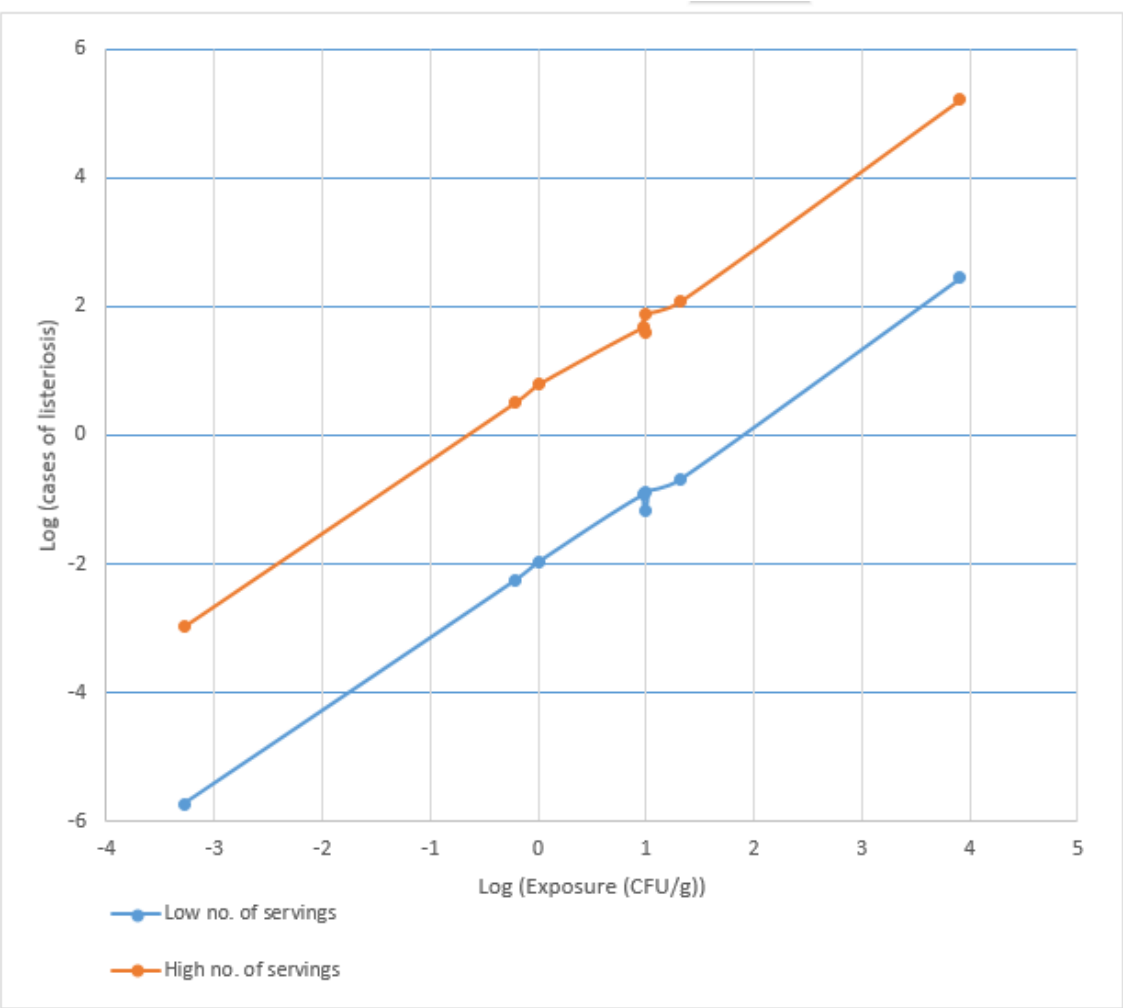

Figure 4.5 Comparison of risk between high and low no. of servings.

al., 2012). In addition, the Food and Drug Administration and Center for Food Safety (FDA) and Applied Nutrition (CFSAN) (2003) reported that, based on their risk assessment model, vegetables are in the low risk food categories due to their low median serving size, contamination level, and growth rate. They reported that the median of estimated number of cases of listeriosis per serving is $2.8x10^{-12}$. However, in the present study, the median of estimated number of cases of listeriosis per serving was between $1.58x10^{-14}$ and $2.38x10^{-6}$ for eight scenarios. The difference between these two results suggest a potential overestimation of the values in the present study.

4.8 Conclusion

This study showed that the quantitative risk assessment model can be used to estimate the effect of mitigation strategies to avoid potential cases of listeriosis in baby spinach and other leafy greens. It is recommended to apply an irradiation step because this decontamination method presented very significant results; it reduced 389.83% of the initial population of *L. monocytogenes* in the leaves from farm to table under cold chain. In addition, the estimated annual cases of listeriosis were reduced 99% when this treatment was used. Besides, the use of irradiation in combination with Modified Atmosphere Packaging (MAP) reduced the relative growth of the pathogen and the estimated number cases of listeriosis. The relative growth of *L. monocytogenes* in fresh baby was 246.15% based on the baseline scenario (no cross-contamination), while it was 66% when the combined treatments (irradiation plus MAP (N_2:O_2 (1:1)) were considered, even though cross-contamination.

Based on these results, cross-contamination increased the risk of outbreaks. Therefore, fresh baby spinach producers should periodically control their processing systems. In addition, cold chain is very important to ensure safety of the produce. The records of time and temperature should be regularly kept during the farm to table chain even though the records after the market are very difficult to obtain. Furthermore, the annual number of servings increased the estimated annual cases of listeriosis.

The developed model supplies a scientific base for risk managers and risk business operators to gain a better understanding of the inhibition of the risk of listeriosis because of fresh baby spinach consumption in spite of important uncertainty association with the predictions.

CHAPTER V

CONCLUSION

This research focused on the quantitative microbial risk assessment of *Listeria monocytogenes* in fresh baby spinach leaves.

The first part of this research evaluated the growth of *L. monocytogenes* and *L. innocua* in fresh baby spinach as well as the reduction of the respective microorganisms under water and chlorine treatments.

1. The reductions of *L. monocytogenes* and *L. innocua* in fresh baby spinach treated by washing (water and chlorine) treatments were significantly different ($P<0.05$).
2. When the initial concentration of total mesophilic bacteria in fresh baby spinach increased, the lag time and maximum growth rate of *L. monocytogenes* decreased by 32.45% and 28.72% whereas that for *L. innocua* increased by 244.22% and 18.22% at 5°C, respectively.
3. When the initial concentration of total mesophilic bacteria in fresh baby spinach increased, the maximum population density of *L. monocytogenes* did not change whereas that for *L. innocua* was reduced by 1.91 log CFU/g at 36°C.
4. *L. innocua* should be tested before it is used as a surrogate for *L. monocytogenes* due to the different conditions affecting the behavior of *L. innocua* and *L. monocytogenes*
5. The Baranyi model and secondary models for maximum growth rate and maximum population density provided accurate descriptions of the growth of *L. innocua* and *L. monocytogenes* at temperature range of 5 to 36°C. However, the

secondary model for lag time did not provide an accurate description as well as maximum growth rate and maximum population density for both microorganisms.

The second part of this research consisted on a quantitative microbial risk assessment for *L. monocytogenes* in fresh baby spinach leaves.

6. *L. monocytogenes* should be considered by producers for risk of listeriosis because it can grow up to very high concentrations under favorable conditions (eg, temperature).
7. Fresh produce should be periodically monitored for presence of pathogens in the processing systems to prevent cross-contamination.
8. The records of time and temperature should be regularly maintained throughout the farm to table chain to monitor temperature abuse.
9. The risk of listeriosis can be reduced by using Irradiation and Modified Atmosphere Packaging (MAP) intervention steps.

CHAPTER VI

RECOMMENDATIONS FOR FURTHER STUDY

Recommendations for future research on validation of *Listeria innocua* as a surrogate for *L. monocytogenes* and the quantitative risk assessment for *L. monocytogenes* in fresh produce are to:

- Test *L. innocua* under different environmental conditions such as temperature, pH, relative humidity, and oxygen and nutrients availability and in several commodities before used as a surrogate for *L. monocytogenes*.
- Evaluate the effect of sub-population of the natural microflora on the growth of *L. innocua* and *L. monocytogenes* in fresh baby spinach.
- Determine the dynamic models to include all parameters, maximum growth rate, lag time, and maximum population density which affected growth of *L. innocua* and *L. monocytogenes* in leafy green vegetables
- Collect data regarding prevalence and initial concentration of *L. monocytogenes* in fresh baby spinach and other each commodities.
- Collect data regarding temperature and time during harvesting, processing, transportation, market and home storage for fresh baby spinach and leafy green vegetables.

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APPENDIX

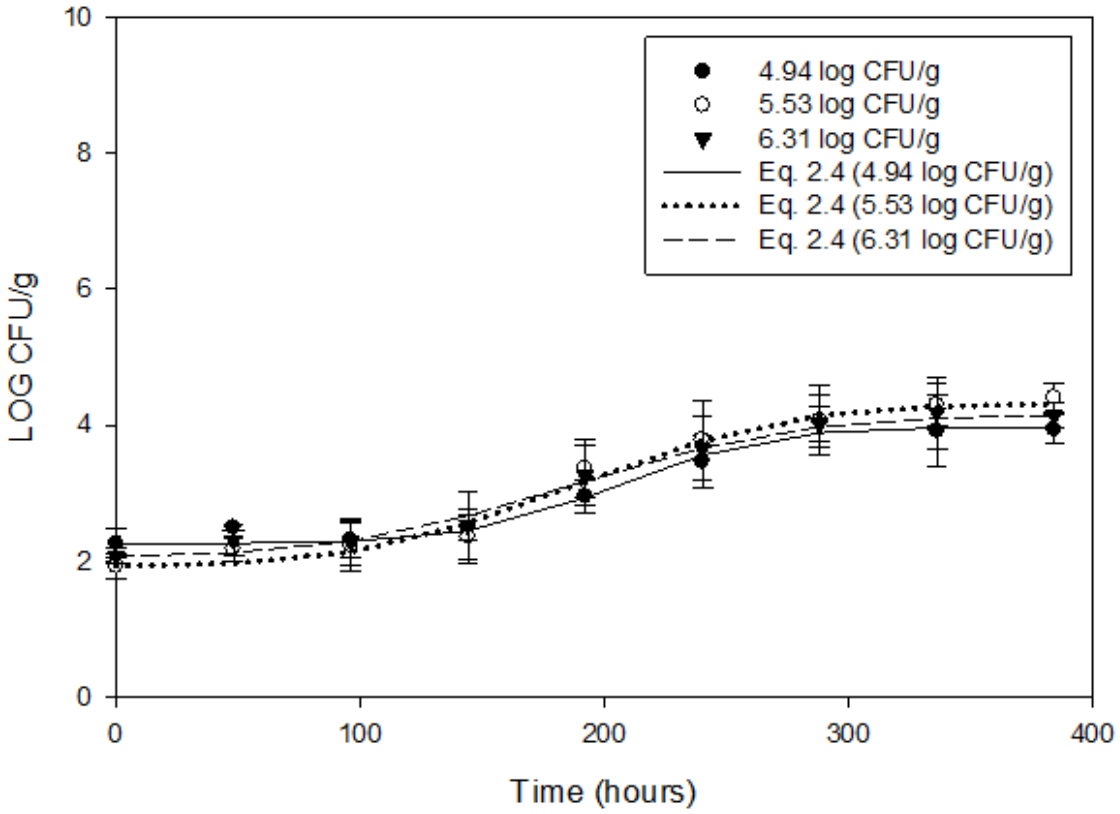

Figure A.1. The observed growth of *Listeria monocytogenes* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 5ºC by fitting Baranyi model (Eq. 2.4).

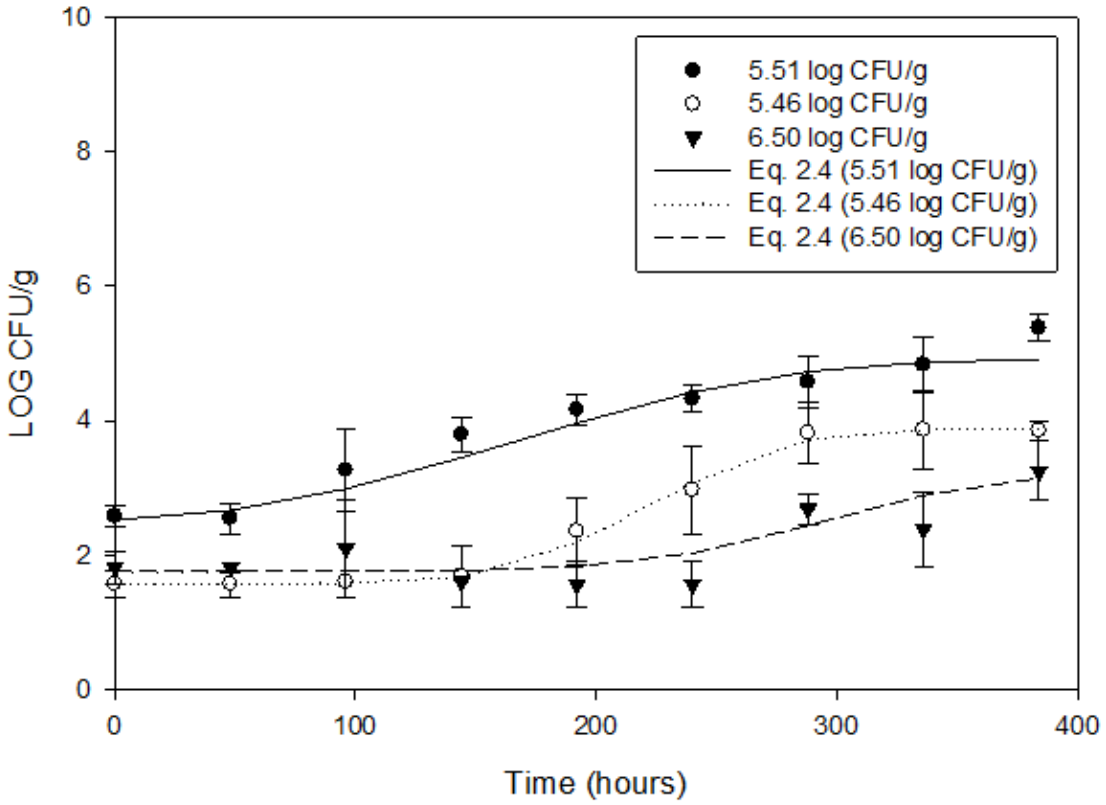

Figure A.2. The observed growth of *Listeria innocua* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 5^{o}C by fitting Baranyi model (Eq. 2.4).

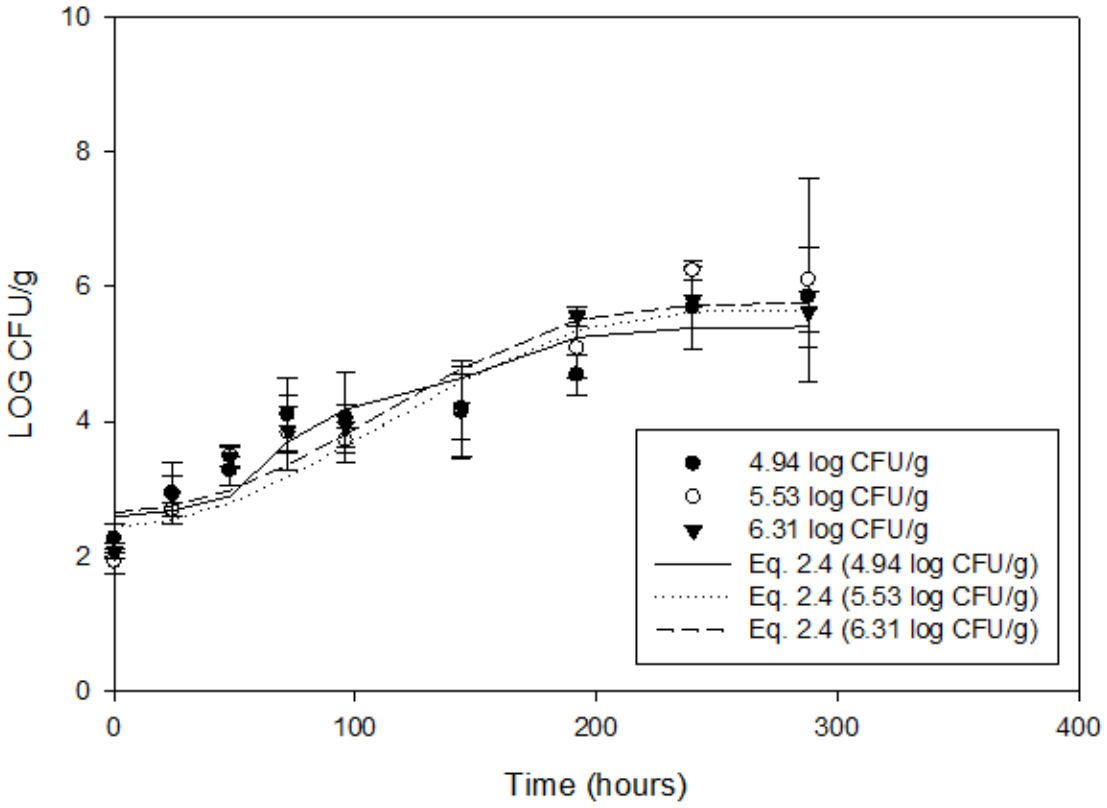

Figure A.3. The observed growth of *Listeria monocytogenes* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 10°C by fitting Baranyi model (Eq. 2.4).

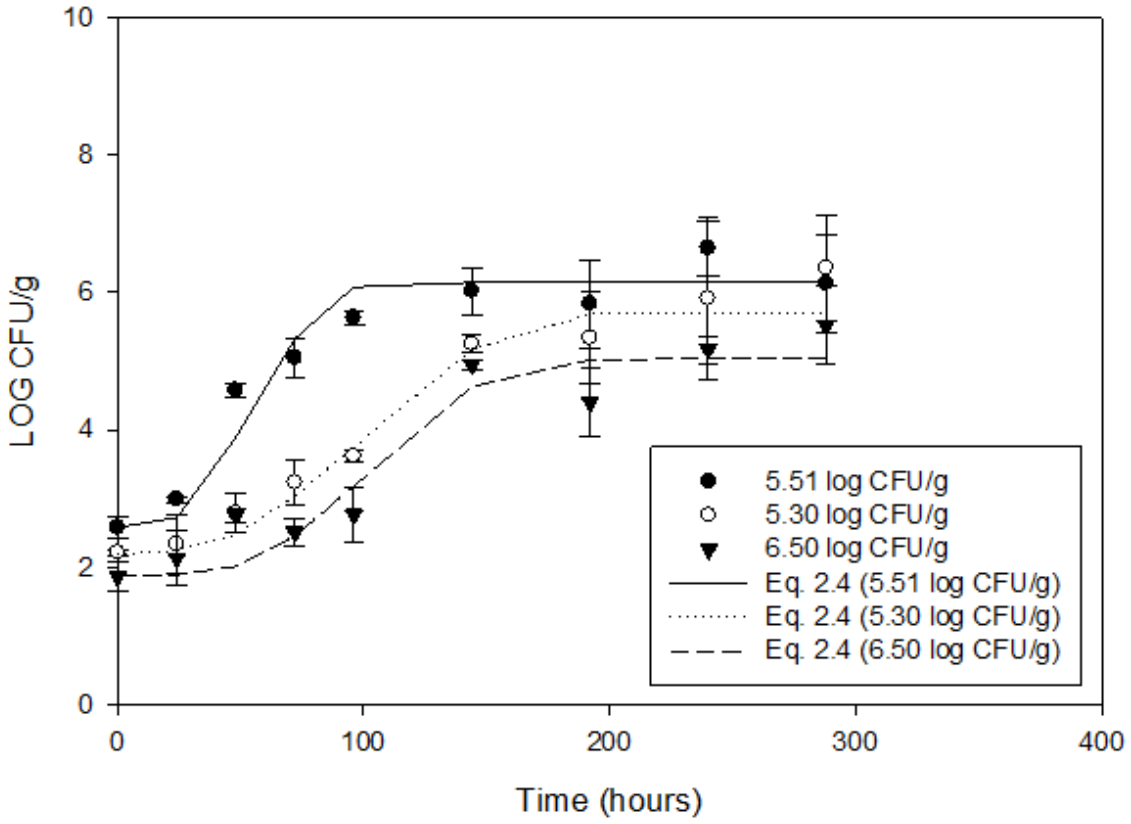

Figure A.4. The observed growth of *Listeria innocua* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 10^{o}C by fitting Baranyi model (Eq. 2.4).

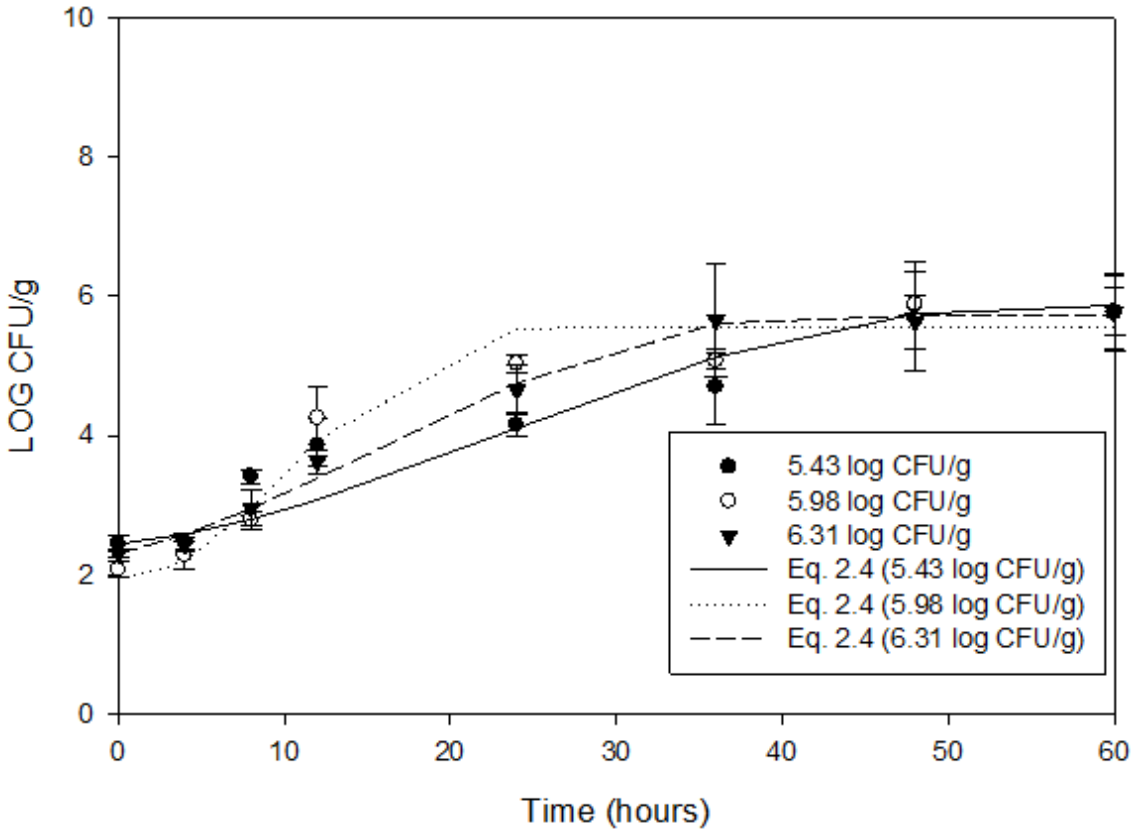

Figure A.5. The observed growth of *Listeria monocytogenes* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 20^{o}C by fitting Baranyi model (Eq. 2.4).

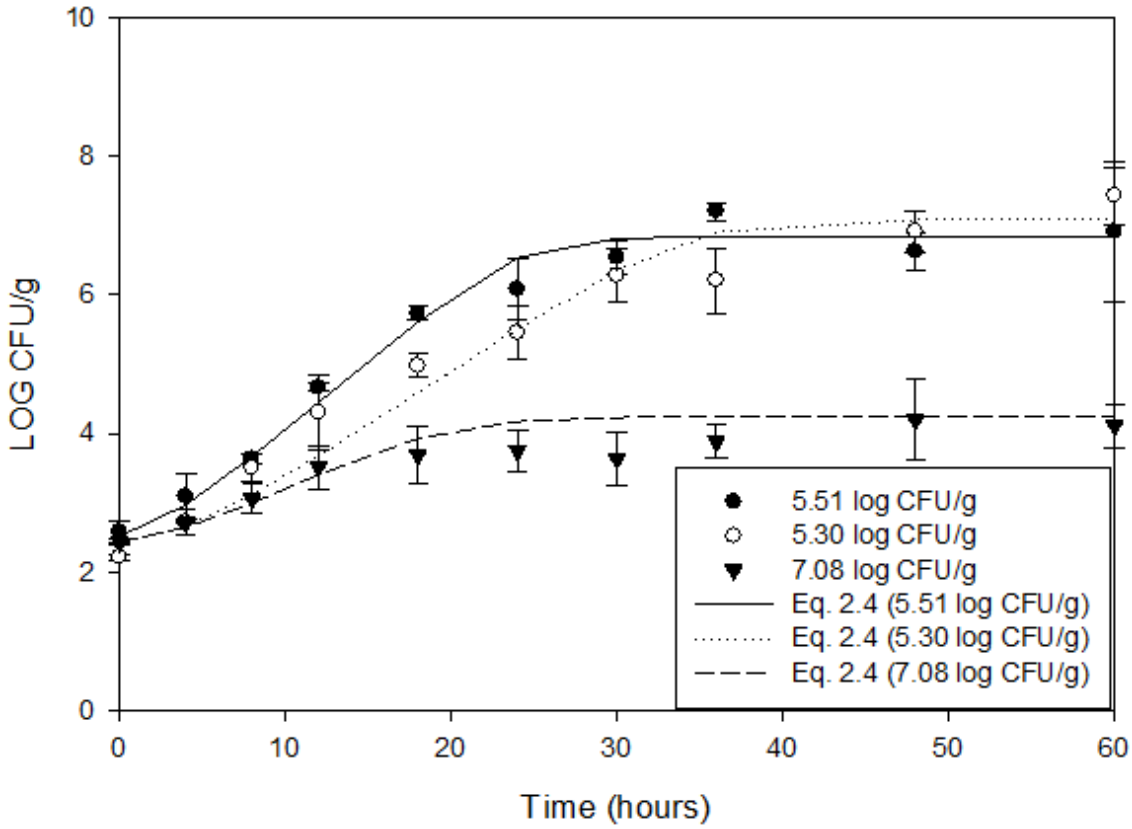

Figure A.6. The observed growth of *Listeria innocua* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 20^{o}C by fitting Baranyi model (Eq. 2.4).

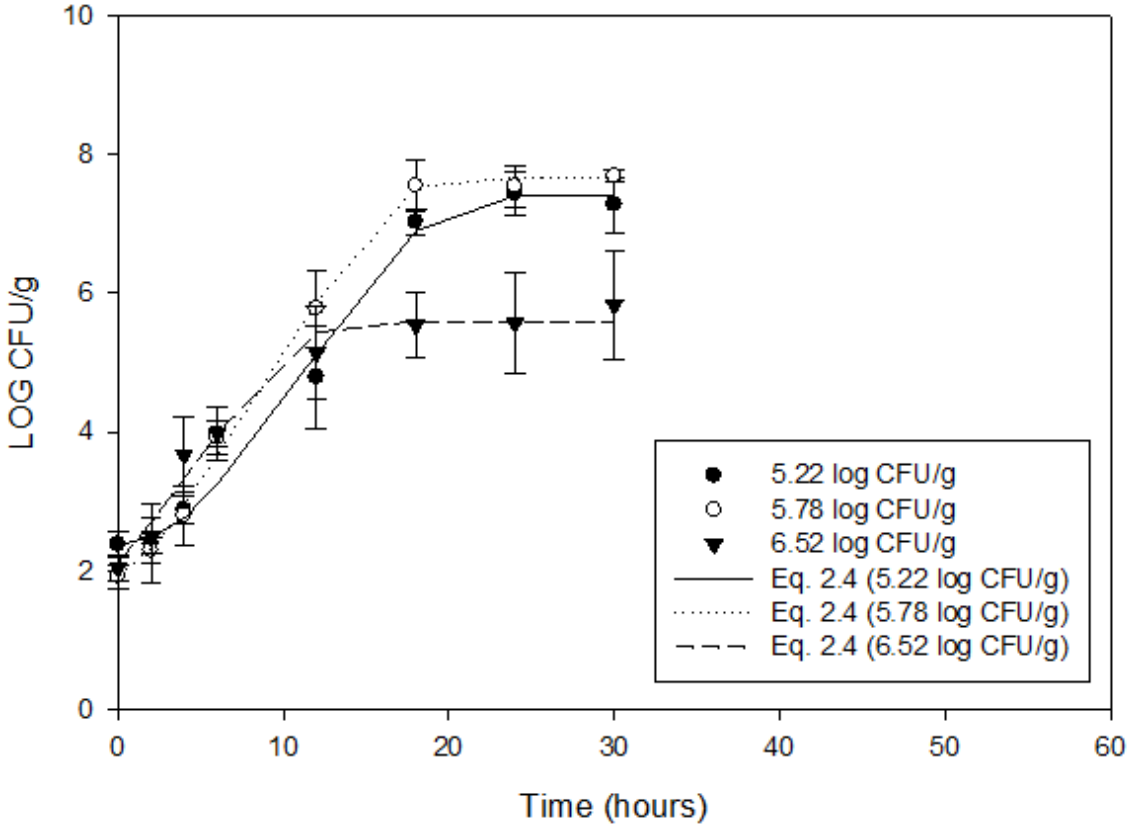

Figure A.7. The observed growth of *Listeria monocytogenes* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 30°C by fitting Baranyi model (Eq. 2.4).

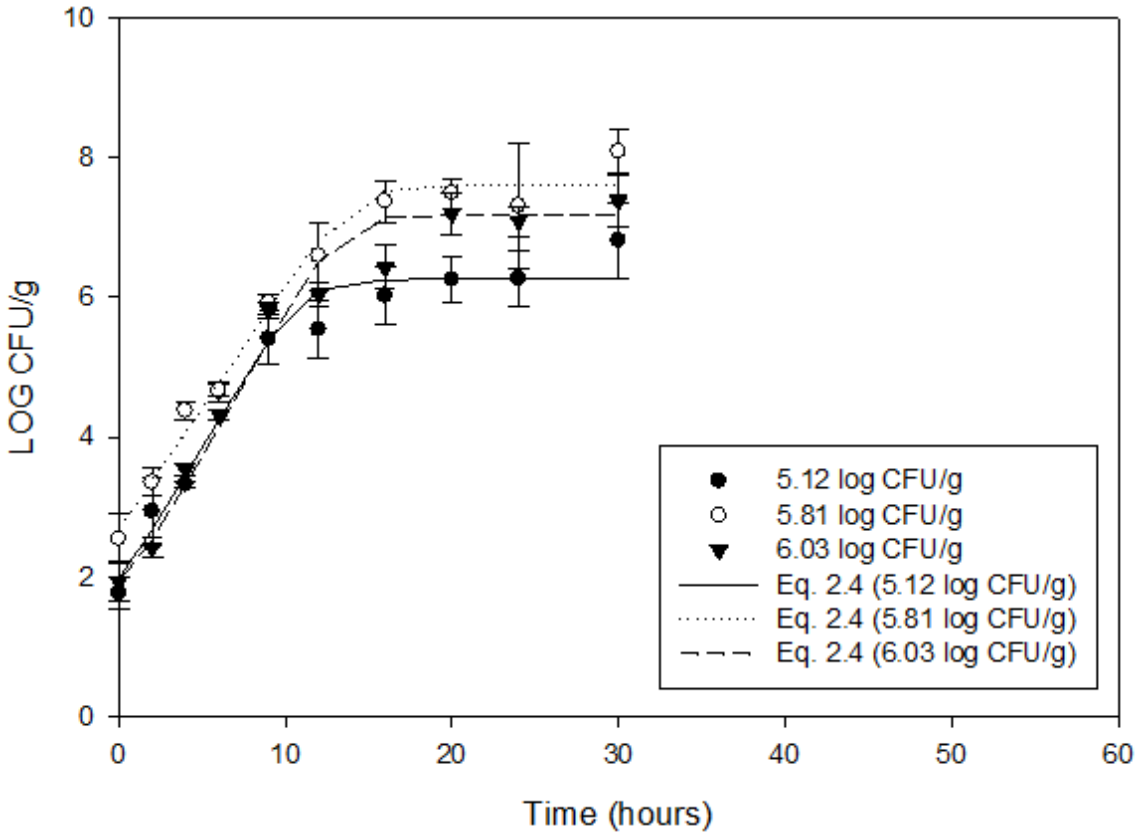

Figure A.8. The observed growth of *Listeria innocua* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 30^{o}C by fitting Baranyi model (Eq. 2.4).

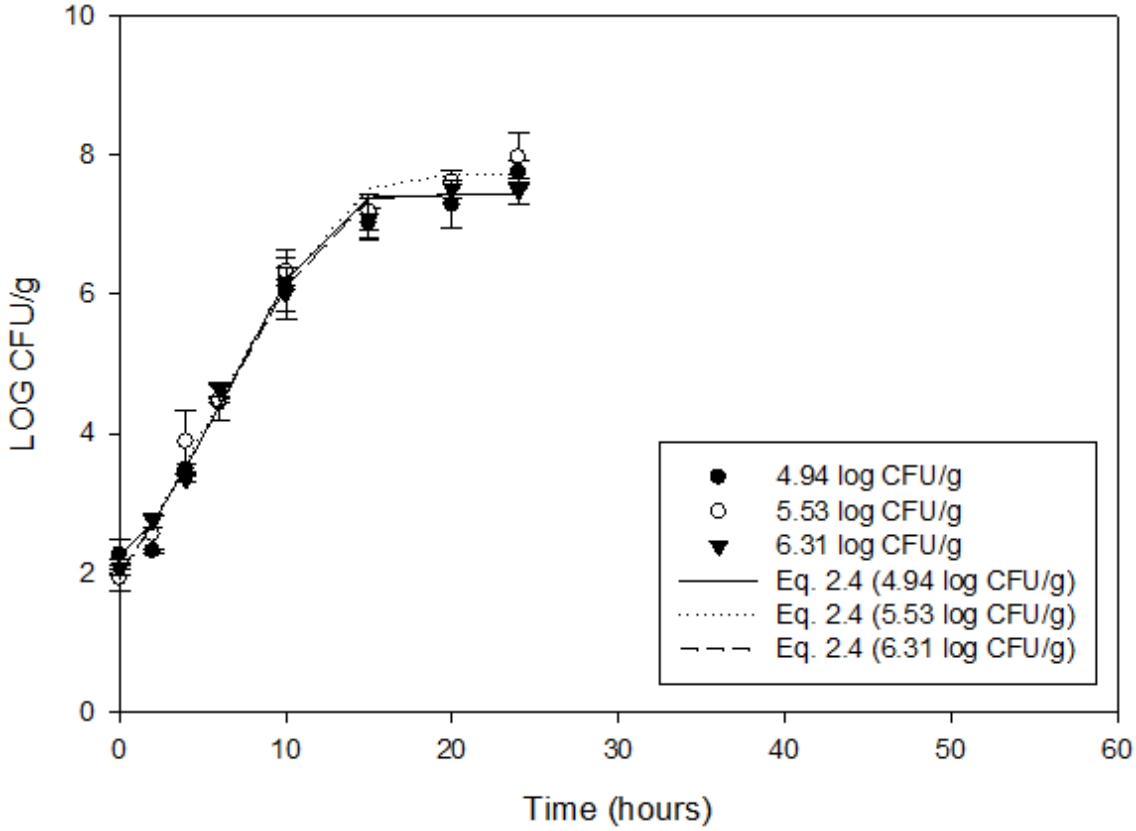

Figure A.9. The observed growth of *Listeria monocytogenes* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 36°C by fitting Baranyi model (Eq. 2.4).

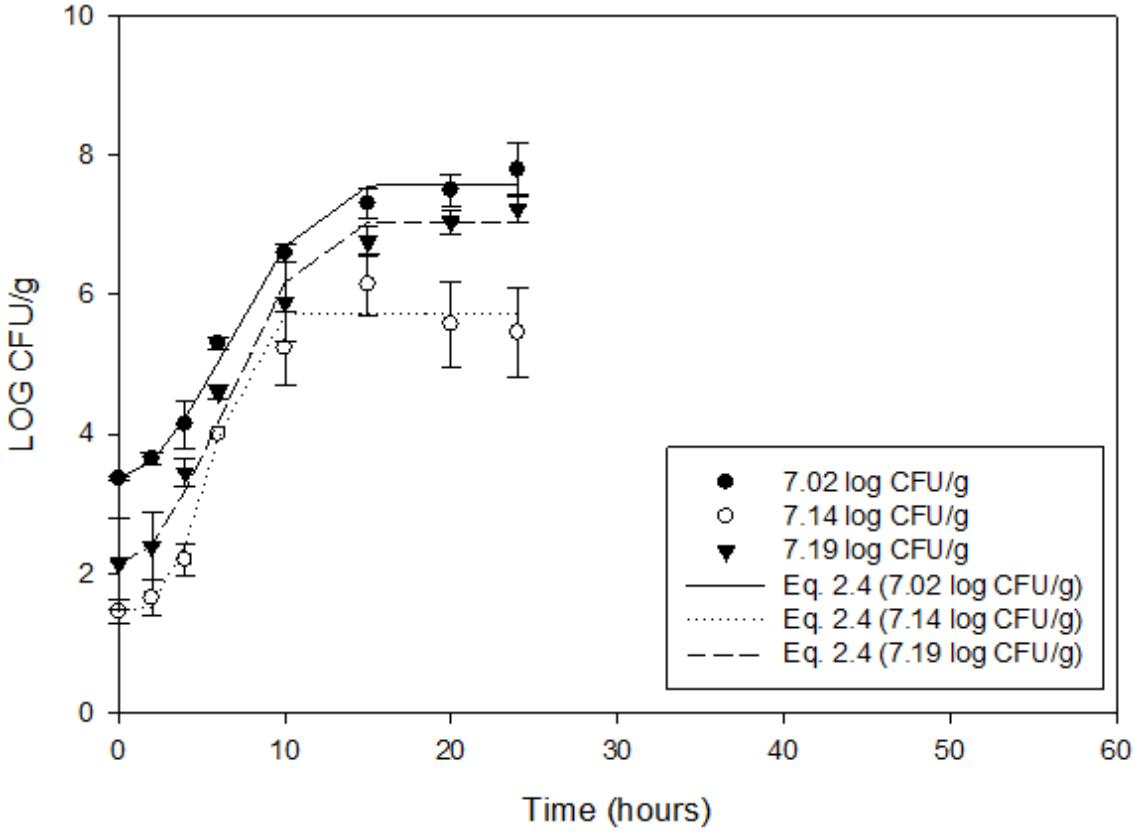

Figure A.10. The observed growth of *Listeria innocua* depend on the initial concentration of total mesophilic bacteria on fresh baby spinach leaves at 36°C by fitting Baranyi model (Eq. 2.4).

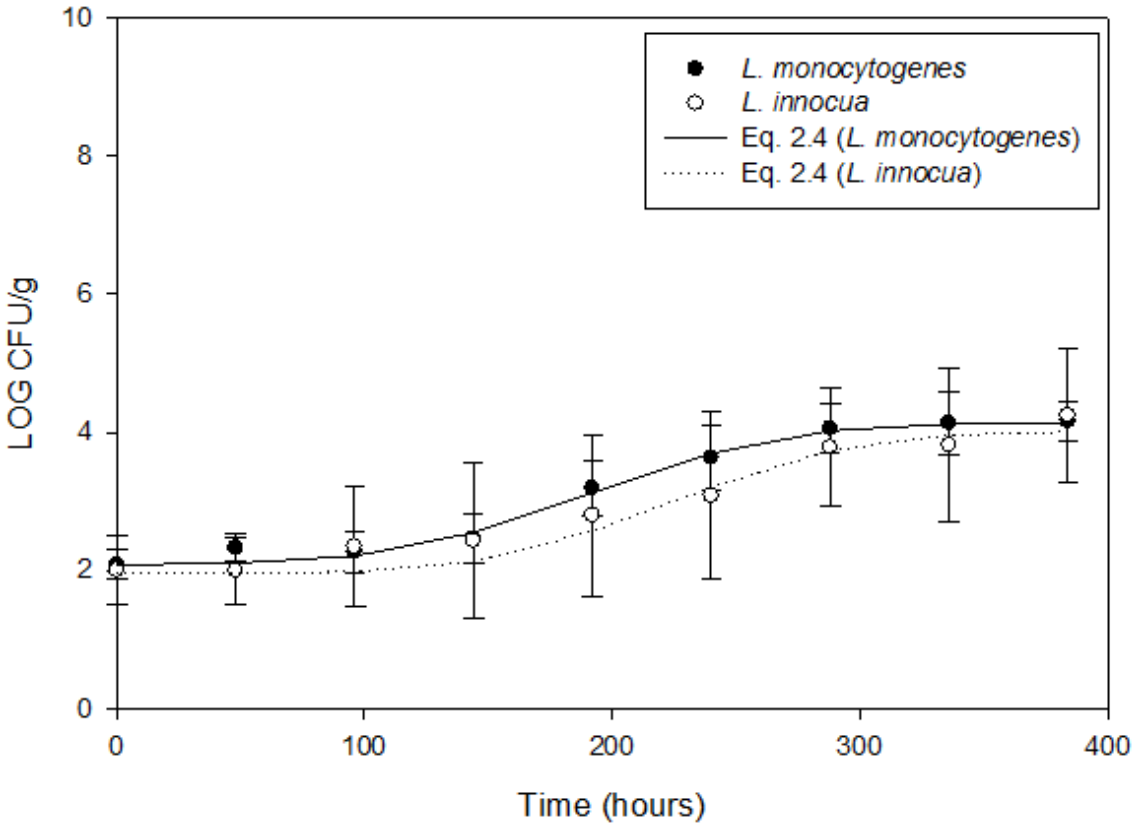

Figure A.11. The observed growth of *Listeria monocytogenes* and *Listeria innocua* on fresh baby spinach leaves at 5^{o}C by fitting Baranyi model (Eq. 2.4).

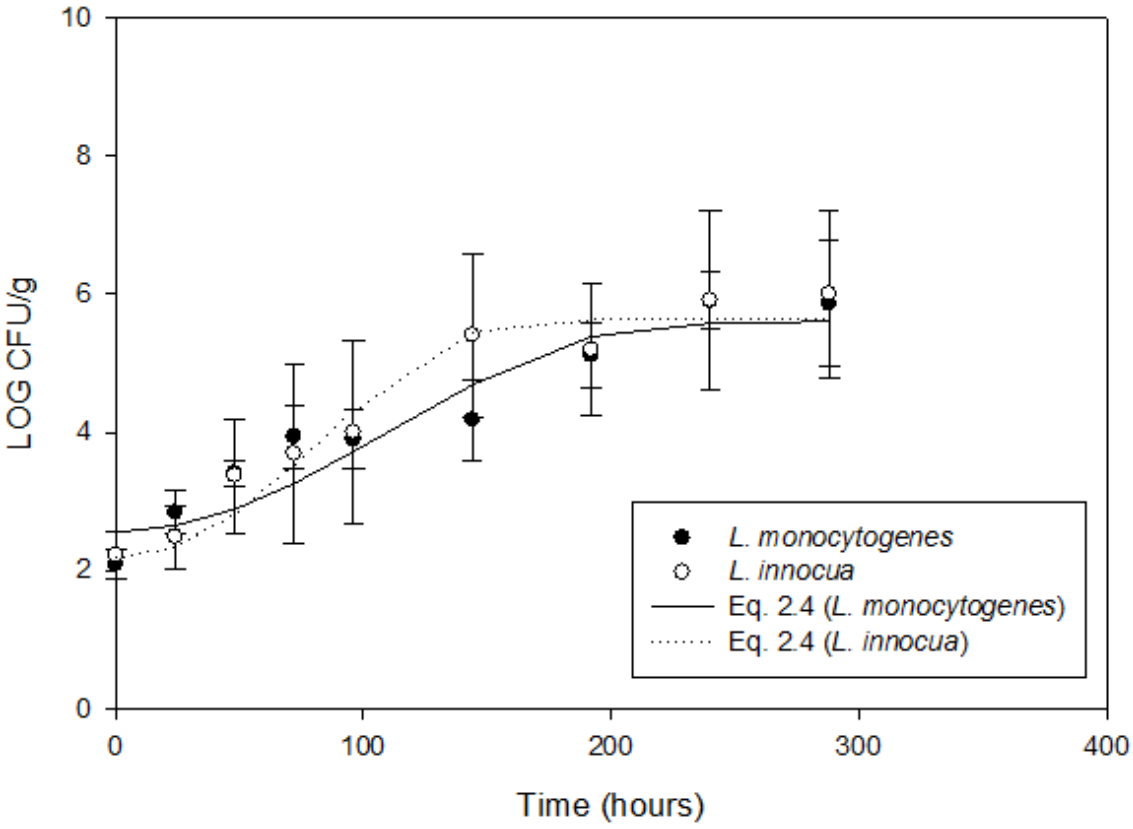

Figure A.12. The observed growth of *Listeria monocytogenes* and *Listeria innocua* on fresh baby spinach leaves at 10°C by fitting Baranyi model (Eq. 2.4).

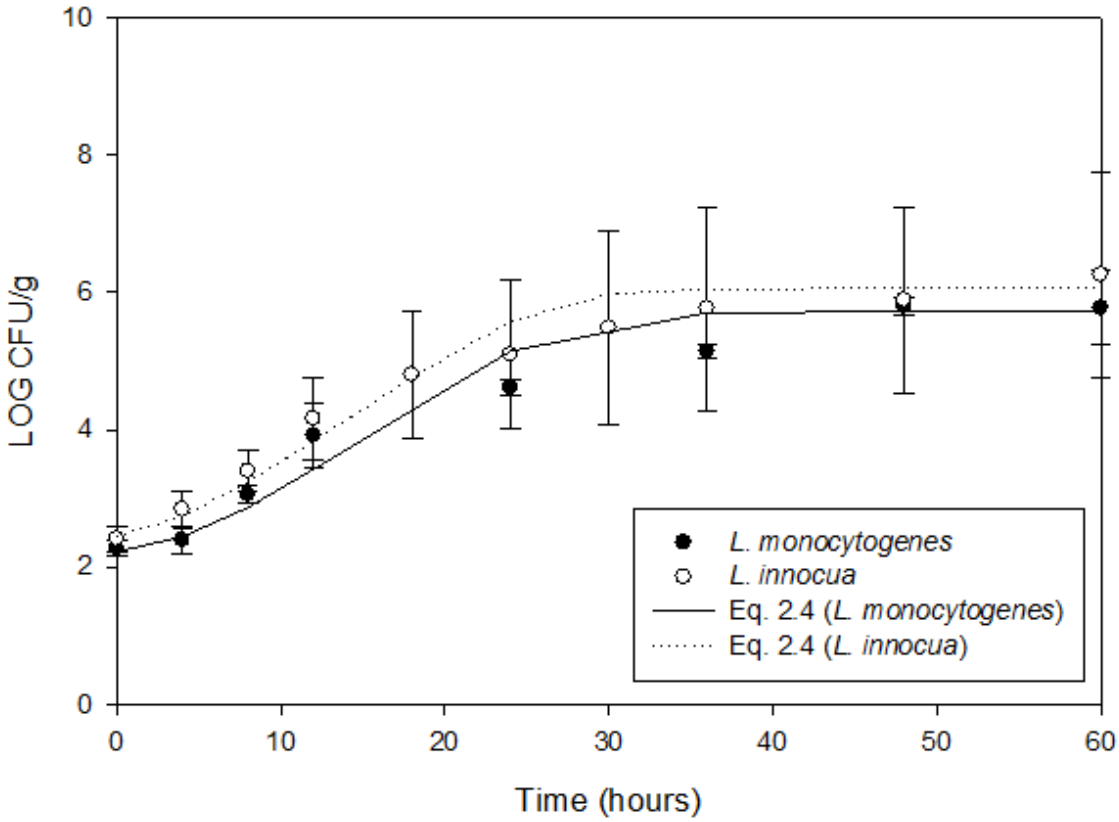

Figure A.13. The observed growth of *Listeria monocytogenes* and *Listeria innocua* on fresh baby spinach leaves at 20ºC by fitting Baranyi model (Eq. 2.4).

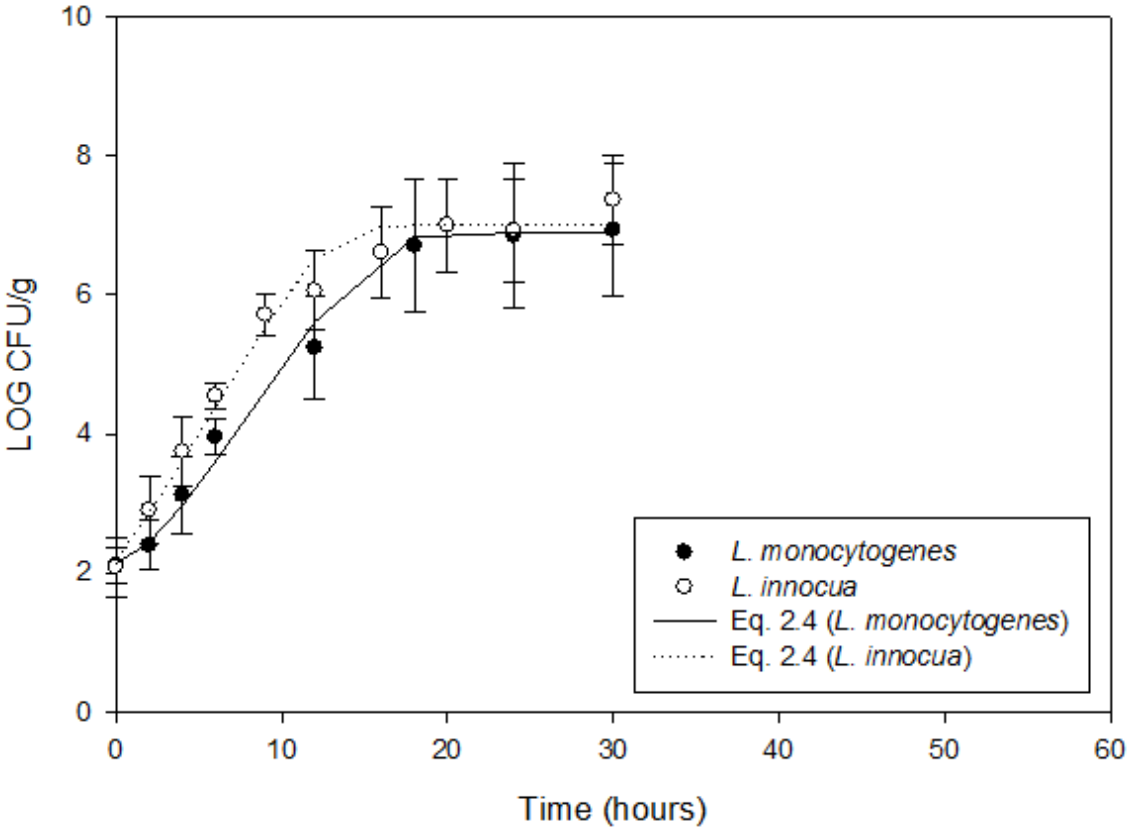

Figure A.14. The observed growth of *Listeria monocytogenes* and *Listeria innocua* on fresh baby spinach leaves at 30ºC by fitting Baranyi model (Eq. 2.4).

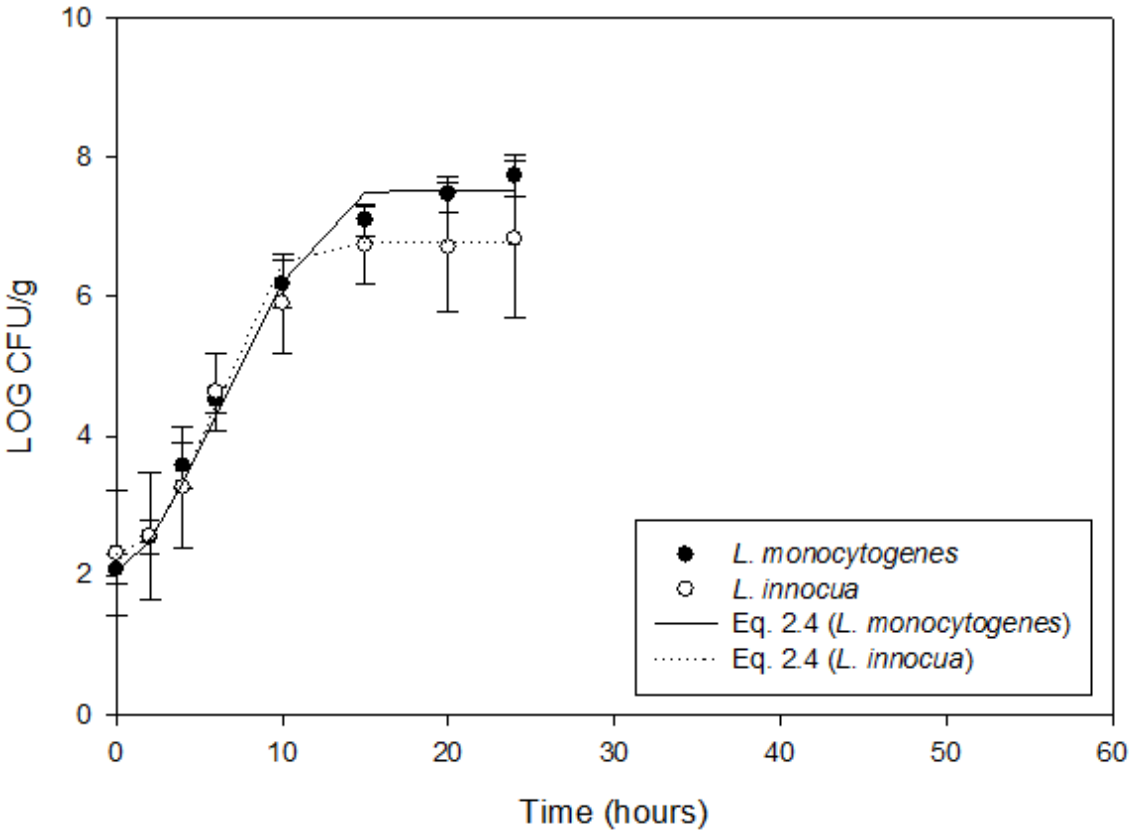

Figure A.15. The observed growth of *Listeria monocytogenes* and *Listeria innocua* on fresh baby spinach leaves at 36°C by fitting Baranyi model (Eq. 2.4).

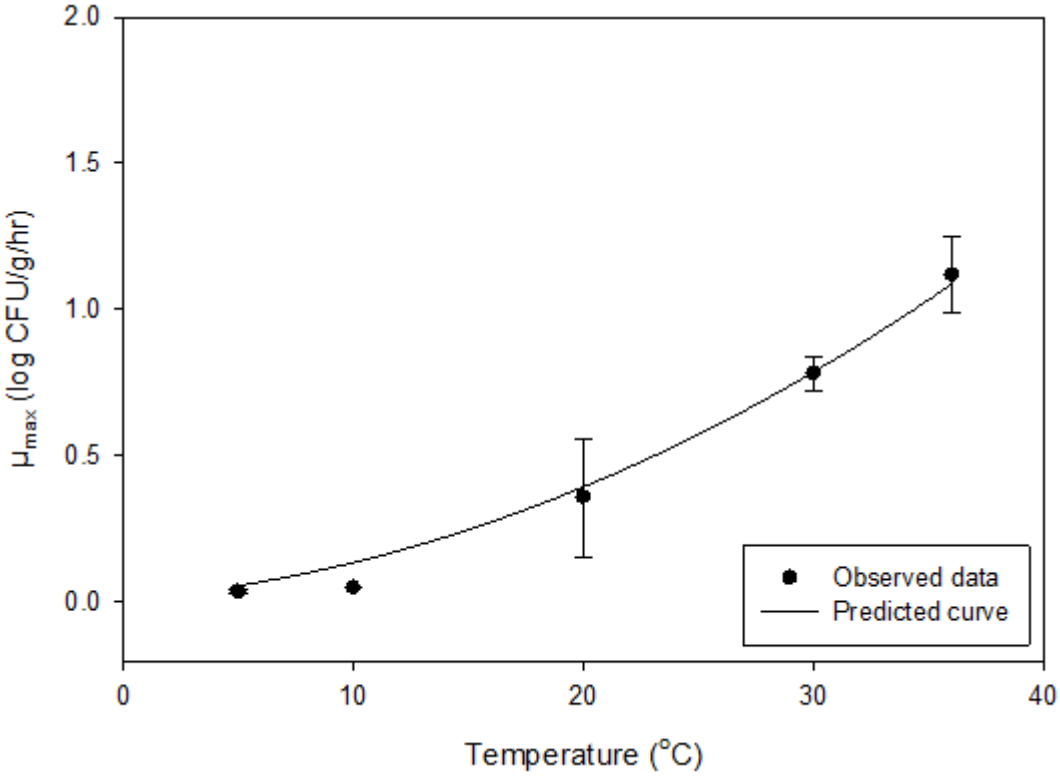

Figure A.16. Maximum growth rate for *Listeria monocytogenes* on fresh baby spinach leaves as a function of temperature.

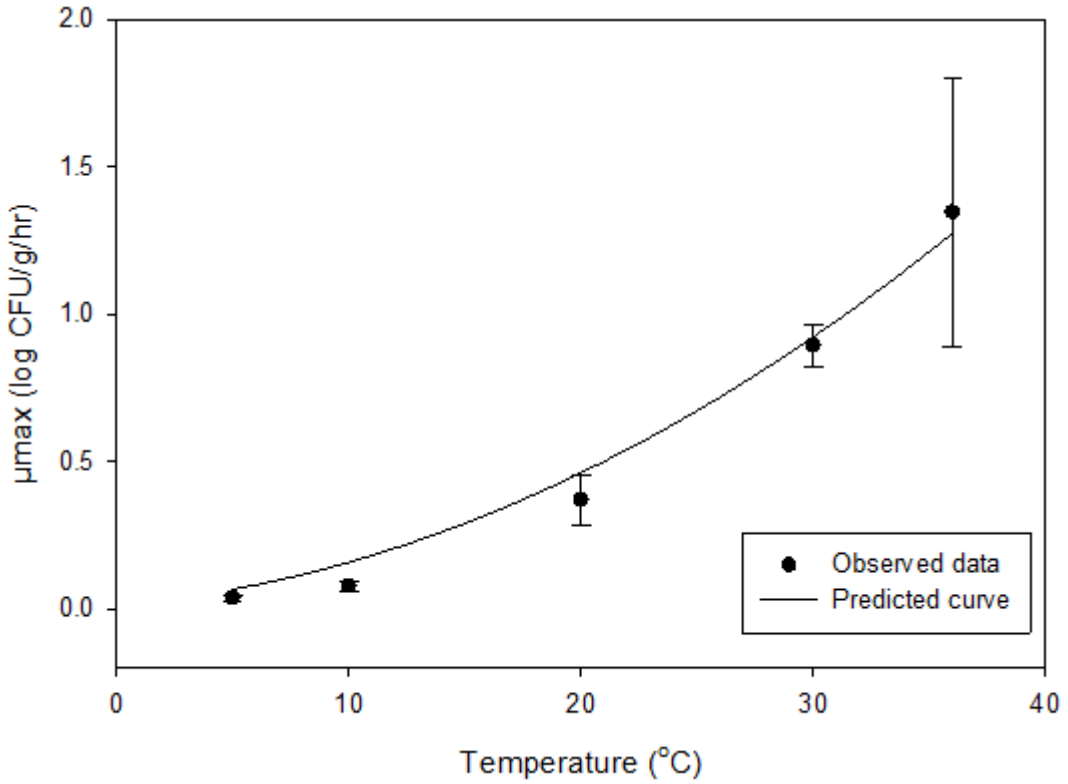

Figure A.17. Maximum growth rate for *Listeria innocua* on fresh baby spinach leaves as a function of temperature.

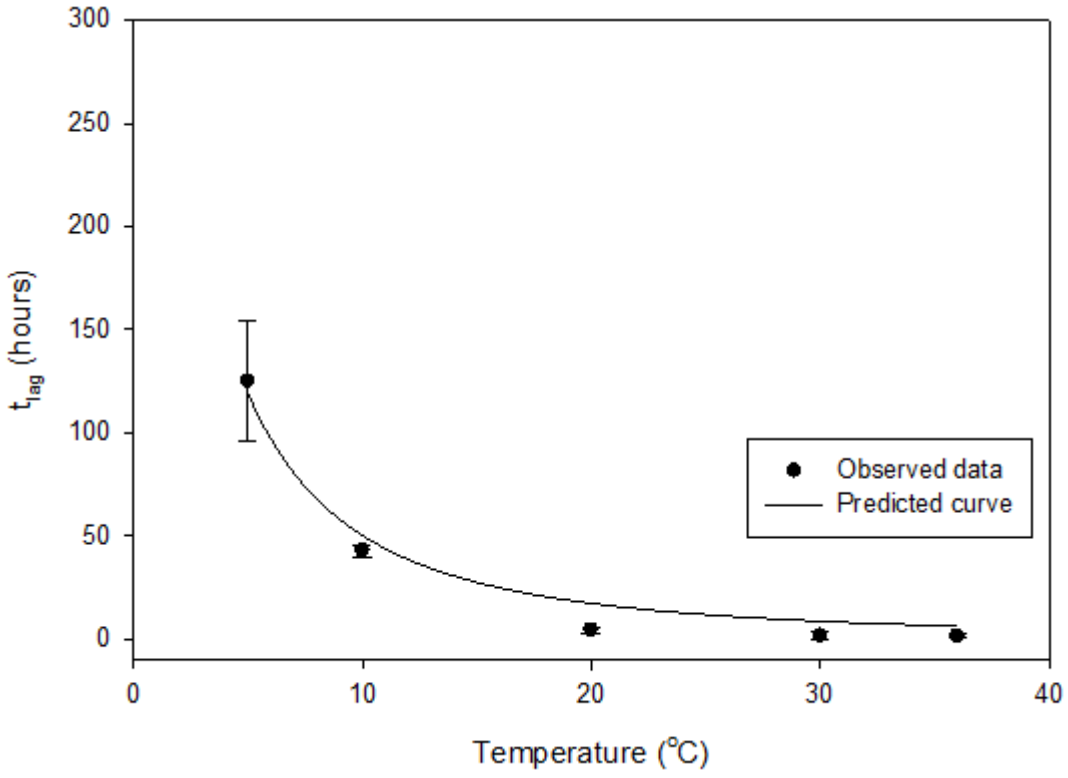

Figure A.18. Lag time for *Listeria monocytogenes* on fresh baby spinach leaves as a function of temperature.

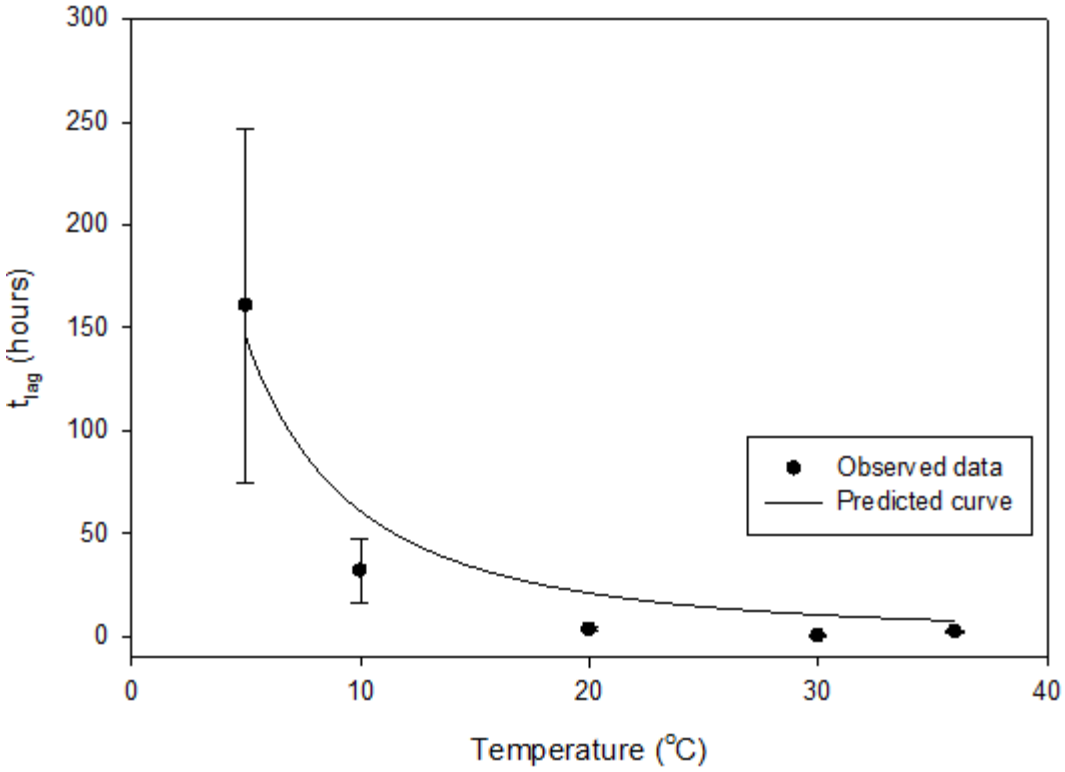

Figure A.19. Lag time for *Listeria innocua* on fresh baby spinach leaves as a function of temperature.

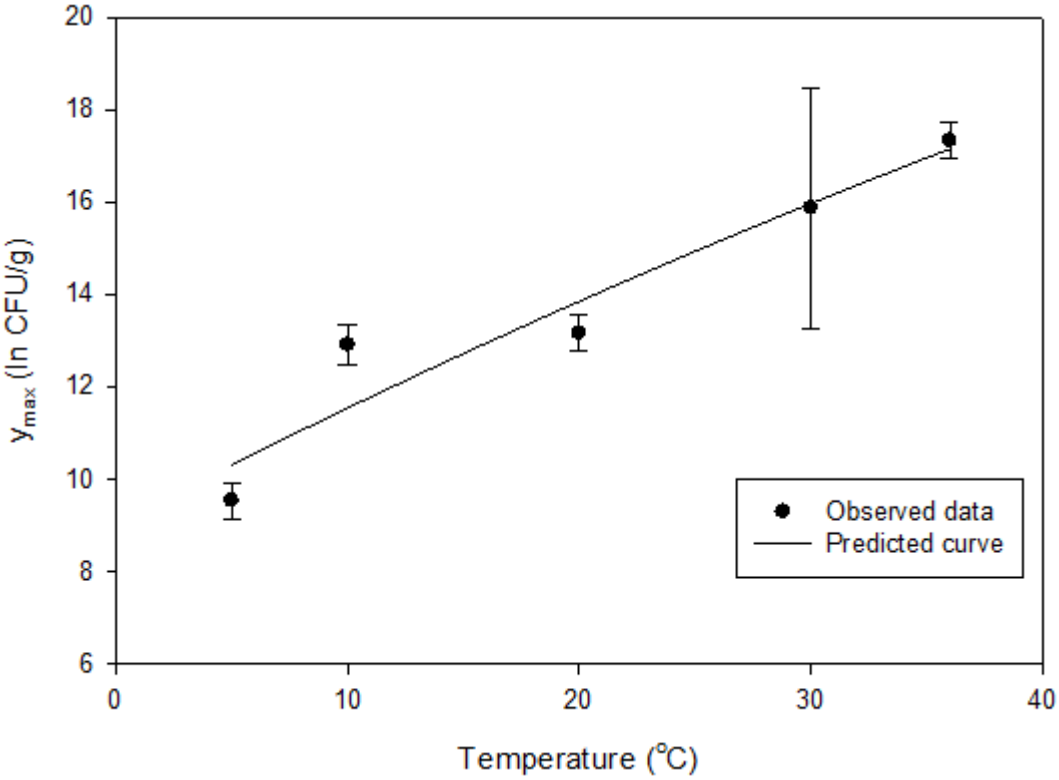

Figure A.20. Maximum population density for *Listeria monocytogenes* on fresh baby spinach leaves as a function of temperature.

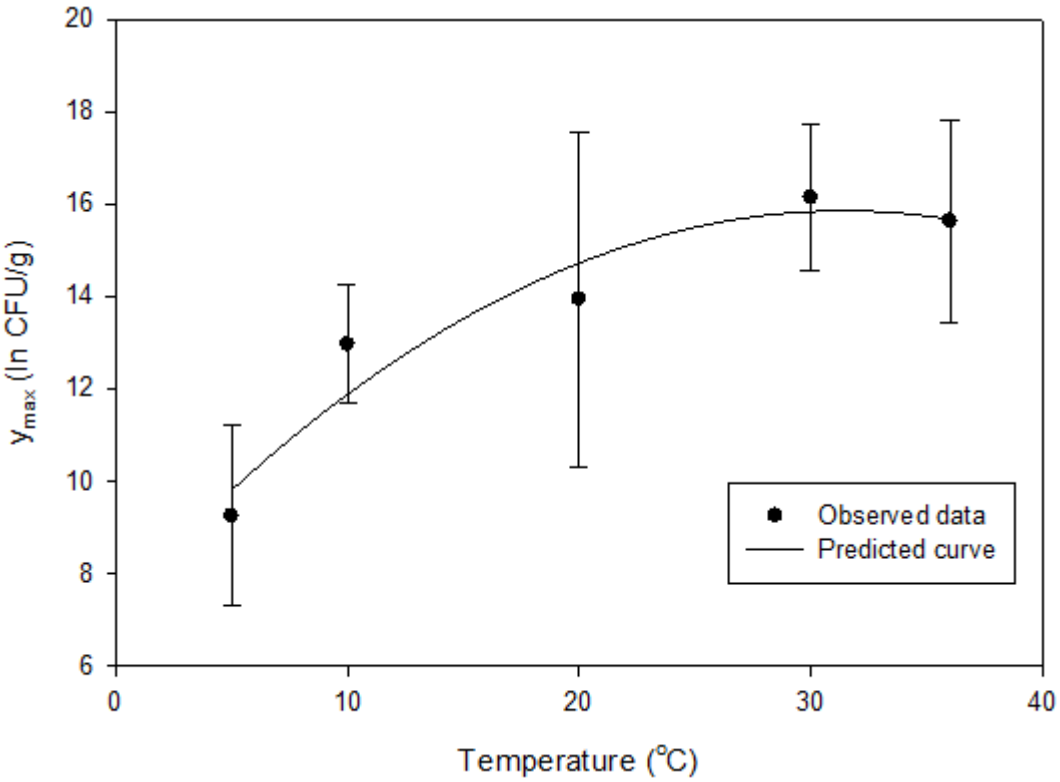

Figure A.21. Maximum population density for *Listeria innocua* on fresh baby spinach leaves as a function of temperature.

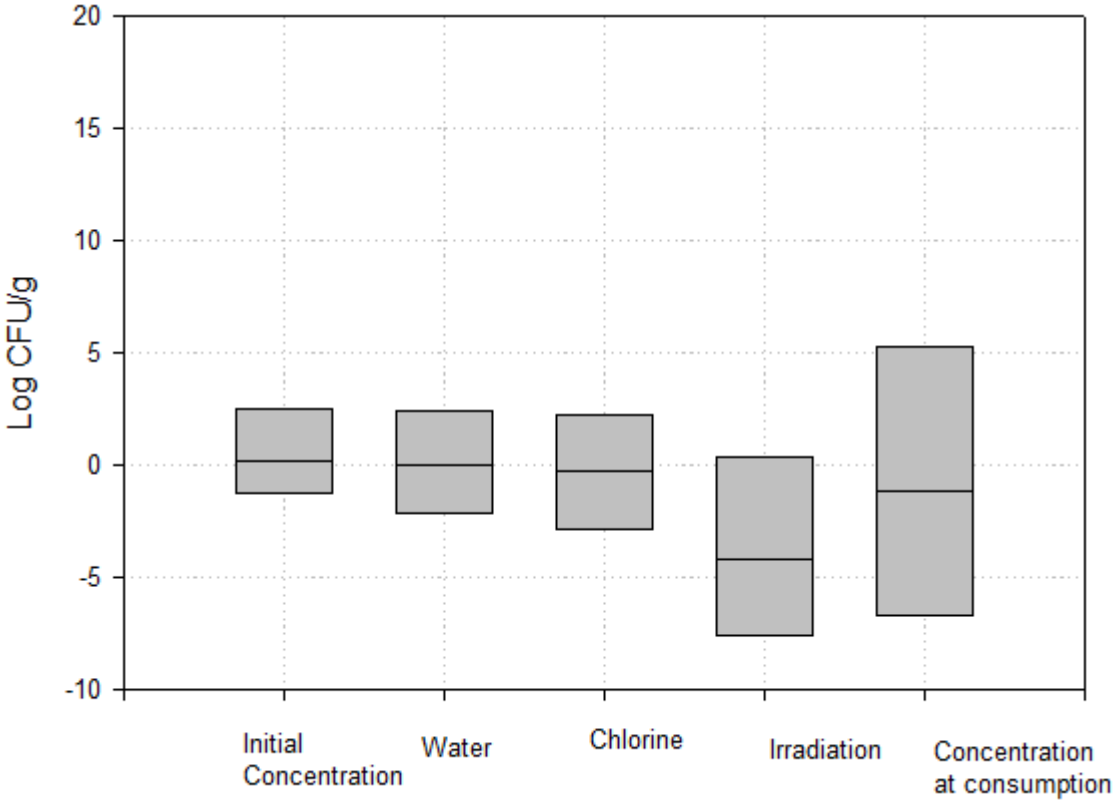

Figure A.22. The change of concentration of *Listeria monocytogenes* from farm to table (low initial concentration).

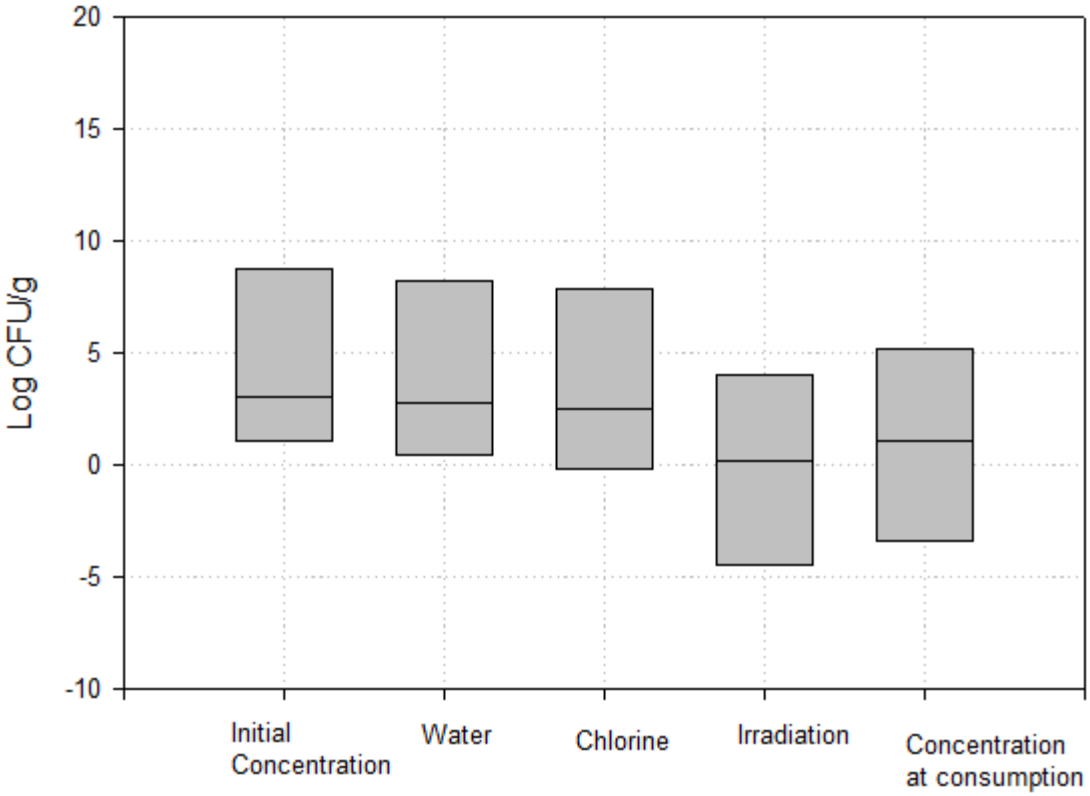

Figure A.23. The change of concentration of *Listeria monocytogenes* from farm to table (high initial concentration).

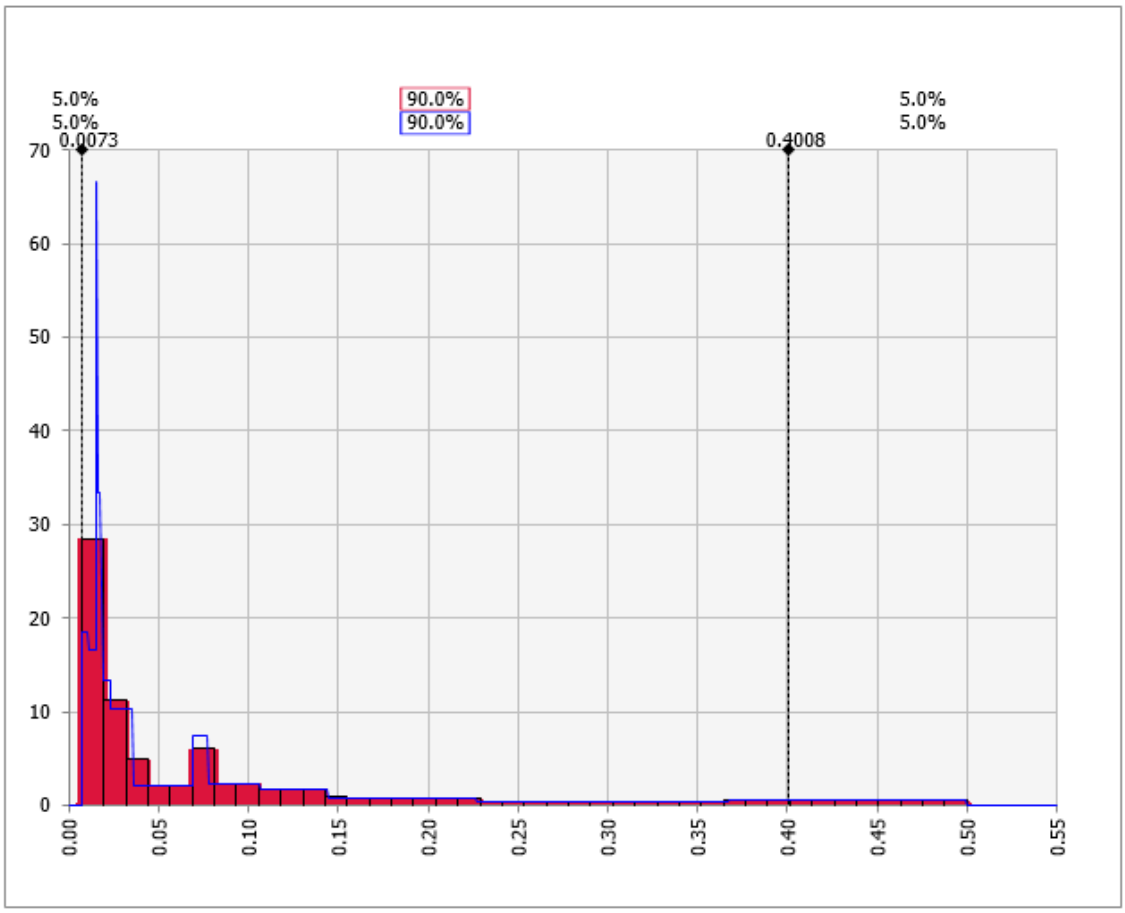

Figure A.24. Probability distribution of the prevalence of *Listeria monocytogenes* on fresh baby spinach leaves.

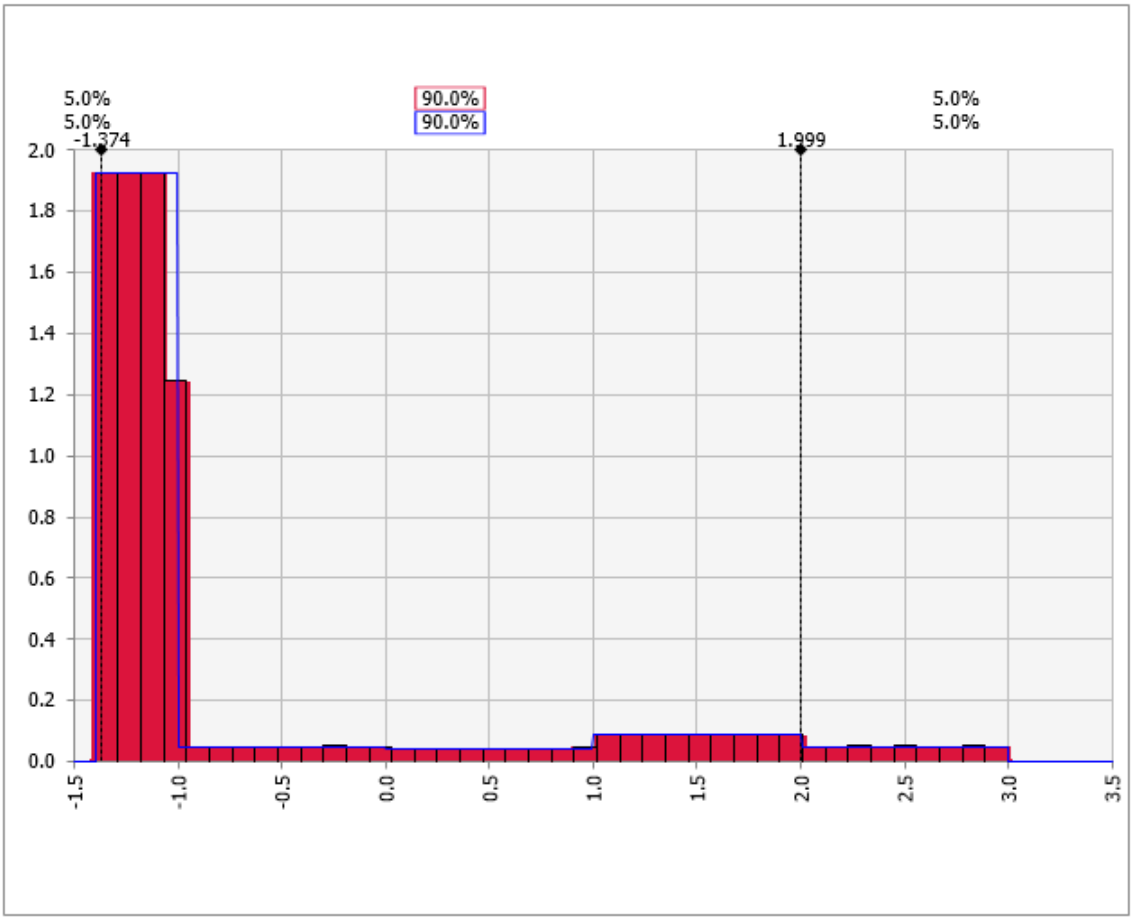

Figure A.25. Probability distribution of the initial concentration of *Listeria monocytogenes* on fresh baby spinach leaves.

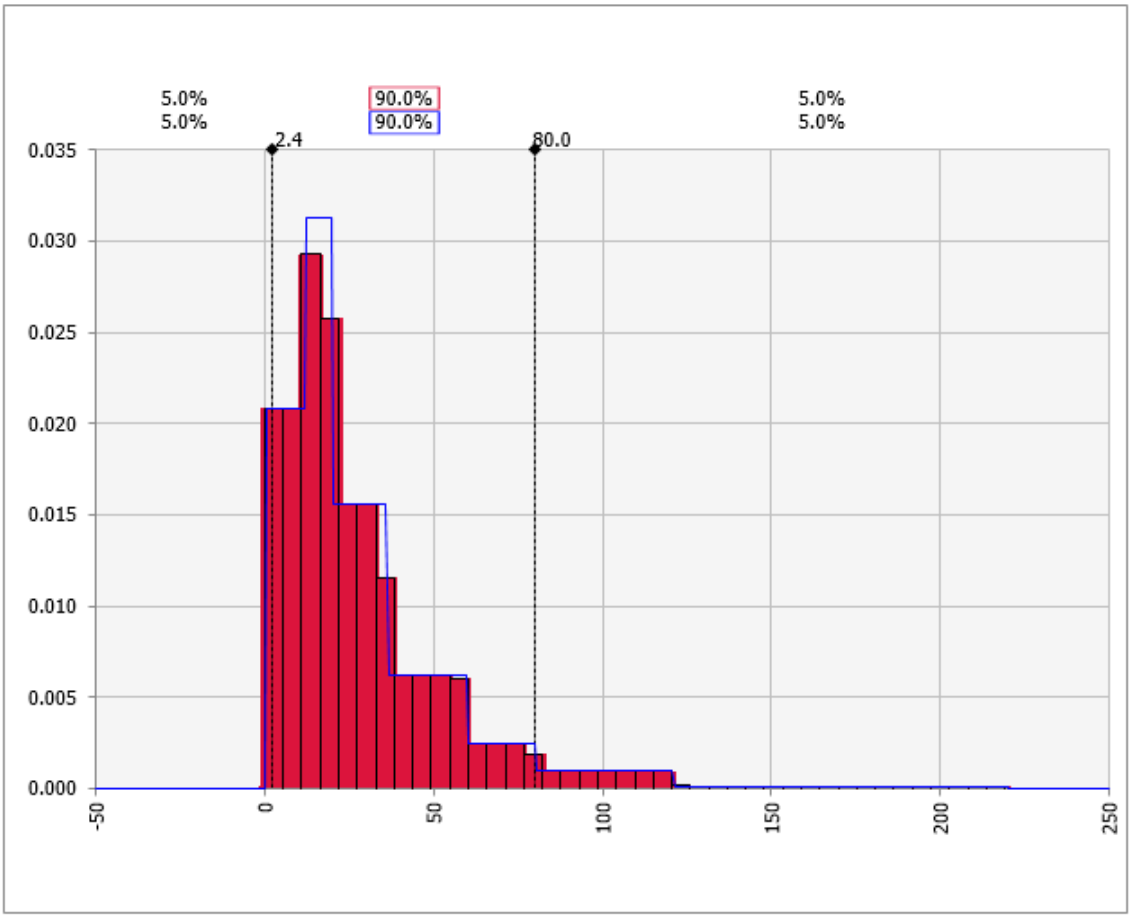

Figure A.26. Probability distribution of the serving size of fresh baby spinach consumed per individual person.

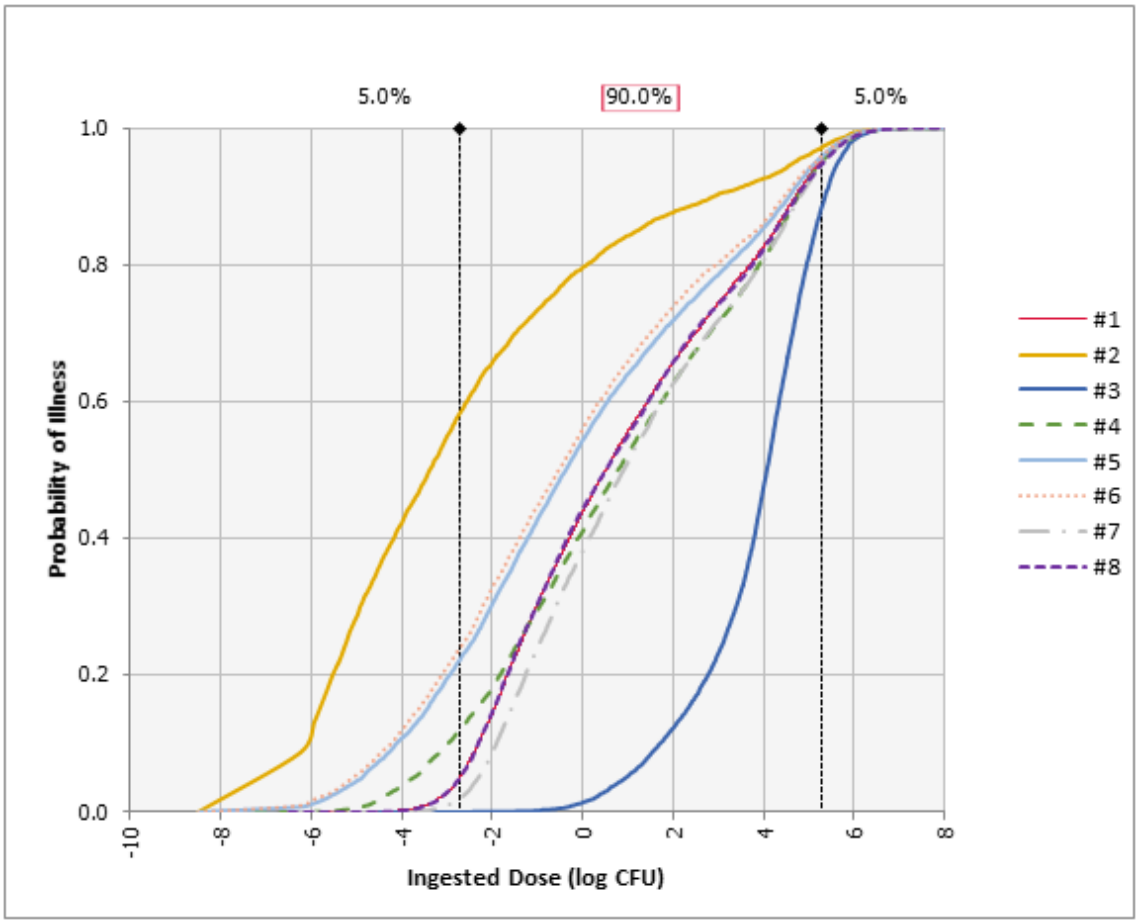

Figure A.27. Probability of infection vs. ingested dose of *Listeria monocytogenes* on fresh baby spinach leaves.

#1: Baseline

#2: Baseline + Irradiation

#3: Baseline + Cross contamination

#4: Baseline + Cross Contamination + Irradiation

#5: Baseline + Cross contamination + MAP (O_2 (100%)) + Irradiation

#6: Baseline + Cross contamination + MAP (N_2O_2 (1:1)) + Irradiation

#7: Baseline +Temperature abuse (21°C, 2 hr) at the table

#8: Baseline + Temperature abuse (25°C, 4 hr) the transportation step

Table A.1 Overview of simulation variables and parameters.

Cell	Unit	Variable	Value	Source
G4	Percent	Prevalence of contamination spinach	RiskCumul(E4,F4,J4:J16,I4:I16)	Table 4.1
G5	Log CFU/g	**Initial Concentrations**	RiskCumul(-1.4,4,{-1,0,1,2,3},{0.77,0.82,0.86,0.95,1})	Carrasco et al. (2010)
G6		**Washing log Reductions**		
G7	Log CFU/g	Water	RiskNormal(0.54,0.16)	
G8	Log CFU/g	Chlorine	RiskNormal(0.42,0.22)	
G9	Log CFU/g	Cross Contamination	RiskUniform(2.12,7.43)	Ding et al. (2013)
G10	Log CFU/g	Pathogen concentration after washing treatments	RiskOutput()+G5-G7-G8+G9	
G11	kGy	Irradiation Dose		
G12	CFU/g	Pathogen concentration after irradiation		
G13	Log CFU/g	Pathogen concentration after disinfection treatments	RiskOutput()+G10	
G14	LN CFU/g	Pathogen concentration after disinfection treatments	RiskOutput()+(G10*LN(10))	
G15		**Transportation and Retail Storage**		
G16	hours	Transportation time	RiskUniform(4,10)	Ding et al. (2013)
G17	^{o}C	Temp, retail	RiskExtvalue(4.9495,2.8227)	Danyluk and Schaffner (2011)
G18	hours	Time, retail	RiskUniform(96,168)	Danyluk and Schaffner (2011)
G19	hours	Total time	RiskUniform(G16,G18)	

Table A.1 Overview of simulation variables and parameters (continued) (This part was modified from Danyluk and Schaffner (2010))

Cell	Unit	Variable	Value	Source
A20		**Home storage**		
A21	^{o}C	Temp, home, mean	4.06	
A22	^{o}C	Temp, difference from mean	RiskExpon(2.31)	
A23	^{o}C	Temp, above or below mean	RiskBinomial(1,0.5)	
A24	^{o}C	Home temp used	RiskOutput()+IF(G23=1,G21+G22,G21-G22)	
A25	^{o}C	Temperature for used	RiskUniform(G24,G17)	
A26	hours	Min. time to first	27.12	
A27	hours	Max. time to first	68.16	
A28	hours	Min. time to first from farm to home	RiskOutput()+(G19+G26)	
A29	hours	Max. time to first from farm to home	RiskOutput()+G27+G19	
A30	hours	Time to first from farm to home	RiskWeibull(G28,G29)	
A31	hours	Min. time to last	41.52	
A32	hours	Max. time to last	191.04	
A33	hours	Min. time to last from farm to home	RiskOutput()+(G31+G19)	
A34	hours	Max. time to last from farm to home	RiskOutput()+(G32+G19)	
A35	hours	Time to last	RiskWeibull(G33,G34)	
A36	hours	Time to used if first is after last	RiskOutput()+IF(G30>G35,G30,0)	
A37	hours	Time from uniform ditribution	RiskUniform(G30,G35)	
A38	hours	Time selected for consumption	RiskOutput()+IF(G36=0,G37,G36)	

Table A.1 Overview of simulation variables and parameters (continued)

Cell	Unit	Variable	Value	Source
G39		**Growth**		
G40		μ_{max} b parameter	0.02645	
G41	^{o}C	μ_{max} T_{min} parameter	-4.26	
G42	1/hours	μ_{max}	RiskOutput()+((G40*(G25-G41))^2)	
G43		t_{lag} c parameters	0.0099	
G44		t_{lag} T_{min} parameter	-4.26	
G45	hours	t_{lag}	RiskOutput()+((G43*(G25-G44))^(-2))	
G46		F(t)	RiskOutput()+(G38+((1/G42)*LN((EXP(-G42*G38))+(EXP(-G45*G42))-(EXP((-G42*G38)-(G42*G45))))))	
G47		y_{max} A1 parameters	-0.0009	
G48		y_{max} A2 parameters	0.2592	
G49		y_{max} A3 parameters	9.0512	
G50	LN CFU/g	y_{max}	RiskOutput()+(((G47*(G25^2))+(G48*G25)+(G49)))	
G51	Log CFU/g	y_{max}	RiskOutput()+(G50/LN(10))	
G52	LN CFU/g	y(t)	RiskOutput()+((G14)+((G42*G46))-(LN(1+(((EXP(G42*G46))-1)/(EXP(G50-(G14)))))))	
G53	Log CFU/g	y(t)	RiskOutput()+(G52/LN(10))	
G54	Log CFU/g	Limit level of if $>y_{max}$	RiskOutput()+(IF(G53<G51,G53,G51))	

Table A.1 Overview of simulation variables and parameters (continued).

Cell	Unit	Variables	Value	Source
G55		**Serving and dose-response**		
G56	g	Serving size	RiskCumul(0,220,H71:H76,J71:J76)	Hoelzer et al. (2012)
G57	CFU/g	Level non Log	RiskOutput()+10^(G54)	
G58		Level per serving	RiskOutput()+G57*G56	
G59		r-value	1.91E-10	Tromp et al. (2010)
G60		Probability of illness	RiskOutput()+(1-(EXP(-G59*G58)))	
G61		Probability of illness (exposure)	RiskOutput()+(G60*G4)	
G62		Log Probability of illness	RiskOutput()+LOG(G61)	

Table A.2. Estimated number cases of listeriosis based on high and low number of annual servings

	The estimated cases of listeriosis per year	
Scenarios	**High no. of servings**	**Low no. of servings**
#1	60	0.08
#2	1.3E-03	1.9E-06
#3	2.0E+05	280.09
#4	97	0.13
#5	8	0.01
#6	4	0.01
#7	150	0.21
#8	48	0.07

#1: Baseline

#2: Baseline + Irradiation

#3: Baseline + Cross contamination

#4: Baseline + Cross Contamination + Irradiation

#5: Baseline + Cross contamination + MAP (O_2 (100%)) + Irradiation

#6: Baseline + Cross contamination + MAP (N_2O_2 (1:1)) + Irradiation

#7: Baseline +Temperature abuse (21^{o}C, 2 hr) at the table

#8: Baseline + Temperature abuse (25^{o}C, 4 hr) the transportation step