A SYSTEMS APPROACH TO DETERMINE THE FATE OF *ESCHERICHIA COLI* O157:H7 ON FRESH AND FRESH-CUT ICEBERG LETTUCE AND SPINACH

by

HELGA J. DOERING

(Under the Direction of Mark Harrison)

ABSTRACT

The systems approach was used to determine the fate of *Escherichia coli* O157:H7 on iceberg lettuce and spinach under conditions that mimic those from field to finished bagged product. Spinach and lettuce were inoculated with *E. coli* O157:H7 and processed and held under conditions typical of those in the fresh or fresh-cut produce industry (washed in chlorine water, packaged, and held to mimic retail distribution). Results showed immediately cooling, washing in chlorine water, and continually practice of the cold chain led to lower populations of *E. coli* O157:H7 and background microflora. At 4°C storage the *E. coli* O157:H7 populations decreased by at least 1.5 logs in retail bags over time up to 18 days. Longer times before cooling down and higher temperatures increased *E. coli* O157:H7 and background microflora populations over time.

INDEX WORDS: Spinach, lettuce, *Escherichia coli* O157:H7, systems approach

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# DEDICATION

This work and thesis are dedicated to my husband, Curtis Lee Higgs for all his love, encouragement and support since we travel our life journey together.

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# Chapter 1

## INTRODUCTION

Lettuce, spinach, and other fresh or fresh-cut produce are a part of a healthy diet. Meeting dietary guidelines for produce consumption may help prevent heart disease, cancer, and diabetes (Matthews, 2006) and may reduce obesity. Over 34% of adults 20 years and older were obese in 2005-2006 (CDC, 2008a). This is one reason leading to recommendation for a change in dietary behavior and a trend to eat more fresh produce. The USDA provides guidance on their web page "My Pyramid" on what to eat and how much to eat in a healthy diet (USDA, 2008).

Occurrences of foodborne illness outbreaks are increasing since more people are eating fresh produce that may be contaminated with human pathogens, like *Escherichia coli* O157:H7. In 2006, *E. coli* O157:H7 outbreaks from spinach and lettuce resulted in 375 illnesses, 192 hospitalizations, and 3 deaths, with several people developing hemolytic uremic syndrome (HUS) (CDC, 2008b; Jay, 2007; Nagin, 2007). The economic cost alone from the 2006 spinach outbreak was estimated to be over $100 million with a decline of business by 30 percent between 2006 - 2008 (Lindsay, 2008).

Since produce is grown in open fields, many factors can contribute to the possible contamination of fresh and fresh-cut produce with human pathogens. Potential sources of contamination at the pre-harvest level are soil, irrigation water, manure, wild and domestic animals, harvest workers, and harvest equipment (Beuchat and Ryu, 1997; Brackett, 1998; Dallaire et al., 2006; Gorny, 2006; Matthews, 2006). At the post-harvest level, wash water, workers, packing materials, process equipment, and transportation vehicles are sources of contamination (Beuchat and Ryu,

1997; Brackett, 1998; Dallaire et al., 2006; Gorny, 2006; Matthews, 2006). Produce can also be contaminated at home when preparing food or in commercial foodservice operations (Beuchat and Ryu, 1997; Brackett, 1998; Dallaire et al., 2006; Gorny, 2006; Matthews, 2006).

Today U.S. consumers enjoy vegetables and fruits year round. This is possible because of new agronomic, processing, preservation, packaging, distributing, and marketing technologies which lead to global trading (Beuchat, 2002). These practices increase transportation time and involve more handling and the use of central distribution centers before produce arrives into retail stores (De Roever, 1999).

Other countries around the world have different standards in sanitation which may influence contamination of produce. In global harmonization, the Codex Alimentarius Food Hygiene Committee of Food and Agriculture Organization (FAO) and the World Health Organization (WHO) are developing standards for production of fresh and fresh-cut produce. They established a code similar to the Food and Drug Administration (FDA) guidance that should be suitable for use in more than 50 countries (U.S. FDA, 2001a).

A systems approach or systems thinking has two basic components. These components are elements and processes. Elements can be measured and linked together. Processes change elements (Anonymous, 2009; Bellinger, 2004; Heylighen, 2000). For example, in an ecosystem of a forest, the elements would be trees, shrubs, birds, insects, etc. and the processes would be growth, mortality, decomposition, etc. (Anonymous, 2009). In our study the elements include spinach, lettuce, *E. coli* O157:H7 cells, temperature, etc. and the processes would be deterioration of spinach and lettuce leaves and changes in *E. coli* O157:H7 populations.

Questions concerning the fate of *E. coli* O157:H7 on leafy greens have been raised. It is not possible to evaluate the fate of the pathogen in actual production, processing and distribution

settings. This project was designed to use the systems approach in evaluating the fate of *E. coli* O157:H7 from harvest through retail distribution. This project was performed in two parts. The first part involved processing and packing of baby spinach, while the second part involved processing iceberg lettuce. The objectives of this study were to determine the ability of *E. coli* O157:H7 to multiply in the presence of normal background microflora on iceberg lettuce and spinach under conditions that mimic those during transportation from harvest field to the cooler, while in the cooler, and during transportation either as cored lettuce product in a nitrogen atmosphere, spinach in open returnable plastic totes, or as finished packaged salads in a low oxygen, high carbon dioxide atmosphere in temperature regimes commonly used, as well as temperatures considered abusive.

In industry, spinach is harvested, cooled, and then transported to a processing facility. In the processing facility spinach is then washed and packaged into retail bags and shipped to distributers. Iceberg lettuce used for fresh-cut is harvested, and transported to a nearby processing facility where it is processed or cooled or cored and than transported under a nitrogen atmosphere to a processing facility, where it is then chopped, washed and packaged into retail bags.

Processing of spinach or lettuce in the laboratory setting started with simulating different harvest temperatures and times and different transportation times for spinach or lettuce. If lettuce was “transported” for a longer time, the cored lettuce heads were packaged into plastic lined totes and placed under a nitrogen atmosphere. Transportation times and temperatures simulated direct from field (0 h) or transportation to the middle of the U.S. (10 h), or transportation to the east coast (72 h). After packaging spinach or lettuce into retail bags, bags

were held at different temperatures but not longer than 18 d. After 18 d produce was decayed, and the end of shelf- life was reached.

Microbiological samples were taken throughout the process. The first samples were taken to determine background microflora. After inoculation of spinach leaves or iceberg lettuce, samples were taken to determine the initial inoculum. During the processing of each green type, samples were taken at certain times after harvesting (0, 10 h), transportation times (0, 10, 72 h), before and after washing, and days of storage in retail bags (0, 2, 5, 10, and 18 days).

# Chapter 2

LITERATURE REVIEW

## Outbreaks

Foodborne diseases cause an estimated 76 million illnesses and 5,000 deaths in the United States each year (Mead et al., 1999). *Campylobacter*, *Salmonella*, and *E. coli* O157:H7 are the most common causes of bacterial foodborne illnesses according to the Center for Disease Control and Prevention (CDC, 2005). The CDC's report *Surveillance for Foodborne-Disease Outbreaks---United States, 1998 -- 2002* listed 140 outbreaks with 4,854 illnesses and 4 deaths associated with *E. coli* (enterohemorrhagic and enterotoxigenic). Among these, 12 outbreaks involved vegetables (Lynch, 2006).

Over the past few decades, foodborne illness outbreaks in the U.S. have been on the rise. In recent years, these outbreaks have shifted from foods of animal origin to those of plant origin. One possibility for this shift may be due to the mandatory HACCP-plans required by the U.S. Department of Agriculture's Food Safety Inspection Services for meat and poultry processors. For U.S. Food and Drug Administrations (FDA) regulated products like juice and seafood, outbreaks are also declining but outbreaks associated with leafy greens, which are not under mandatory HACCP or HACCP-like regulations, are increasing (U.S. FDA, 2008a).

Unpublished data from the CDC for 1982 to 1996, reported 68% of *E. coli* O157:H7 outbreaks were from food or beverages. From 1993 to 1996, 27% of these outbreaks were food

items of non-bovine origin, compared to 13% of outbreaks from 1982 to 1992 (Ackers et al, 1998).

Sivapalasingam et al. (2004) prepared a review entitled *Fresh Produce: A Growing Cause of Outbreaks of Foodborne Illness in the United States, 1973 through 1997* from the CDC Foodborne Outbreak Surveillance System which began in 1966. During this 24 year period, there were 25 lettuce–associated outbreaks reported with 2,078 illnesses, 181 hospitalizations, and 6 deaths. The most common pathogen was *Salmonella* for the produce associated outbreaks involving salad, lettuce, juice, melon, and sprouts. More recently, the leading causative pathogen has shifted from *Salmonella* to *E. coli* O157:H7. With the change in distribution and globalization, increase centralized production, and improved local and national surveillance systems, more multi-state outbreaks involving different pathogens have been reported since the 1990's (Sivapalasingam et al., 2004).

In 1995, in an outbreak of *E. coli* O157:H7 involving leaf lettuce, 40 people were identified with laboratory-confirmed *E. coli* O157:H7 infection. Fifty-two patients had bloody diarrhea, 13 were hospitalized, and 1 patient developed hemolytic uremic syndrome (HUS) (Ackers et al., 1998).

In 2006, large multiple multi-state outbreaks involving iceberg lettuce and spinach contaminated with *E. coli* O157:H7 occurred (CDC, 2008b). The largest outbreak in 2006, involved spinach, affected 26 U.S. states resulting in 205 illnesses, and some patients developed HUS, and 3 people died (U.S. FDA, 2006; California Food Emergency Response Team, 2007).

With these outbreaks in the past 2 decades, FDA developed Good Agricultural Practices (GAPs) for the fresh produce industry in mid-1990s. FDA published a guide *Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables* for

growers, packers, and shippers of fresh or fresh-cut produce in 1998 (Bihn and Gravani, 2006; Hontz and Scott, 2006). In February 2008, FDA published a new improved guidance for industry, *Guide to Minimize Microbial Food Safety Hazards of Fresh-Cut Fruits and Vegetables* (U.S. FDA, 2008a).

Consumers who buy fresh or fresh-cut produce feel insecure when outbreaks and recalls are made public. In a Gallup survey about *U.S. Produce Safety-Consumer Confidence*, approximately 60% of Americans do avoid eating certain foods or brands as a result of food safety advisory or product recalls (Cooper and Morales, 2008). Economic costs add up because consumers do not like to buy any type of recalled food or food which is involved in an outbreak (Bihn and Gravani, 2006). Other costs, like medical costs, lost in productivity, and premature death caused by *E. coli* O157 STEC, was $405 million in 2003, which add to the economic costs (Frenzen et al., 2005).

**Pre-harvest**

The journey of produce from farm to table is extensive. Even before the seed is planted in the field, some contamination sources already exist. The farmer must consider where to grow the produce and its proximity to irrigation water, wild and domestic animals, drift and run-off from adjacent farms, and prior land-use history (Brackett, 1998; U.S. FDA, 2001b). These factors can contribute to high microbiology populations and exposure to human pathogens.

Soil is not an important source of human pathogens (De Roever, 1999). However, naturally occurring microorganisms from the soil can be found on fresh produce such as *Clostridium* species, (including *C. botulinum* and *C. perfringens)*, *Bacillus cereus, Listeria monocytogenes, L. innocua* (predominate in feces)*, L. ivanovi, and L. seeligeri* (most common in soil) (Beuchat and Ryu, 1997), and coliforms of non-fecal sources that include *Enterobacter* spp.

and *Klebsiella* spp. (De Roever, 1999). Foodborne bacteria in soil have to overcome many environmental stresses, like fluctuations in temperature, humidity, osmotic potential, water, nutrient availability, pH, and microbial antagonism, before attaching to the surface of leafy greens (De Roever, 1999, Solomon et al., 2006). Contamination of the soil can result from how the land was previously used. If the land was used as a feedlot or pasture for grazing animals, then the soil may contain undesired microorganisms and human pathogens (Brackett, 1998; Gorny, 2006). Floodwater after a heavy, long rain can also be a source for contamination, since water can carry wild animal feces to the soil or produce (Brackett, 1998).

Wild animals such as birds, deer, dogs, rodents, amphibians, insects, and reptiles can be carriers of human pathogens (Brackett, 1998; Gorny, 2006). Since these wild animals roam freely, it is difficult to track them and prohibit them from entering the crops in the field. *E. coli* O157:H7 can be found in feces of wild birds like starlings and gulls (Heaton and Jones, 2008). Feces from domestic birds, like geese, have been carriers of *E. coli* O157:H7 isolated from a lettuce field (Matthews, 2006). Contamination from some domestic animals, like dairy cattle (Heaton and Jones, 2008) may be easier to control. For example, the location of a pasture could be below the produce field to prevent run-off after a heavy rain from contaminating the crop with feces from the cattle. Shedding of *E. coli* O157:H7, other human pathogens, and overall microbial loads are higher in animals during spring and summer (Beuchat and Ryu, 1997; Heaton and Jones, 2008; Matthews, 2006; U.S. FDA, 2001b).

**Manure**

For thousands of years, farmers have applied fertilizers to increase crop yield. Cow manure is often used to fertilize fields, and if the manure is not properly composted, *E. coli* O157:H7 can be introduced into the soil where it can remain and contaminate the produce as it

grows (De Roever, 1999). If untreated manure is applied to the land, *E. coli* O157:H7 can survive for more than one month depending on type of soil (Jackson et al., 2007). Studies show that *E. coli* O157:H7 can survive in manure for up to 49 days when stored at 37°C, up to 56 days at 22°C (Wang et al., 1996), and up to 15 days on lettuce contaminated with manure inoculated with *E. coli* O157:H7 and then stored at 4°C (Beuchat, 2002). In another study, fecal samples were spiked with *E. coli* O157:H7 and the soil tested positive for the pathogen after approximately 50 days of composting (Gale, 2004).

The age of cattle can influence the amount of shedding *E. coli* O157:H7. Younger cattle and weaning calves have higher prevalence of *E. coli* O157:H7 in feces than older cattle. Studies show that the diet may even have an influence on the shedding of *E. coli* O157:H7 by cattle. Grain fed cattle have a 1,000-fold increase in acid-resistant *E. coli* compared to hay fed cattle. A study using different feeds inoculated with *E. coli* O157:H7 found that corn-fed or cottonseed/barley fed steers resulted in fewer positive cattle than those given only barley (U.S. FDA, 2001b). Different kinds of feed influence the pH during digestion which can contribute to the survival of *E. coli* O157:H7. Also farm management seems to play a role in whether livestock is infected with *E. coli* O157:H7, e.g., a larger herd size, open pile manure storage, and/or use of equipment to handle manure and feed (U.S. FDA, 2001b).

**Irrigation Water**

Water is used to irrigate crops as well as for mixing pesticides and fungicides used to protect the plants. *E. coli* O157:H7 can survive in water and this poses a risk if contaminated water is used (Gillian et al., 1999). Farmers use different sources of water, such as rivers, lakes, and municipal water (treated and untreated), deep well water, and reclaimed water depending on availability. Deep well water generally has a lower microbial load than surface water. Surface

water such as rivers or lakes have a higher microbial load because of run-off, which can be loaded with raw manure from pastures, feces from wild animals, sewage spill, or from a dump site (Gorny, 2006).

Irrigation water can be applied in different ways such as drip irrigation or spray irrigation. Drip irrigation has less of an effect on the leaves of leafy greens since there is minimal splashing on the edible parts of produce. Overhead irrigation systems water the whole plant and may splash portions of the surrounding soil onto the edible portion of the produce (Bihn and Gravani, 2006; Brackett, 1998). In one study lettuce plants irrigated with *E. coli* O157:H7 contaminated water, 30 days before harvest, tested positive at harvest (Heaton and Jones, 2008). Another study with wastewater showed that if wastewater was applied immediately before harvest, the produce tested positive for pathogens, but when the wastewater was applied during the growing stage, the produce tested negative for pathogens (Brackett, 1998).

Water can also be applied to help prevent damage to cold-sensitive plants from frost (Bihn and Gravani, 2006). Water to be used to mix pesticide and fungicide should be potable and free of human pathogens, since the effect of the pesticide or fungicide may not kill human pathogens (Bihn and Gravani, 2006; De Roever, 1999; Gorny, 2006; Heaton and Jones, 2008; Matthews, 2006; Suslow et al., 2003).

**Post-harvest**

The method of harvesting depends on the type of product and the machinery used by the company. Sometimes produce such as iceberg lettuce is picked and cored by hand in the field. From the field, lettuce can travel on a conveyor belt to a large bin for transportation. Once the bins are full, the fresh produce is transported by trucks to processing plants. In the processing

plant, the produce may be introduced into water fumes and transported to cutting, slicing, or shredding devices before final retail packaging. Once this occurs, the product is now considered fresh-cut produce. In other situations, as with baby spinach, a harvest machine may pick the leaves that are transported to bins that will ultimately be taken to a packing shed.

In the shed, the leafy greens are washed, spun dried, weighed and packaged prior to being held at refrigeration temperatures. The fresh-cut produce may then travel by refrigerated truck to retail stores where the consumer can purchase the fresh or fresh-cut produce. The variables that are encountered during harvesting and post-harvesting can result in many situations where the produce can become contaminated making the tracking of products nearly impossible.

**Biofilms**

Biofilms can develop on plant surfaces and on harvest and processing equipment if they are not properly cleaned and sanitized (Bihn and Gravani, 2006). Biofilms harbor many species of bacteria, fungi and yeast. Bacteria in biofilms have a higher rate of survival and growth (Heaton and Jones, 2008; Beuchat, 2002) and can lead to contamination of fresh and fresh-cut produce and add to the microbial population on the produce.

At harvest, leafy greens by nature have a diverse microbial population from the environment in which the plants were grown. Factors that affect what kind of microorganisms found on the surface of the plant are determined by surface morphology, internal tissue composition, and metabolic activities of the produce itself (Beuchat, 2002). The number of microbes on the leafy surfaces is influenced by environmental factors, like drought, field conditions, cultivation techniques, ultraviolet light, as well as intrinsic factors like the physical structure of the plant itself, pH, availability of growth substrates, and antimicrobial factors.

Some microorganisms are inadvertently introduced if untreated irrigation water or manure is used (Dallaire et al., 2006, Harris et al., 2003).

During handling, harvesting, cutting, slicing, and shredding of produce, the leaf tissue is susceptible to damage which makes the leaf more vulnerable for entry of foodborne and spoilage microorganisms into the tissue. Microorganisms can easily colonize on natural plant surfaces because of natural openings, harvest and trimming wounds, and handling injuries (Gorny, 2001). Washing, application of antimicrobials, storage atmospheres and temperatures, and contact with workers are some of the factors that contribute to the survival of microorganisms (Dallaire et al., 2006). On damaged leafy green tissue, nutrients are leached at the site of damage providing nutrients to microorganisms. Microbial growth may alter the ecological environment. Factors for survival and growth of pathogens are influenced by metabolic capabilities, intrinsic and extrinsic factors, and at any point of production, processing, distribution, and preparation (Beuchat, 2002; De Roever, 1999; Gillian and O'Breine, 1999). Even if microbial populations on the tissue surface are effectively reduced (e.g., with chlorine), the sanitizer may not penetrate deep into the produce tissue, so harmful and spoilage microorganisms harbored internally may not be deactivated (Solomon et al., 2006).

**Wash water**

Water is mainly used to transport produce in flumes from point A to point B, for washing, and cooling. In industry, water is re-circulated and is re-used so the quality of water is important as the produce comes in contact with the water during processing. Properly disinfected water can reduce microbial populations in water and on produce. During processing of leafy vegetables (e.g., lettuce and spinach), the leaves are washed mainly to remove debris and loose soil on or around the leaves and to reduce background microflora populations (Gorny, 2001).

Studies have shown that microbial populations are initially reduced by about 1 log when washed with tap water (Brackett and Splittstoesser, 2001; Rodgers et al., 2004). To prevent introduction of microorganisms to the produce, it is important that the wash water be clean and contain low numbers of microorganisms. To achieve low microbial counts, a sanitizer like chlorine, ozone, organic acids or ultraviolet light is often used (Gorny, 2001). The wash water used in processing must meet the same microbial standards for drinking water advised/regulated by the U.S. Environmental Protection Agency (EPA) or World Health Organization (WHO) (U.S. FDA, 2006).

The antimicrobial activity of chlorine compounds depends on the amount of hypochlorous acid (HOCl), a form of free available chlorine in water that is present. The antimicrobial action of free active chlorine to reduce pathogens depends also on the contact time. Hypochlorous acid is the most effective disinfectant against a wide range of bacteria (Beuchat, 1998; Gorny, 2001). Free chloride is dependent on pH, amount of organic and inorganic matter, presence of metals, and temperature of the water. Lettuce, especially chopped or shredded, and spinach release organic material from their tissues which react with the free chlorine in wash water and thus, reduce the effectiveness of chlorine to kill bacteria. The pH of water should be between 6.0 and 7.5 to ensure chlorine activity and to minimize corrosion of equipment (Gorny, 2001). If the level is above pH 7.5, very little chlorine occurs in the active HOCl form and instead, more inactive hypochlorite ($OCl^-$) is formed. If the pH is less than 6.0, chlorine gas may be formed which can in return be harmful to employees. The temperature of water also affects the ability of HOCl to sanitize. At temperatures around 4°C, HOCl has maximum solubility in water. Guidelines recommend maintaining the wash water temperature 10°C higher than that of produce to prevent a temperature-generated pressure differential which can play a role in

microbial infiltration in produce (Beuchat, 1998; Fonseca, 2006; Parish et al., 2003). All these factors influence the effectiveness of free chlorine to reduce microbial population in wash water and on produce.

**Monitoring of wash water**

Wash water should be monitored regularly for proper pH and temperature needed for chlorine to be most effective and detailed records should be kept. Chlorine can be measured in various ways ranging from free chlorine, total chlorine, available chlorine, and oxidation reduction potential (ORP). Quantity of free chlorine, often measured as parts per million (ppm) of free chlorine, can easily be determined by a standard ppm test kit such as a drop test kit or titration test. ORP, measured in millivolts (mV), can be measured with a variety of hand held probes and an electrochemical test. Measuring chlorine in terms of ppm or ORP values do not provide an actual value of free chlorine in water. Instead, ORP is a measurement of activity level, like pH values, rather than a measurement of concentration in a solution. At a constant pH, the ORP and free chlorine measurements have a straight line relationship (Topping, 2002). ORP is used in the produce industry to maintain standardized water disinfection parameters. The produce industry uses such parameters as critical control points, and the levels are monitored and adjusted with automation as much as possible. Often, the ORP is constantly being monitored and an alarm will sound when the ORP is out of the desired range to alert quality control personnel to take action. According to the International Fresh-Cut Produce Association's *Food Safety Guidelines for the Fresh-Cut Produce Industry*, for very clean water, 3 to 5 ppm free chlorine will provide more than adequate microbial control for free-floating bacteria with a very short contact time. In this type of situation, the ORP would likely have a measurement of 650-700 mV if the pH of the water is in the range of 6.5-7.0 (Gorny, 2001).

**Human hygiene**

Humans are involved at all points of growing, harvesting, processing, and handling. Human workers can carry microbial pathogens on skin, hair, or hands, and in their digestive systems or respiratory tracts, and may unknowingly contaminate produce (U.S. FDA, 2008a). If workers are not properly trained in personal hygiene, or have a gastrointestinal infection while handling produce, the produce can be contaminated with human pathogens (Beuchat and Ryu, 1997; Bihn and Gravani, 2006; De Roever, 1999).

**Control of *E. coli* O157:H7 on Produce**

What can be done to reduce foodborne microorganisms on fresh and fresh-cut produce, such as leafy greens, which are grown in fields? The environment is difficult if not impossible to control. The FDA's regulations in 21 CFR Part 110 GMPs (Good Manufacturing Practices) and *Guidance to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables* (U.S. FDA, 2008) were developed to help reduce the microbial load on produce. These FDA documents focus on preventive control programs for fresh-cut processors. Employees should be trained in personal hygiene including hand washing techniques, when to wash hands, wearing clean work clothes, hair nets, and reporting any illnesses or health issues to prevent cross-contamination. Employees should be taught how to clean and sanitize food surfaces, equipment, floors, walls, and other structures in a processing facility to reduce or eliminate microorganisms. Buildings and work flow should be designed so that the produce travels from the dirtiest areas to the cleanest areas to prevent contamination (Bihn and Gravani, 2006; U.S. FDA, 2008a). FDA also recommends that a pest control program be established since pests, rodents, insects, etc., are potential carriers of many foodborne bacteria. Minimizing the number of pests greatly reduces cross-contamination of human pathogens onto produce and ready-to-eat foods (Bihn and

Gravani, 2006; U.S. FDA, 2008a). FDA recommends that processing water comply with applicable federal, state, and local requirements. The use of antimicrobial chemicals helps to minimize microbial contamination in processing water. As mentioned in the section above, washing the produce helps reduce microbial loads (Bihn and Gravani, 2006; U.S. FDA, 2008a). FDA also recommends that the produce be pre-cooled and stored at refrigeration temperatures to minimize microbial loads. FDA recommends that packaging material used for fresh and fresh-cut produce be free of damage or not defective to prevent contamination (U.S. FDA, 2008a). Modified atmosphere packaging (MAP) affects the environment within the package by reducing the levels of available oxygen which helps to maintain quality of some fresh produce and extend shelf-life. However, not all microorganisms respond the same to MAP (U.S. FDA, 2008a). The FDA guidelines stress the importance of record keeping to trace back the product, especially if a recall is implemented (Bihn and Gravani, 2006; U.S. FDA, 2008a).

Water quality, manure quality, sanitation, and hygiene of harvest workers are points that can be monitored in the field. Harvest workers should have access to a clean toilet and hand-washing facilities close to the field. Irrigation water should be regularly tested for *E. coli* O157:H7, especially if surface water is used for irrigation. Well water or deep wells should be capped and properly constructed to prevent contamination. Well water should be tested at least once a year to monitor microbiological quality. The National Organic Standards recommend a 120-day interval between application of raw manure and time of harvest. Field sanitation should apply not only to harvesting machines, but to all harvest equipment (totes, baskets, bags, and bins) and all equipment should be well constructed and easy to clean and sanitize (Bihn and Gravani, 2006).

**Reduction of *E. coli* O157:H7 during processing**

Natural microflora and pathogens on leafy greens are not easy to kill. During the entire process from farm to retail, the washing step can reduce microbial levels. Microorganisms use natural openings, wounds or other damages to move into the leave (Beuchat, 2002; De Roever, 1999; Gillian and O'Breine, 1999).

Chlorine is mainly used in the fresh or fresh-cut produce industry to sanitize the wash water and to reduce surface level of microorganisms by approximately 1 log (Rodgers et al., 2007; Brackett and Splittstoesser, 2001). One study showed a 2-3 log reduction in microbial populations when shredded iceberg lettuce was washed in warm water (45ºC) for 3 min (Delaquis et al., 1999).

Another disinfectant for fruits and vegetables is chlorine dioxide ($ClO_2$). $ClO_2$ can be used in a wider pH range than chlorine, is less affected by organic matter, and does not react with ammonia to form chloramines (Beuchat, 1998; Singh et al., 2002). $ClO_2$ does not react with phenolic compounds to produce foul-smelling or tasting chlorophenols like chlorine does. $ClO_2$ has 2.5 times the oxidation capacity of chlorine (Singh et al., 2002). The biggest disadvantage of $ClO_2$ is that it has to be generated on-site, and if it is made at a too high concentration, it can explode.

A study compared different sanitizers (chlorine dioxide, peracetic acid, and ozone) to reduce *E. coli* O157:H7 on surfaces of green leaf lettuce (whole and shredded). Rodgers et al., (2003) showed that peracetic acid at 80 ppm for 5 min reduced microbial populations approximately 4 logs, chlorine dioxide at 3 ppm for 5 min approximately 4 logs, chlorine dioxide at 5 ppm for 5 min approximately 5 logs, and ozone at 3 ppm for 5 min approximately 6 logs. Another study combined $ClO_2$ and ultrasound at 170-kiloHertz. With a concentration of 40 ppm

$ClO_2$ and ultrasonic rate at 170-kiloHertz for 10 min, *E. coli* O157:H7 populations were reduced by approximately 2.3 logs (Huang et al., 2006).

Organic acids (e.g., acetic, citric succinic, malic, tartaric, benzoic, and sorbic acids) are the major organic acids naturally found in many fruits and vegetables. These acids act by reducing the internal pH of microbial cells by the internal ionization of the undissociated acid molecule within the cell, or by disruption of substrate transport by alteration of cell membrane permeability (Beuchat, 1998). In a study using lactic acid, citric acid, acetic acid and ascorbic acid at concentrations of 0.5 and 1.0% showed a reduction of *E. coli* O157:H7 between 1–2 logs (Akbas and Ölmez, 2007).Washing or rinsing produce with vinegar or lemon juice will reduce some types of pathogenic bacterial populations. The aerobic population was reduced by 3-6 logs depending on the concentration of the vinegar and the length of time the leafy salad greens were exposed to the vinegar. However, the flavor and aroma of the produce may be affected by exposure to acetic acid in solutions such as vinegar or lemon juice (Beuchat, 1998).

Hydrogen peroxide ($H_2O_2$) is also used to reduce microbial population. Hydrogen peroxide may improve the microbiological quality and extend the shelf-life of minimally processed fruit and vegetable products (Beuchat, 1998). However, previous studies with $H_2O_2$ on shredded lettuce induced severe browning (Beuchat, 1998; Fonseca, 2006).

Ozone has been used to kill microorganisms in drinking water for nearly a century (Beuchat, 1998). A disadvantage of ozone is that it has to be generated on-site. Another disadvantage is that it is a strong oxidizer, when metal and other types of surfaces come in contact with ozone it will corrode or deteriorate (Beuchat, 1998). Ozone's biocidal effect is a result of the combination of high oxidation potential reacting 3,000 times faster with organic matter than chlorine (Singh et al., 2002). Kim et al., (1999) used bubbling ozone in wash water

and noted the natural microflora on shredded lettuce decrease approximately 1.5 logs within a 3 min treatment.

Essential oils and extracts from some herbs and spices have antimicrobial properties. Singh et al., (2002) used thyme oil, ozone and chlorine dioxide. They inoculated romaine lettuce with a cocktail of *E. coli* O157:H7 and used different inoculation methods, incubation times, and single or multiple washings with thyme oil, ozone and chlorine dioxide. They reported a reduction of approximately 1–3 logs of *E. coli* O157:H7 depending on treatment. Best results were achieved with thyme oil (0.1% for 5 min) which yielded a 3.3 log reduction when used with a drop inoculation technique and 24 h incubation time (Singh et al., 2002).

Ionizing irradiation [e.g., gamma rays from cobalt-60 ($^{60}$Co) or cesium-137 ($^{137}$Cs)] of raw fruits and vegetables extends shelf-life by destroying large numbers of molds and yeasts, bacteria, and pathogens present on produce (Beuchat, 1998). FDA published in August 2008 a final rule allowing irradiation of iceberg lettuce and spinach with radiation up to 4.0 kilogray (kGy) to reduce pathogens (U.S. FDA, 2008b). The International Atomic Energy Agency (IAEA) did a research project on fruits and vegetables with irradiation (IAEA, 2006). Hammad et al., (2006) inoculated fresh-cut lettuce with *E. coli*. Irradiation with cobalt-60 ($^{60}$Co) with an irradiation dose of 2 kGy reduced *E. coli* approximately 5 logs by Hammad et al., (2006). Niemira (2007, 2008) internalized *E. coli* O157:H7 in baby spinach and iceberg lettuce, with the vacuum perfusion method, and then used cesium-137 ($^{137}$Cs) as a gamma radiation source. *E. coli* O157:H7 populations on baby spinach was reduced by approximately 3 logs with 1.5 kGy, while that on iceberg lettuce was reduced by approximately 5 logs with 1.5 kGy.

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# Chapter 3

## A SYSTEMS APPROACH TO DETERMINE THE FATE OF *ESCHERICHIA COLI* O157:H7 ON FRESH AND FRESH-CUT ICEBERG LETTUCE AND SPINACH[1]

[1] Helga J. Doering, Mark A. Harrison, Ruth A. Morrow, William C. Hurst, and William L. Kerr, 01/15/2009 submitted to Journal of Food Protection®

**Abstract**

The systems approach was used to determine the fate of *Escherichia coli* O157:H7 on iceberg lettuce and spinach under conditions that mimic those from field to finished bagged product. Spinach and lettuce were inoculated with *E. coli* O157:H7 and processed and held under conditions typical of those in the fresh or fresh-cut produce industry (washed in chlorine water, packaged, and held to mimic retail distribution). Results showed immediately cooling ($p < 0.0001$), washing in chlorine water ($p < 0.0001$), and continually practice of the cold chain ($p < 0.0001$) led to lower populations of *E. coli* O157:H7 and background microflora. At 4°C storage the *E. coli* O157:H7 populations decreased by at least 1.5 logs in retail bags over time up to 18 days. Longer times before cooling down and higher temperatures increased *E. coli* O157:H7 and background microflora populations over time. In retail bags, stored at higher temperature (25 or 32°C), microbial populations increased rapidly and the product quality quickly declined.

INDEX WORDS: Spinach, lettuce, *Escherichia coli* O157:H7, systems approach

## Introduction

Occurrences of foodborne illness outbreaks are increasing since more people are eating fresh produce that may be contaminated with human pathogens, like *Escherichia coli* O157:H7. In 2006, *E. coli* O157:H7 outbreaks from spinach and lettuce resulted in 375 illnesses, 192 hospitalizations, and 3 deaths, with several people developing hemolytic uremic syndrome (HUS) (CDC, 2008b; Jay, 2007; Nagin, 2007). The economic cost alone from the 2006 spinach outbreak was estimated to be over $100 million with a decline of business by 30 percent between 2006 - 2008 (Lindsay, 2008).

Since produce is grown in open fields, many factors can contribute to the possible contamination of fresh and fresh-cut produce with human pathogens. Potential sources of contamination at the pre-harvest level are soil, irrigation water, manure, wild and domestic animals, harvest workers, and harvest equipment (Beuchat and Ryu, 1997; Brackett, 1998; Dallaire et al., 2006; Matthews, 2006; Gorny, 2006). At the post-harvest level, wash water, workers, packing materials, process equipment, and transportation vehicles are sources of contamination (Beuchat and Ryu, 1997; Brackett, 1998; Dallaire et al., 2006; Matthews, 2006; Gorny, 2006). Produce can also be contaminated at home when preparing food or in commercial foodservice operations (Beuchat and Ryu, 1997; Brackett, 1998; Dallaire et al., 2006; Matthews, 2006; Gorny, 2006).

Good Agricultural Practices, Good Manufactures Practices and Hazard Analysis Critical Control Points are plans to reduce the potential for contamination of fresh and fresh-cut produce. Growers and processors have the possibility to use these plans to reduce microbial contaminations on their produce. One common intervention practice is to sanitize wash or flume water in process facilities to reduce pathogen contamination on produce. Still sanitizers can

reduce but not eliminate pathogens on produce (Behrsing et al., 2000; Delaquis et al., 2002; Lang et al., 2004; Lee et al., 2008; Li et al., 2001a; Velazquez et al., 2009).

Due to the pathogenic nature of *Escherichia coli* O157:H7, this project was done under laboratory settings. Green fluorescent protein-expressing (GFP) *E. coli* O157:H7 strains were used for inoculation on iceberg lettuce and spinach. This gave a better traceability during the whole process.

This project was designed to use the systems approach in evaluating the fate of *E. coli* O157:H7 from harvest through retail distribution and was performed in two parts. The first part involved processing and packing of baby spinach, while the second part involved processing iceberg lettuce. The objectives of this study were to determine the ability of *E. coli* O157:H7 to multiply in the presence of normal background microflora on iceberg lettuce and spinach under conditions that mimic those during transportation from harvest field to the cooler, while in the cooler, and during transportation either as cored lettuce product in a nitrogen atmosphere, spinach in open returnable plastic totes, or as finished packaged salads in a low oxygen, high carbon dioxide atmosphere in temperature regimes commonly used, as well as temperatures considered abusive.

## Materials and Methods

### Bacteria and preparation of inocula

Four strains of green fluorescent protein-expressing (GFP) *E. coli* O157:H7 (ATCC 4388, 123995, E0122, and 124546 obtained from Dr. Larry Beuchat, Center for Food Safety, University of Georgia, Griffin) were used in this study. Each strain was activated by three successive transfers from fresh stock cultures (maintained at -80$^{o}$C) into 9 ml tryptic soy broth

(Becton Dickinson, Sparks, MD) containing 50 µg / ml of ampicillin (TSB+A) (ampicillin sodium salt; Sigma Aldrich, St. Louis, MO) and incubated for 24 h at 37°C.

Before inoculating spinach, the *E. coli* O157:H7 strains were centrifuged (Allegra™ X-22R Centrifuge, Beckman Coulter, Fullerton, CA) separately at 4,000 x g for 10 min. The supernatant was removed and cells were washed with 9 ml of 0.1% peptone (Becton Dickinsion) and recentrifuged twice. Then the cells were resuspended in 2 ml of 0.1% peptone for each strain and pooled together to form the initial inoculum cocktail to obtain a suspension containing $10^9$ colony forming units (cfu) *E. coli* O157:H7/ml. Before inoculating lettuce, the unwashed *E. coli* O157:H7 strains were pooled together equally to yield a suspension containing $10^9$ cfu/ml.

**Microbial analysis**

For each sample time, 25 g of inoculated lettuce were placed into a sterile stomacher bag in 225 ml of D/E neutralizing broth (Becton Dickinson) and stomached (Stomacher®400 Circulator, Seward Laboratory Systems Inc., Bohemia, NY) for 2 min at normal speed. Twenty-five grams of uninoculated spinach were placed into a sterile stomacher bag in 225 ml of 0.1% peptone (Becton Dickinson) and stomached (Stomacher®400 Circulator) for 2 min at normal speed. And one *E. coli* O157:H7 inoculated spinach leaf was placed into a sterile stomacher bag in 100 ml of 0.1% peptone (Becton Dickinson) and stomached (Stomacher®400 Circulator) for 2 min at normal speed. Appropriate serial dilutions were prepared in 9 ml portions of 0.1% peptone. Samples were spiral plated (Autoplate® 4000, Spiral Biotech, Norwood, MA) onto plate count agar (PCA; Becton Dickinson) plates incubated at 37±2°C for 24 h to enumerate aerobic mesophilic bacteria (Viswanathan and Kaur, 2001), onto PCA plates incubated at 4±2°C for 8-10 d for aerobic psychrotrophic bacteria, onto violet red bile agar + 4-methylumbelliferyl-β-D-gluconide agar (VRB + MUG) (Becton Dickinson) plates (only done with the lettuce

samples) incubated at 32±2°C for 24 h for coliforms and generic *E. coli*, and onto dichloran rose bengal chlortetracycline agar plates (Becton Dickinson) incubated at 25±2°C for 5-7 days for yeasts and molds. After these were prepared, to enumerate lactic acid bacteria, 41 ml of 4x lactobacillus MRS broth (MRS; Becton Dickinson) were added to the stomacher bags and mixed by vigorous shaking for 1 min. Serial dilutions were made in 9 ml portions of regular strength MRS before 1 ml portions were plated onto Petrifilm™ Aerobic Count Plates (3M, Minneapolis, MN). Petrifilm was incubated in anaerobe jars with gas packs (GasPak Plus™, Becton Dickinson) at 37±2°C for 48 h. GFP-expressing *E. coli* O157:H7 was enumerated by plating onto tryptic soy agar (Becton Dickinson) with 50 µg/ml ampicillin (Sigma Aldrich) (TSA+A). After incubation, fluorescent colonies were enumerated using a UV light, method described by Islam et al. (2004) (Spectronics Corporation, Westbury, NY). For each plating medium, colony forming units were enumerated and the plate counts were converted to $\log_{10}$ values before statistically analyzed. Figures 3.1 and 3.2 show the sampling process for lettuce and spinach.

For the lettuce samples, in the event the GFP-expressing *E. coli* O157:H7 populations were below the detection limit ($< 4.0 \times 10^1$) on the TSA+A plates, an enrichment step was performed. One ml of the blended lettuce samples were transferred to 9 ml portions of universal preenrichment broth (UPB; Becton Dickinson) to enrich for *E. coli* O157:H7. After the UPB was incubated at 37°C for 24 h, 10 µl portions of UPB were streaked onto TSA+A plates, incubated by 37°C for 24 h, to check for presumptive *E. coli* O157:H7. The UPB was reincubated for an additional 24 h at 37°C, and if no growth occurred on the streaked TSA+A plate, another portion of the UPB was streaked onto a second TSA+A plate as a secondary confirmation.

**Lettuce and spinach source and inoculation with *E. coli* O157:H7**

Fresh iceberg lettuce and baby spinach were harvested from fields in Salinas Valley, California on at least 6 separate occasions for each type of green. Due to the sample numbers and portion sizes, treatments were scheduled so 1 replication of 1 'equilibration' temperature (25 or 32°C) was done at a time. The lettuce and spinach were harvested under normal conditions except the lettuce heads were not cored in the field and none of the greens were washed with water. Instead, the greens were cooled and packaged with freezer packs for overnight delivery to the Department of Food Science and Technology in Athens, GA.

Upon receipt, greens were portioned into totes (Agricultural Containers #CP 242013, Buckhorn Inc., Milford, OH) and placed into temperature and relative humidity controlled incubators (HotPack Benchtop Humidity Chambers, Model 435315, Philadelphia, PA) to equilibrate to either 25 or 32°C. Relative humidity was maintained at 70±5% RH. Using these temperatures allowed for a comparison of two harvest conditions (one that might be desirable and one that is abusive). After equilibration, outer leaves of the iceberg lettuce heads were removed and the heads cored by hand. Investigators wore gloves to minimize introduction of microorganisms. For the iceberg lettuce, exterior leaves and those from the next layer underneath were sampled to determine the background microflora. For spinach, individual leaves were sampled.

To inoculate lettuce with *E. coli* O157:H7, a sterile, 42 L tank (Nalgene Labware, Rochester, NY) was filled with 24 L of room temperature distilled water and inoculated with 8 ml of the pooled GFP-expressing *E. coli* O157:H7 strains, approximately $10^4$ cfu GFP-expressing *E. coli* O157:H7/$cm^2$. Cored lettuce was submerged into the tank for approximately 1 min and then placed in sterile stainless steel pans on sterile stainless steel wire racks inside a

biological safety cabinet (Bio Safety Cabinets, NuAire, Class II Type A2, Plymouth, MN) to allow the excess water to drain. After drying, samples were analyzed microbiologically to enumerate the various microbial groups described previously. Then one-third of the portions in each replication were immediately vacuumed cooled to 4±2°C, before sampling microbiologically and processed as described below.

Samples were chilled in a pilot scale vacuum cooler constructed at the University of Georgia. The chamber was constructed from two PVC tubes (2.14m x 0.31 m) allowing approximately 20 heads of lettuce to be cooled at one time. Air was evacuated by a Trivac B D40, two-storage rotary vacuum pump (Oerlikon-Leybold, Cologne, Germany). Water from air was condensed in a cold trap at -30°C before entering pump. The operating pressure was 75 cm of mercury, allowing the lettuce to be cooled to 4°C within 20 minutes.

The rest of the lettuce was held at either 25 or 32°C for 10 h before vacuum cooling. Samples were microbiologically analyzed before and after cooling. The 10 h time interval was chosen to represent a delay between harvesting and processing. After cooling, these portions were placed into proprietary low oxygen transmission rate plastic liners inside plastic totes. Heads placed in the liners were flushed with nitrogen gas to achieve a final oxygen concentration of 4% (Mocon® / Modern Control, Inc., Pac Check™ Model 650, Dual Head Space Analyzer, Flo Smart™, Patent 5,212,993, Minneapolis, MN), the liners closed and the totes held at the appropriate temperature (4 or 12°C) for 10 or 72 h before being sampled microbiologically and processed as described below.

To inoculate spinach with *E. coli* O157:H7, individual leaves were spot inoculated (10µl per leaf) resulting in approximately $10^5$ cfu GFP-expressing *E. coli* O157:H7/$cm^2$. The petioles of the inoculated leaves were marked with red paint to allow for identification of the inoculated

leaves from uninoculated ones. The leaves were placed into the biological safety cabinet and the excess liquid was allowed to dry over the course of 30 min. After drying, inoculated spinach leaves in each replication were equally divided and placed in the middle of the uninoculated spinach leaves in the totes. One-third of the totes per replication representing 0 h before processing were immediately cooled with circulating air to approximately 4±2ºC within 6 h and processed immediately as described below. Other totes were held at either 25 or 32ºC for 10 h prior to being cooled with circulating air to 4±2ºC before processing as described below. These conditions were to represent 2 different harvesting temperature conditions with a delayed time between harvesting and processing.

**Iceberg lettuce processing and storage**

Lettuce was chop-cut (3-5 cm pieces) with a food slicer (Chef's Choice®, ©EdgeCraft Corp., Avondale, PA), microbiologically sampled, and 2,100 g portions were washed in 20 L municipal water with added ice to create chilled water of approximately 4$^{o}$C with or without chlorine (approx 54 ppm free chlorine, pH 7.0-10.0, 710-900 mV ORP). For each treatment a separate tank was used. Lettuce was agitated for 1 min to simulate fresh-cut operations. Prior to washing, the temperature, oxidation-reduction potential (ORP) and the pH of the water were measured (waterproof pH/ORP & Temperature Meter, Hanna Instruments, HI98121, Woonsocket, RI). If needed, pH was adjusted with citric acid (J.T. Baker, Phillipsburg, NJ). Samples were dewatered by draining and centrifuged for 45 sec in a 20 L restaurant-style salad spinner (Matfer Bourgeat, Van Nuys, CA). After dewatering, samples were taken for microbiological analysis and three-700 g portions were bagged into 2,260 g bags (Cryovac PD-900 Bag, Duncan, SC) The $O_2$ permeability of these bags was 3,000 cc/m$^2$/24 h at 23ºC at 1 atm, while the $CO_2$ permeability was 9,800 cc/m$^2$/24 h at 23ºC at 1 atm. The bags were sealed with a

clip, and the bags were placed in storage at temperature 4, 25 or 32ºC. Samples were analyzed on 0, 2, 5, 10 and 18 days to determine the microbial populations by the enumeration methods previously described. Storage times and temperatures included abusive conditions (25 or 32ºC) that could occur during distribution of the retail product. In some instances the product stored at the higher temperatures became noticeably spoiled (microbiologically and visually) before the end of the 18 d storage period, so sampling times at those temperatures were not pursued.

**Spinach processing and storage**

The spinach wash treatments were similar to that described above for lettuce except the inoculated and uninoculated spinach leaves were washed separately to facilitate the selection of the appropriate leaves for sampling. For the uninoculated spinach, approximately 800 g of leaves were added to each wash tank. After washing, the leaves were dewatered by centrifugation and divided into 250 g portions and placed into retail macroperforated bags (Cryovac). The same steps were done with the inoculated leaves with the exception that they were placed into separate retail bags. The bags were closed with a clip, placed in storage at 4, 25, and 32ºC, and microbiologically sampled on days 0, 2, 5, and 10.

**Statistical Analysis**

Throughout the analyses, we use linear models to analyze $\log_{10}$ (Z) where Z is the count of mesophilic, psychrotrophs, yeasts and molds, coliforms, *E. coli* O157:H7, or lactic acid bacteria on lettuce or spinach. The experiment has a factorial design, and explanatory variables (factors) includes field temperatures (25 and 32°C), holding time before product cooling (0 and 10 h; precool), holding time after cooling (0, 10 and 72 h; postcool), transportation temperatures (4 and 12°C; for lettuce only), with or without chlorine wash (wash), product storage temperatures (4, 25 and 32°C), and days of storage. Since each explanatory variable has only 2

or 3 levels, these variables are treated as categorical variables (also unknown as class variables in SAS), and factorial ANOVA, a special case of linear models, is used to analyze the data. Factorial ANOVA allows one to predict the value of a response variable with a combination of independent categorical variables and to test hypotheses about the means. In the factorial ANOVA model, the error terms of each observation are assumed to independently follow the same normal distribution with mean 0 and an unknown variance $\sigma^2$. Counts of the six groups of bacteria have highly skewed distributions, meaning that the normality assumption is violated and that the variances of the error terms are not the same. Logarithmic transformed responses are closer to being normally distributed. Thus, modeling $\log_{10}(Z)$ with factorial ANOVA is more appropriate.

To make tables, we first examined the interactions between days of storage and other factors. If all these interaction terms are not significant, then least square means are computed for days 0, 2, 5, 10, and 18. However, if one interaction term, say the interaction between days of storage and postcool, is significant (which means that the three curves of $\log_{10}(Z)$ over time corresponding to the three different post-cool levels are not parallel), then least square means are computed for days 0, 2, 5, 10, and 18 given each level of postcool. Since it is of interest to examine whether $\log_{10}(Z)$ changes over time, multiple pair-wise comparisons are made within each column of the tables, and the Tukey's method is used to adjust the p-values of these multiple comparisons. By 'multiple comparison', we mean that a set of comparisons are considered as a whole. For example, if we consider the multiple comparison of day 0 vs. day 2, day 0 vs. day 5, and day 2 vs. day 5, then we should view the 3 pairs as a whole and test them simultaneously rather than treating them as 3 separate tests. There are a number of statistical

methods that can be used to adjust the p-values obtained from the 3 separate tests, and the Tukey's approach is one commonly used method.

## Results

### Microbial changes on iceberg lettuce

When considering the effects of field temperature, cooling, transportation temperature and wash treatment on *E. coli* O157:H7 counts prior to storing lettuce in retail bags, field temperature ($p = 0.0013$), length of time lettuce was held at field temperature before it was vacuum cooled (0 or 10 h) ($p < 0.0001$), length of time between cooling and washing step (0, 10, or 72 h) ($p = 0.0028$) and washing ($p < 0.0001$) are important factors that affect *E. coli* O157:H7 populations, while transportation temperature (4 or 12$^{o}$C) was not significant ($p = 0.2$). Specifically, a lower field temperature (25°C) and a shorter length of time (0 hour) lettuce was held before it was vacuum cooled reduced *E. coli* O157:H7 populations, as compared to a higher field temperature (32°C) and a longer length of time (10 h). Washing method was an important step. Washing with chlorine was significantly better than washing without chlorine, and washing without chlorine was significantly better than no washing in terms of reducing *E. coli* O157:H7 populations. To compare different storage temperatures, storage days were fixed at day 2 and 5. For lettuce stored in retail bags, on day 2 and 5 of the storage period, *E. coli* O157:H7 populations were mainly affected by length of time lettuce was held at field temperature before it was vacuum cooled (0 or 10 h) ($p < 0.0001$), whether lettuce was washed or not ($p = 0.0392$), and storage temperature of the bagged product ($p < 0.0001$). The quicker the lettuce was cooled and washing with chlorine were associated with the lowest *E. coli* O157:H7 populations. The length of time between cooling and washing step (0, 10, or 72 h) was not as significant ($p = 0.0856$). As expected, storage of lettuce at 4$^{o}$C was much more effective in inhibiting growth of

*E. coli* O157:H7 compared to storage at 25 or 32°C. Lettuce stored at 25 or 32°C was of such poor quality by the second day, samples were not analyzed after the fifth day. In contrast, the quality of lettuce stored at 4°C allowed for sampling through 18 days. Field temperature and transportation temperature (4 and 12°C) were not important, once the storage period started.

To determine the ability of *E. coli* O157:H7 to multiply during storage, the data was analyzed by fixing storage temperature at 4 and 25°C, respectively. When stored at 4°C, the length of time lettuce was held at field temperature before it was vacuum cooled (0 or 10 h) ($p < 0.0001$), length of time between cooling and washing step ($p = 0.0015$), washing ($p < 0.0001$), and storage day in retail bags ($p < 0.0001$) have significant effects on *E. coli* O157:H7 populations. Over 18 days at 4°C, the longer lettuce, washed with chlorine, was stored, the less *E. coli* O157:H7 was found on samples (day 0 > day 2 > day 5 > day 10 > day18) (Table 3.1). The general trend for lettuce not washed with chlorine was the same. When stored at 25°C, length of time lettuce was held at field temperature before it was vacuum cooled (0 or 10 h) ($p < 0.0001$), washing ($p < 0.0342$) and storage day ($p < 0.0001$) were important, whereas length of time between cooling and the washing step becomes insignificant ($p = 0.2309$) (Table 3.2). At 25°C, *E. coli* O157:H7 increased in numbers over storage time (day 0 < day 2 < day 5) (Table 3.3). By day 2 at 32°C the lettuce was grossly spoiled and was not sampled past this point.

Prior to the storage in retail bags, important factors that affected psychrotrophic bacteria populations were length of time between cooling and washing step (0, 10, 72 h) ($p < 0.0001$), transportation temperature (4 and 12°C) ($p = 0.0028$) and washing ($p < 0.0001$). Shorter time between cooling and washing resulted in a reduction in the number of psychrotrophic bacteria (0 h < 10 h < 72 h). The lower transportation temperature (4°C), the lower the number of psychrotrophs compared to the higher transportation temperature (12°C). Psychrotrophic

populations were lower on lettuce washed with chlorine compared to washing without chlorine or unwashed. Field temperature and length of time lettuce was held at field temperature before it was vacuum cooled (0 or 10 h) did not significantly affect the psychrotrophic populations.

Again the days of storage were fixed at 2 and 5 to compare different storage temperatures. For lettuce in retail bags and analyzed on day 2 of the storage period, psychrotrophic bacteria counts were affected by length of time lettuce was held at field temperature before it was vacuum cooled (0 or 10 h) ($p = 0.0006$), length of time between cooling and washing step (0, 10, 72 h) ($p < 0.0001$), transportation temperature (4 and 12°C) ($p = 0.037$), wash treatment ($p = 0.0016$), and storage temperature ($p < 0.0001$). Field temperature was not important ($p = 0.4806$). The effects of length of time between cooling and washing, transportation temperature, and wash are similar to those in the above paragraph. A shorter holding time at field temperatures and a lower storage temperature resulted in less psychrotrophic growth. For samples analyzed on day 5, similar results were noted.

Psychrotrophic populations increased during storage at both 4 and 25°C. Lettuce stored at 25 or 32°C had psychrotrophic populations in excess of log 7 by day 2 of storage and were considered to be spoiled based on the size of the psychrotrophic populations and the visual appearance of the lettuce (Tables 3.4 to 3.6). The quicker the lettuce was vacuum cooled, the quicker the lettuce was washed after being cooled, and the lower transportation temperature resulted in lower populations of aerobic mesophilic bacteria, lactic acid bacteria, coliform (non-*E. coli*), and yeasts and molds. Shorter precool time, shorter postcool time, and lower transportation temperature led to lower populations of aerobic mesophilic bacteria, lactic acid bacteria, coliform (non-*E. coli*), and yeasts and molds. Lower field temperature resulted in lower populations of all the groups except psychrotrophic and yeasts and molds. Washing significantly

reduced levels of each group, with washing with chlorine being more effective than washing without chlorine. Storage temperature had a significant effect ($p < 0.0001$) on each microbial group with greater amounts of growth at the higher temperatures. The overall trend at higher temperature and longer storage time microbial populations increased ($4^oC < 25^oC < 32^oC$) (Tables 3.7 to 3.12). There were significant increases in the population size for all the groups except the lactic acid bacteria for the first 10 days when the product was stored at $4^oC$ compared to 25 or $32^oC$. There was no significant increase in the numbers of lactic acid bacteria on samples stored for the first 10 days.

**Microbial changes on baby spinach**

Prior to storage in retail bags, field temperature ($p = 0.0003$), length of time the spinach was held at field temperature before it was cooled by circulating air ($p = 0.0019$) and washing ($p < 0.0001$) significantly affected *E. coli* O157:H7 levels, while length of time between cooling and washing ($p = 0.7878$) was not important. Spinach held for a minimum time before cooling had lower levels of *E. coli* O157:H7. Washing with chlorine significantly reduced *E. coli* O157:H7 to a greater degree than washing without chlorine.

To compare 4 and $25^oC$ storage temperatures, days of storage was fixed at 2. *E. coli* O157:H7 populations were mainly affected by the length of time the spinach was held at field temperature before it was cooled by circulating air ($p = 0.0231$), washing ($p < 0.0001$) and storage temperature of retail bagged product (4 or 25°C) ($p < 0.0001$). The effect of field temperature (25 or 32°C) was not significant ($p = 0.9785$). The *E. coli* O157:H7 populations were significantly lower at $4^oC$ storage temperature, compared to storage at $25^oC$.

Spinach samples were not analyzed after 10 days of storage due to the visible degradation of the produce. As with the lettuce, the longer the bags were stored at 4°C, the less *E. coli*

O157:H7 populations were found on the samples (day 0 > day 2 > day 5) ($p < 0.0001$). *E. coli* O157:H7 populations were about the same for days 5 and 10. When stored at 25°C, *E. coli* O157:H7 populations increased in numbers over storage time (day 0 < day 2) ($p = 0.0475$) (Table 3.13).

Prior to the storage in retail bags, field temperature ($p < 0.0001$), length of time spinach was held at field temperature before it was cooled by circulating air ($p < 0.0001$), length of time between cooling and washing step (0, 10, 72 h) ($p = 0.005$) and washing ($p < 0.0001$) significantly affect psychrotrophic bacteria. Lower field temperature (25°C), shorter length of time (0 hour) spinach was held at field temperature before it was cooled by circulating air, and shorter length of time between cooling and washing step (0 h and 10 h, as opposed to 72 h; the difference between 0 h and 10 h was not significant), and washing resulted in reduction of psychrotrophic bacteria populations. Washing with chlorine was more effective in reducing psychrotrophic bacteria than washing without chlorine.

When comparing spinach stored in retail bags on day 2, field temperature ($p < 0.0001$), length of time between cooling and washing step (0, 10, 72 h) ($p = 0.0019$) and storage temperature of retail bags (4 or 25°C) ($p < 0.0001$) significantly affected the number of psychrotrophic bacteria. In particular, at 4°C storage temperature, psychrotrophic bacteria populations were significantly lower than those on spinach stored at 25°C on day 2. Over the course of the period spinach was stored in retail bags, psychrotrophic bacteria populations increased over time, at both 4 and 25°C storage temperature (Tables 3.14 to 3.16).

The quicker the spinach was cooled by circulating air, the quicker the spinach was washed after being cooled, and transportation temperature of 4°C resulted in lower populations of aerobic mesophilic bacteria, lactic acid bacteria, and yeasts and molds. Shorter pre-cool time,

shorter post-cool time, and transportation temperature of 4°C led to lower populations of aerobic mesophilic bacteria, lactic acid bacteria, and yeasts and molds. Prior to storage the aerobic mesophilic bacteria, lactic acid bacteria, and yeasts and mold populations were significantly affected by field temperature ($p < 0.0001$), length of time spinach was held at field temperature before it was cooled by circulating air ($p < 0.0001$), length of time between cooling and washing step ($p < 0.0001$) and wash treatment ($p < 0.0417$). Lower field temperature (25$^{o}$C), the quicker the spinach was cooled (0 hour), shorter length of time between cooling and washing step (0 hour and 10 hours, < 72 hours), and washing with chlorine resulted in lower levels of each group. Comparing populations of these groups at day 2 of storage showed that in addition to field temperature, the length of time before and after cooling, wash treatment, and storage temperature ($p < 0.0001$) significantly affected populations with lower populations on spinach stored at 4$^{o}$C. Populations of mesophilic bacteria, lactic acid bacteria and yeasts and molds increased over time, at both 4 and 25$^{o}$C storage temperatures (Tables 3.17 to 3.20).

**Discussion**

In this study, a systems approach was used to analyze what happens to *E. coli* O157:H7 populations on lettuce and spinach from harvest to storage in retail bags. A systems approach analyzes a series of steps and looks at changes after each step. In the past, various studies involving fresh and fresh-cut produce, and *E. coli* O157:H7 or background microflora were analyzed at a particular step in the overall process (Behrsing et al., 2000; Bracket and Splittstoesser, 2001; Jay et al., 2000; Johnston et al., 2006; Li et al., 2008).

In our study, we took samples of iceberg lettuce or spinach at two represented field temperatures and determine the background microflora of lactic acid bacteria (<$10^3$ cfu/g), coliforms (<$10^5$ cfu/g), yeasts and molds (<$10^5$ cfu/g), psychrotrophs (<$10^6$cfu/g), or mesophiles

($<10^6$cfu/g). These values from our study were similar to previous studies (Bracket and Splittstoesser, 2001, Jay et al., 2000). In the field study done by Johnston et al., (2006), researchers collected samples of leafy greens in different packaging sheds. Samples were taken throughout processing. Their findings of total aerobic bacteria on field samples (before processing) ranged from $10^5$ to$10^7$ cfu/g and in retail samples ranged from $10^4$ to$10^6$ cfu/g. Background microflora was taken before washing, after the rinse step, and just before distribution. There was no statistically significant change during processing in the Johnston et al., (2006) study.

One study found that vacuum cooling romaine lettuce allowed *E. coli* O157:H7 to infiltrate into the leaves (Li et al., 2008). In their study, romaine lettuce was incubated at 28ºC for 30 min, and then inoculated with *E. coli* O157:H7 using a cotton swab. Then the lettuce was vacuum cooled to approximately 10 to 12ºC. After vacuum cooling, the lettuce leaf surface was surface treated with 1.2% sodium hypochlorite and sterile distilled water to achieve an 8 log reduction on the lettuce leaf surface. *E. coli* O157:H7 cells infiltrated the lettuce leaf tissue. A laser scanning microscopy showed that *E. coli* O157:H7 cells had a higher penetration into the tissue when vacuum cooled compared to when no vacuum was used to cool the leaves. After one week in storage at 4ºC, the vacuum cooled lettuce had 2 logs cfu/g more *E. coli* O157:H7 cells than non-vacuum cooled lettuce. During vacuum cooling, pressure disrupted the cell wall of the lettuce and the water in the vacuoles evaporated through the stomata. This modified the stomata significantly from the non-vacuum cooled lettuce (Li et al., 2008).

Lettuce was vacuum cooled in our study. Our results showed that *E. coli* O157:H7 populations overall decreased after vacuum cooling on lettuce unlike the study conducted by Li et al. (2008). In some cases *E. coli* O157:H7 populations increased < 1 log after vacuum cooling

on lettuce like in the study conducted by Li et al. (2008). This is probably due that *E. coli* O157:H7 cells were not evenly distributed in the lettuce leaf.

In our study we simulated transportation after cooling of lettuce (4 or 12ºC) and spinach only at 4ºC. Lettuce was packed into totes lined with a low oxygen transmission rate plastic liner and nitrogen flushed. We found that *E. coli* O157:H7 populations remained constant after 10 h of simulated transportation conditions but then started to increase after 72 h. The nitrogen flush seems to retard the growth of *E. coli* O157:H7 populations while the background microflora, except of lactic acid bacteria, was not affected by nitrogen flushing.

Cooled lettuce and spinach washed in municipal water either with or without added chlorine resulted in a decrease in *E. coli* O157:H7 and the background microflora populations by 1-2 $\log_{10}$ cfu/g. This reduction depended on whether chlorine was added and the temperature or pH of the water. Washing of leafy greens most likely reduces surface bacteria. Since *E. coli* O157:H7 can penetrate into damaged lettuce or spinach tissue (Li et al., 2008), wash water cannot reach *E. coli* O157:H7 or background microflora to kill them. The washing time for spinach and lettuce in this study was one minute in agitated water. Many studies have examined the effects of chlorinated wash water and most of these studies showed a reduction of approximately 1-2 $\log_{10}$ cfu/g. For example, in one study Cos lettuce was cooled to 4ºC and inoculated with generic *E. coli*. Immediately after inoculation the lettuce was dipped into 50 or 100 mg/L chlorine solution or deionized water for 0.5, 2, or 5 min. The population reductions in this experiment were between 1.5 to 2.8 $\log_{10}$ cfu/g depending on chlorine solution and time (Behrsing et al., 2000). In our study, the reductions were approximately $<1 - 3$ $\log_{10}$ cfu/g depending on many different parameters described in the materials and methods.

In a different study, iceberg lettuce was treated with warm chlorinated water (50 or 20ºC; 20 mg/L chlorine) for 90 s, then immediately submersed into 10ºC cold water for 30 s, and stored for up to18 days at 5 or 15ºC. The reduction of background microflora was approximately 1-2 $\log_{10}$ cfu/g depending on treatment and time. The background microflora increased over storage time to > 7 $\log_{10}$ cfu/g depending on treatment (Li et al., 2001b).

In our study, after the leafy greens were washed, they were packaged into Cryovac bags and stored at different temperatures (4, 25, or 32ºC). At every temperature, the background microflora population increased over the storage period. At 4ºC storage, *E. coli* O157:H7 populations decreased by approximately 1.5 $\log_{10}$ cfu/g between day 0 and day 18 depending on treatment and other factors during processing. Background microflora outcompeted or slowed down the *E. coli* O157:H7 population growth. With the abusive temperatures of 25 or 32ºC, *E. coli* O157:H7 populations increased over the storage period. There are at least two studies that came to the same conclusion about *E. coli* O157:H7 populations. One study did a model prediction with iceberg lettuce with fluctuating temperatures from farm to table. Iceberg lettuce was inoculated with a cocktail of *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes*, to initial populations of approximately 3 $\log_{10}$ cfu/g and held in a programmable incubator to simulate the fluctuated temperature during farm to table (Koseki and Isobe, 2005). To obtain real temperature from farm to table, temperature was measured with an inserted temperature data logger in the center of a lettuce head. The temperature data logger was inserted during harvesting and removed in the grocery store. The lettuce traveled a distance of 400 km over a 72 h period. The temperature fluctuated from harvest to retail store by approximately 15ºC. Starting with harvest by approximately 15ºC, when the lettuce was cooled the temperature dropped to approximately 4ºC. During storage the temperature was between 4 - 8ºC. During

transportation the temperature was between 4 - 15ºC. At display in the retail store the temperature was between 4-15ºC with equilibration by approximately 8ºC in the end. The researchers found that, if *E. coli* O157:H7 was inoculated onto lettuce which was equilibrated to 15ºC field temperature, the maximum population density (MPD) of *E. coli* O157:H7 decreased during storage where the temperature fluctuated due to competitive background microflora. The growth rate of *E. coli* O157:H7 was 0.03 $\log_{10}$ cfu/g over 72 h. The same researchers found that if lettuce equilibrated to 25ºC was inoculated with *E. coli* O157:H7, the MPD of *E. coli* O157:H7 increased during storage with fluctuating temperature. The growth rate of *E. coli* O157:H7 was 1.23 $\log_{10}$ cfu/g over 72 h (Koseki and Isobe, 2005).

Another study examined shredded lettuce inoculated with *E. coli* O157:H7. The initial inoculum in the bag with shredded lettuce was 4 $\log_{10}$ cfu/g and the bags were stored at 4 or 8ºC for up to 12 days. Bags held at 4ºC did not show significant increases of *E. coli* O157:H7 populations, whereas bags held at 8ºC showed an increase of *E. coli* O157:H7 population by approximately 2 log over a 12 days storage period (Francis and O'Beirne, 2001). In our study, we came to the same conclusion. At 4ºC storage, *E. coli* O157:H7 populations decreased significantly (p-value < 0.0001) over storage up to 18 days. At 25ºC storage, *E. coli* O157:H7 populations increased significantly (p-value < 0.0001) over storage up 5 days. The growth rate of *E. coli* O157:H7 populations was approximately 2 $\log_{10}$ cfu/g.

In a study done by Abdul-Raouf et al., (1993) iceberg lettuce and other vegetables were inoculated with *E. coli* O157:H7. They bagged the lettuce into modified atmosphere bags (3% oxygen and 97% nitrogen) or in ambient atmosphere conditions, and stored them for up to 14 days at 5, 12, or 21ºC. *E. coli* O157:H7 populations decreased over the storage period of 14 days when the bags were stored at 5ºC. *E. coli* O157:H7 populations increased on shredded lettuce

when stored at 12 or 21ºC. *E. coli* O157:H7 populations were detected at each combination parameter. The composition of the gaseous atmosphere did not influence *E. coli* O157:H7. Background microflora (psychrotrophic, mesophilic bacteria) increased over time regardless of which gaseous atmospheres were used.

The findings from our study show similar results. Lettuce and spinach were bagged into Cryovac bags which develop a passive modified atmosphere over the storage period. At 4ºC storage, *E. coli* O157:H7 populations decreased. At 25 or 32ºC storage, *E. coli* O157:H7 populations increased. *E. coli* O157:H7 populations were always present. Background microflora increased over storage time at 4, 25 or 32ºC up to 18 days.

The importance of this research was to show that many different factors influence the growth of the background microflora and fate of *E. coli* O157:H7 populations. The systems approach showed that microorganisms and pathogens were not completely removed through the entire process. Although some steps did retard or reduce the microbial growth.

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# Chapter 4

## CONCLUSION

The studies of spinach and iceberg lettuce showed that washing with chlorine reduced *E. coli* O157:H7 and background microflora populations. The whole process parameter showed, if fresh produce was harvested at 25°C, immediately cooled, chopped, washed with chlorine, and transported quickly at low transportation temperature, and consumed as soon as possible the product will have a low level of background microflora. If *E. coli* O157:H7 populations were present, *E. coli* O157:H7 populations decreased in many cases by at least 1.5 logs over storage period with low temperature.

Fresh, bagged salads and salad mixes have no kill step in their processing. The main microbial inactivation step is the wash treatment with chlorine. It is important to work during the whole process with GAP's, GMP's and HACCP-like regulations, and with a low temperature chain (cooling chain) without interruption to ensure that background microflora, and pathogens, if present, are low in population.

Table 3.1. Least square means for *E. coli* O157:H7 populations ($log_{10}$) on lettuce stored at 4°C. The interaction between storage days and after vacuum cooling was significant, if lettuce was washed without chlorine, meaning that the three curves of $log_{10}$ (*E. coli* O157:H7 populations on lettuce) over time corresponding to the three different after cooling levels are not parallel.[1]

| Days | Chlorine | No Chlorine | | |
|---|---|---|---|---|
| | No Interaction[2] | 0 h[2] | 10 h | 72 h |
| 0 | 3.43 | 3.77[a] | 3.49[c] | 3.86[e] |
| 2 | 3.21 | 3.43[ab] | 3.80 | 3.68[e] |
| 5 | 2.83 | 3.50[ab] | 3.45[cd] | 3.13[f] |
| 10 | 2.53 | 3.30[b] | 3.19[d] | 2.94[f] |
| 18 | 2.09 | 3.17[b] | 2.60 | 2.82[f] |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript letters were not statistically different at significance level of 0.1.

[2] Time after-vacuum cooling.

Table 3.2. Least square means for *E. coli* O157:H7 populations ($log_{10}$) on lettuce stored at 25°C. Given the washing method (with or without chlorine), interaction between storage days and length of time before vacuum cooling was significant, meaning that the two curves of $log_{10}$ (*E. coli* O157:H7 populations on lettuce) over time corresponding to the two different before vacuum cooling levels were not parallel[1].

| Days | Chlorine | | No Chlorine | |
|---|---|---|---|---|
| | 0 h[2] | 10 h | 0 h[2] | 10 h |
| 0 | 2.39 | 4.46 | 2.61 | 4.80 |
| 2 | 4.43 | 5.26[a] | 4.70[b] | 5.52[c] |
| 5 | 5.06 | 5.34[a] | 4.95[b] | 5.63[c] |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Time before vacuum cooling.

Table 3.3 Least square means for *E. coli* O157:H7 populations ($log_{10}$) on lettuce stored at 32°C. Interaction between storage days and length of time before vacuum cooling was significant[1].

| Days | Chlorine | | No Chlorine | |
|---|---|---|---|---|
| | 0 h[2] | 10 h | 0 h[2] | 10 h |
| 0 | 2.39 | 4.46 | 262 | 4.80 |
| 2 | 5.32 | 6.18 | 5.30 | 6.12 |

[1] Comparisons were made within each column. Least square means were statistically different at significance level 0.1 (and at level 0.5).

[2] Time before vacuum cooling.

Table 3.4 Least square means for psychrotroph populations ($log_{10}$) on lettuce stored at 4°C. Storage temperature was adjusted for factors including field temperature, holding time before and after vacuum cooling, and transportation temperature. The washing method (with or without chlorine) showed no significant interaction between storage days and other factors[1].

| Days | Chlorine | No Chlorine |
|---|---|---|
| 0 | 5.13 | 5.62 |
| 2 | 5.90 | 6.34 |
| 5 | 6.86 | 7.27 |
| 10 | 7.59 | 7.74 |
| 18 | 7.96 | 8.10 |

[1] Multiple pair-wise comparisons were made within each column. Each pair was statistically different at significance level 0.1.

Table 3.5 Least square means for psychrotroph populations ($log_{10}$) on lettuce stored at 25°C. Given the washing method (with or without chlorine) interaction between after vacuum cooling was significant. The three curves of $log_{10}$ (*E. coli* O157:H7 populations on lettuce) over time corresponding to the three different time before vacuum cooling levels were not parallel[1].

| Days | Chlorine | | | No Chlorine | | |
|---|---|---|---|---|---|---|
| | 0 h[2] | 10 h | 72 h | 0 h[2] | 10 h | 72 h |
| 0 | 4.71 | 5.14 | 5.55 | 5.23 | 5.49 | 6.16 |
| 2 | 7.32 | 7.71 | 7.63 | 7.47 | 7.69 | 7.77[a] |
| 5 | 8.15 | 8.10 | 8.07 | 8.15 | 8.09 | 8.08[a] |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Time before vacuum cooling.

Table 3.6 Least square means for psychrotroph populations ($log_{10}$) on lettuce stored at 32°C. If lettuce was washed with chlorine, the interaction between storage days and before vacuum cooling was significant. If lettuce was washed without chlorine, the interaction between storage days and after vacuum cooling was significant.

| Days | Chlorine | | No Chlorine | | |
|---|---|---|---|---|---|
| | 0 h[1] | 10 h | No Interaction[1] | | |
| | No Interaction[2] | | 0 h[2] | 10 h | 72 h |
| 0 | 5.06 | 5.21 | 5.23 | 5.49 | 6.16 |
| 2 | 7.78 | 7.29 | 7.44 | 7.48 | 7.69 |

[1] Time before vacuum cooling.

[2] After- vacuum cooling.

Table 3.7 Least square means for mesophilic populations ($log_{10}$) on lettuce stored at 4°C. If lettuce was washed with chlorine, the interaction between storage days and time before vacuum cooling was significant. If lettuce was washed without chlorine, the interaction between before vacuum cooling and storage days and the interaction between after vacuum cooling and storage days were both significant[1].

| Days | Chlorine | | No Chlorine | | | | | |
|---|---|---|---|---|---|---|---|---|
| | No Interaction[2] | | 0 h[2] | | 10 h | | 72 h | |
| | 0 h[3] | 10 h | 0 h[3] | 10 h | 0 h | 10 h | 0 h | 10 h |
| 0 | 5.07 | 5.80 | 5.05$^{c}$ | 5.92 | 5.41$^{g}$ | 6.00 | 6.15$^{k}$ | 6.34$^{m}$ |
| 2 | 5.73 | 6.32 | 5.37$^{c}$ | 6.68$^{f}$ | 5.89$^{g}$ | 6.53$^{i}$ | 6.59$^{k}$ | 6.36$^{m}$ |
| 5 | 6.50 | 6.71$^{b}$ | 6.83$^{d}$ | 6.85$^{f}$ | 6.99$^{h}$ | 6.71$^{i}$ | 7.51$^{l}$ | 6.98$^{n}$ |
| 10 | 6.96$^{a}$ | 6.77$^{b}$ | 7.02$^{de}$ | 6.51$^{f}$ | 7.32$^{h}$ | 7.25$^{j}$ | 7.65$^{l}$ | 6.90$^{n}$ |
| 18 | 7.09$^{a}$ | 6.88$^{b}$ | 7.37$^{e}$ | 7.29 | 7.49$^{h}$ | 6.95$^{ij}$ | 5.89$^{k}$ | 6.94$^{n}$ |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] After- vacuum cooling.

[3] Time before vacuum cooling.

Table 3.8 Least square means for mesophilic populations ($log_{10}$) on lettuce stored at 25°C. Given the washing method (with or without chlorine), the interaction between storage days and time before vacuum cooling was significant[1].

| Days | Chlorine | | No Chlorine | |
|---|---|---|---|---|
| | 0 h[2] | 10 h | 0 h[2] | 10 h |
| 0 | 5.06 | 5.79 | 5.53 | 6.09 |
| 2 | 7.41 | 7.56 | 7.61 | 7.72 |
| 5 | 8.25 | 8.25 | 8.05 | 8.16 |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Time before vacuum cooling.

Table 3.9 Least square means for mesophilic populations ($log_{10}$) on lettuce stored at 32°C. If lettuce was washed with chlorine, the interaction between storage days and time before vacuum cooling was significant. If lettuce was washed without chlorine, storage days did not interact with other factors[1].

| Days | Chlorine | | No Chlorine |
|---|---|---|---|
| | 0 h[2] | 10 h | No Interaction[2] |
| 0 | 5.06 | 5.79 | 5.82 |
| 2 | 7.86 | 8.18 | 8.08 |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Time before vacuum cooling.

Table 3.10 Least square means for mold and yeast populations ($log_{10}$) on lettuce stored at 4°C. Interaction between storage days and after vacuum cooling was significant, if lettuce was washed without chlorine[1].

| Days | Chlorine | No Chlorine | | |
|---|---|---|---|---|
| | No Interaction[2] | 0 h[2] | 10 h | 72 h |
| 0 | 4.97 | 5.01 | 5.43 | 6.05 |
| 2 | 5.88 | 5.73 | 6.22 | 6.45 |
| 5 | 6.65 | 6.93[a] | 6.88 | 7.10 |
| 10 | 7.40 | 7.10[a] | 7.68[b] | 7.55[c] |
| 18 | 7.70 | 7.77 | 7.75[b] | 7.66[c] |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] After vacuum cooling.

Table 3.11 Least square means for mold and yeast populations ($log_{10}$) on lettuce stored at 25°C. Interaction between storage days and after vacuum cooling was significant, if lettuce is washed without chlorine[1].

| Days | Chlorine | No Chlorine | | |
|---|---|---|---|---|
| | No Interaction[2] | 0 h[2] | 10 h | 7 2 h |
| 0 | 4.97 | 5.01 | 5.43 | 6.06 |
| 2 | 7.16 | 7.17[a] | 7.32[b] | 7.37[c] |
| 5 | 7.73 | 7.33[a] | 7.55[b] | 7.15[c] |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] After vacuum cooling.

Table 3.12 Least square means for mold and yeast populations ($log_{10}$) on lettuce stored at 32°C. Interaction between storage days and after vacuum cooling was significant, if lettuce was washed without chlorine.

| Days | Chlorine | No Chlorine | | |
|---|---|---|---|---|
| | No Interaction[1] | 0h | 10h | 72h |
| 0 | 4.97 | 5.02 | 5.43 | 6.03 |
| 2 | 7.25 | 7.05 | 7.02 | 7.13 |

[1] After vacuum cooling.

Table 3.13 Least square means for *E. coli* O157:H7 populations ($log_{10}$) on spinach were adjusted for factors including field temperature, holding time before and after forced air cooling. Given the washing method (with or without chlorine), there was no significant interaction between storage days and other factors[1].

| Days | Chlorine | | | No Chlorine | | |
|---|---|---|---|---|---|---|
| | 4°C | 25°C | 32°C | 4°C | 25°C | 32°C |
| 0 | 2.26 | 2.26[b] | 2.26 | 2.89 | 2.89 | 2.89 |
| 2 | 1.95 | 2.41[b] | nd[2] | 2.42[c] | 3.54 | nd[2] |
| 5 | 1.69[a] | nd | nd | 2.13[cd] | nd | nd |
| 10 | 1.67[a] | nd | nd | 2.02[d] | nd | nd |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] nd = not determined.

Table 3.14. Least square means for psychrotroph populations ($log_{10}$) on spinach stored at 4°C. Factors were adjusted including holding time before and after forced air cooling. Given the washing method (with or without chlorine), the interaction between storage days and field temperature was significant[1].

| Days | Chlorine | | No Chlorine | |
|---|---|---|---|---|
| | 25°C[2] | 32°C | 25°C[2] | 32°C |
| 0 | 4.30 | 5.74 | 4.51 | 6.39 |
| 2 | 5.28 | 6.78 | 5.85 | 6.93 |
| 5 | 6.95 | 7.64 | 7.30[a] | 7.67 |
| 10 | 7.59 | 8.15 | 7.37[a] | 8.62 |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Field temperature.

Table 3.15. Least square means for psychrotroph populations ($log_{10}$) on spinach stored at 25°C. If spinach was washed with chlorine, the interaction between field temperature and storage days were significant; if spinach was washed without chlorine, the interaction between storage days and field temperature and the interaction between storage days and time before forced air cooling was both significant[1].

| Days | Chlorine | | No Chlorine | | | |
|---|---|---|---|---|---|---|
| | No Interaction[2] | | 25°C[2] | | 32°C | |
| | 0 h[3] | 10 h | 0 h[3] | 10 h | 0 h | 10 h |
| 0 | 4.53 | 5.52 | 4.08 | 4.95 | 5.98 | 6.80[a] |
| 2 | 7.18 | 7.27 | 7.13 | 6.61 | 7.59 | 7.05[a] |
| 5 | nd[4] | nd | nd[4] | nd | nd | nd |
| 10 | nd | nd | nd | nd | nd | nd |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Field temperature.

[3] Time before forced air cooling.

[4] nd = not determined.

Table 3.16. Least square means for psychrotroph populations ($log_{10}$) on spinach stored at 32°C. Note that on day 0, least square means for $log_{10}$ (psychrotroph populations on spinach) were the same for different storage temperatures since the storage temperature had not become effective yet.

| Days | Chlorine | No Chlorine |
|---|---|---|
| 0 | 5.02 | 5.45 |
| 2 | nd[1] | nd[1] |
| 5 | nd | nd |
| 10 | nd | nd |

[1] nd = not determined

Table 3.17. Least square means for mesophilic populations ($log_{10}$) on spinach, which were adjusted for factors including field temperature, holding time before and after forced air cooling. Given the washing method (with or without chlorine), there was no significant interaction between storage days and other factors[1].

| Days | Chlorine | | | No Chlorine | | |
|---|---|---|---|---|---|---|
| | 4°C[2] | 25°C | 32°C | 4°C[2] | 25°C | 32°C |
| 0 | 5.79 | 5.79 | 5.79 | 6.24 | 6.24 | 6.24 |
| 2 | 6.49 | 8.20 | nd[3] | 6.90 | 8.47 | nd[3] |
| 5 | 7.64 | nd | nd | 7.66 | nd | nd |
| 10 | 8.08 | nd | nd | 8.31 | nd | nd |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Storage temperature.

[3] nd = not determined.

Table 3.18. Least square means for mold and yeast populations (log10) on spinach stored at 4°C. If spinach was washed without chlorine, the interaction between storage days and field temperature was significant[1].

| Days | Chlorine | No Chlorine | |
|---|---|---|---|
| | No interaction[2] | 25°C[2] | 32°C |
| 0 | 4.42 | 3.69 | 5.84 |
| 2 | 5.16 | 5.15 | 6.45[a] |
| 5 | 6.47 | 5.81 | 6.91[ab] |
| 10 | 7.18 | 7.07 | 7.28[b] |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Field temperature.

Table 3.19. Least square means for mold and yeast populations (log10) on spinach stored at 25°C. If spinach was washed without chlorine, the interaction between storage days and field temperature was significant[1].

| Days | Chlorine | No Chlorine | |
|---|---|---|---|
| | No interaction[2] | 25°C | 32°C |
| 0 | 4.41 | 3.69 | 5.85 |
| 2 | 6.68 | 6.23 | 7.08 |
| 5 | nd[3] | Nd[3] | nd |
| 10 | nd | nd | nd |

[1] Multiple pair-wise comparisons were made within each column. Least square means with the same superscript were not statistically different at significance level 0.1.

[2] Field temperature.

[3] nd = not determined.

Table 3.20. Least square means for mold and yeast populations (log10) on spinach stored at 32°C. Note that on day 0, least square means for $\log_{10}$ (mold and yeast populations on spinach) were the same for different storage temperatures since the storage temperature had not become effective yet.

| Days | Chlorine | No Chlorine |
|---|---|---|
| 0 | 4.41 | 4.77 |
| 2 | nd[1] | nd[1] |
| 5 | nd | nd |
| 10 | nd | nd |

[1] nd = not determined.

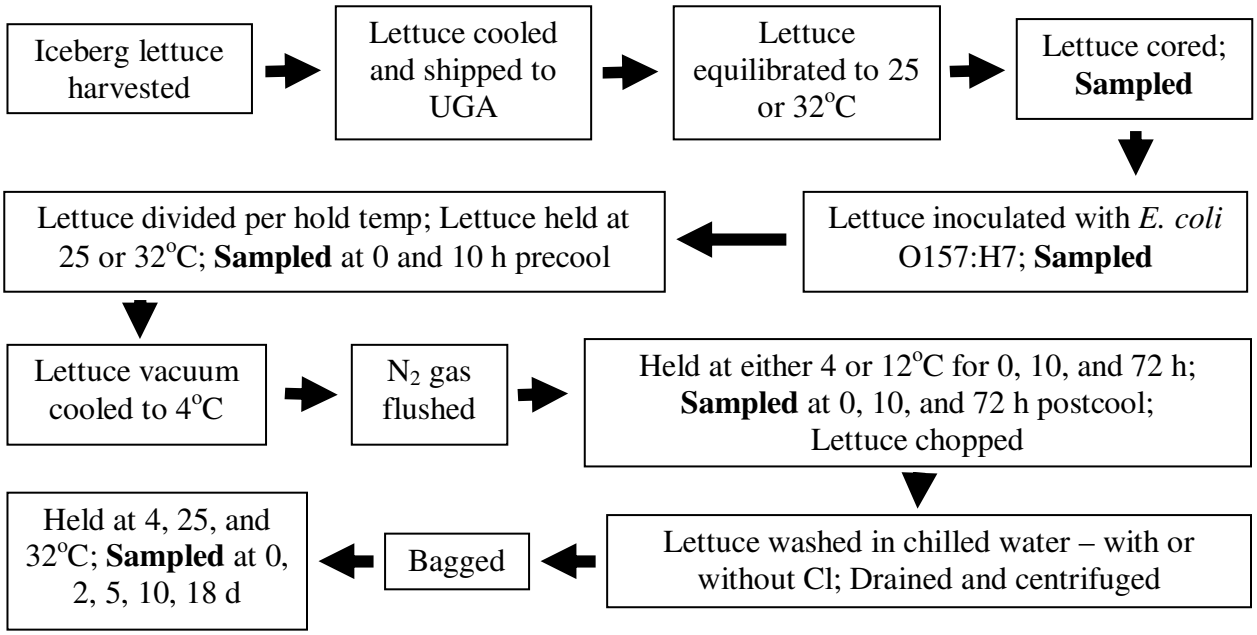


Figure 3.1 Processing, handling and sampling plan for iceberg lettuce.

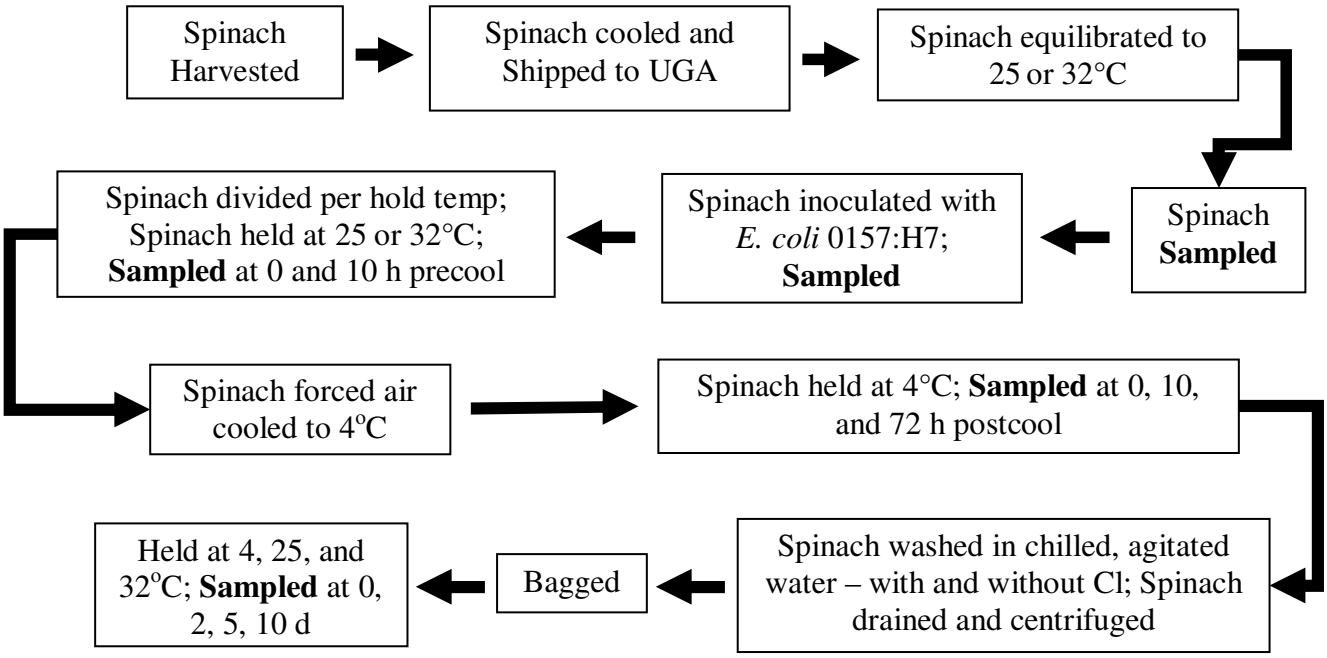


Figure 3.2 Processing, handling and sampling plan for spinach.