Survival of *Escherichia coli* O157:H7 on cut and whole surfaces of spinach and leaf lettuce, packaged under modified atmospheric conditions

By

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ABSTRACT:

Numerous food-borne outbreaks of *Escherichia coli* O157:H7 have been linked to leafy greens in recent years. An overwhelming amount of lettuce and spinach on the market is sold in modified atmosphere packaging as ready to eat salad mixes. The objectives of this study were to determine the effects of modified atmosphere, storage temperature, and inoculum size on survival of *E. coli* O157:H7 on cut and whole leaf lettuce and spinach. *E. coli* O157:H7 H1730 was inoculated onto cut and whole leaves of leaf lettuce and spinach. Samples were held under normal atmospheric conditions or in a modified atmosphere package at either 4ºC or 10ºC to simulate display and abuse temperatures. Leaves were sampled at Days 0, 1 and every other day until visual spoilage occurred (7 days for lettuce, 9 days for spinach). *E. coli* O157:H7 was able to survive at 4° and 10°C regardless of atmosphere and inoculum size for 7 days on cut and whole lettuce and 9 days on cut and whole spinach. Overall, numbers of *E. coli* O157:H7 increased (1 log) throughout the storage period on spinach, and decreased on lettuce (1-1.5 log). Significantly higher ($P \leq 0.05$) numbers of *E. coli* O157:H7 were found on lettuce and spinach stored at 10ºC than when stored at 4ºC. There were no significant differences ($P \geq 0.05$) in Numbers of *E. coli* O157:H7 with respect to atmosphere, leaf type or inoculum size. If contamination of lettuce or spinach with *E. coli* O157:H7 occurred, the pathogen may survive well under typical packaging and storage conditions.

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DEDICATION

I dedicate this work to my grandfather, Louis A. Mumford, for being my number one fan and guardian angel.

Attribution

Several colleagues and coworkers aided in the writing and research behind several chapters of this thesis. A brief description of their background and their contributions are included here.

Asst. Prof. Renee R. Boyer- Ph.D. (Department of Food Science and Technology, Virginia Tech) is the primary Advisor and Committee Chair. Dr. Boyer provided constant assistance and guidance throughout this research work. Furthermore, Dr. Boyer also provided funds for all of the supplies involved with this project.

Chapter 3: Effect of storage temperature and atmosphere on the survival of *Escherichia coli* O157:H7 on cut and whole leaf lettuce and spinach

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Appendix: Use of GFP fluorescence assay to quantify *E. coli* O157:H7 numbers from lettuce and spinach samples

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CHAPTER 1

INTRODUCTION AND JUSTIFICATION:

Fresh produce consumption has increased dramatically over the last two decades. From 1982 to 1997, per capita consumption of fresh produce increased from 91.6 to 121.1 kg per annum, an increase of 32% (FDA, 2001). This increase may be attributed to an increased consumer demand for fresh fruits and vegetables, as well as the reduced seasonality associated with most fresh fruits and vegetables (Ana Allende, Y. L., James L. McEvoy, Francisco Artes, Chien Y. Wang, 2004). Coupled with this increase in produce consumption has been a greater number of food-borne illnesses linked to fresh produce (FDA, 2001). This increase in outbreaks can be attributed to these changes in consumption patterns and more global distribution of produce as well as changes in agricultural practices and processing (Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001). Lettuce and spinach, specifically, have been common vehicles associated with the food-borne pathogen *Escherichia coli* O157:H7. Consequently, routes of contamination, and methods to remove this pathogen has become a significant issue and major concern for the fresh-cut produce industry (Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001, Warriner, K., Ibrahim, F., Dickinson, M., Wright, C. and Waites, W.M., 2003).

Fresh-cut produce are defined as all fruits and vegetables that have been physically altered, but remain in the fresh state. This can include cut carrots, broccoli and bagged salads to name a few. These commodities are prepared and transported in a ready-to-eat condition for convenience to the consumer. This added convenience is made possible in part, through the use of modified atmosphere packaging (MAP) along with maintaining low storage temperatures. These two variables help to enhance the freshness

of fruits and vegetables and extend their shelf life (M.A Del Nobile, B., A., Benedetto, E. and Massignan, L. , 2005). These two factors slow the metabolism of the produce so that it stays fresh for an extended period of time (Yoonseok Song, N. V., and Kit L. Yam, 2001).

Fresh-cut produce is still living and requires energy which occurs through the process of respiration (M.A Del Nobile, B., A., Benedetto, E. and Massignan, L. , 2005). The respiration rate of produce commodities vary significantly. Higher respiration rates correlate to the product being more perishable (Yoonseok Song, N. V., and Kit L. Yam, 2001). The act of cutting or "processing" fresh cut produce increases the respiration rate further due to a greater surface area being exposed to the atmosphere. This allows oxygen to diffuse into the interior cells more rapidly (Yoonseok Song, N. V., and Kit L. Yam, 2001). Temperature also affects respiration rate of produce. When the temperature is decreased, the respiratory metabolism of the produce will also decrease (Yoonseok Song, N. V., and Kit L. Yam, 2001). The majority of fresh produce maintains of ideal quality and freshness at temperatures near 0ºC (Mou, B., 2008). Respiration of produce can also be decreased by modifying the atmosphere in which the product is stored. When the concentration of oxygen falls below 10% respiration begins to slow (Yoonseok Song, N. V., and Kit L. Yam, 2001). By maintaining produce in an atmosphere with less than 10% oxygen, the product's shelf life can be extended.

A concern regarded MAP of fresh produce involves the delay of spoilage aerobic microorganisms. This will additionally increase the amount of time for pathogenic bacteria to grow on the product. By increasing the shelf life of fresh cut produce utilitizing MAP, it is creating an environment with a low O_2 level that will inhibit aerobic

spoilage microorganisms as well as potentially encourage growth of facultative anaerobes (FDA, 2001). Reducing O_2 is ideal for maintaining the freshness of a product, but can create a situation for increased growth of pathogenic bacteria such as *E. coli* O157:H7. Oxygen levels below 1% can lead to anaerobic respiration, thereby allowing growth of facultative anaerobic pathogens (FDA, 2001). Oxygen in MAP is usually recommended to be kept very low (1-5%) in order to counteract the respiration rate of fresh cut produce. This reduction of O_2 aids in prolonging the shelf life of produce by delaying the product's respiration (FDA, 2001). Carbon dioxide in MAP has been shown to have an inhibitory effect on spoilage microorganisms at levels of 10-20%, however, at increased CO_2 levels browning and deterioration of product quality may occur (FDA, 2001).

The implementation of MAP has provided many positive contributions to the food industry by allowing a variety of products to remain on the shelf for a longer period of time while maintaining quality. Extension of shelf life may allow increased growth of pathogens compared to products stored at normal atmospheres. These issues can be addressed through the use of permeable films, in which spoilage should occur before the production of toxins from pathogens (FDA, 2001). Therefore, while MAP may inhibit spoilage microorganisms, which warn consumers when a product is not suitable for consumption, pathogens will have adequate time to grow with no visual indication.

The objectives of this study were to determine the survival of *E. coli* O157:H7 on whole and cut portions of spinach and leaf lettuces packaged under passively modified atmospheric conditions and held at two temperatures (4°C and 10°C). Analysis of whole and cut portions was completed because it is common for lettuce and/or spinach to be cut before packaging. Additionally, slight damage of leaves could be a risk factor for *E. coli*

O157:H7 survival. The packaging used and storage temperatures selected mimic typical industry practices. According to industry, lettuce is typically packaged in PD 900 bags (O_2 transmission rate = 200 cc/100 sq. in. and a CO_2 transmission = 632 cc/100 sq. in.) flushed with 100% Nitrogen to remove as much O_2 as possible and the atmosphere is able to equilibrate passively through respiration of the product. Spinach is typically packaged in PD 961 bags which have an O_2 transmission rate of 450 cc/100 sq. in. The increased respiration rate of spinach will consume O_2 in the package more quickly; therefore the package is sealed under atmospheric O_2 concentrations.

CHAPTER 2:

Literature Review

Escherichia coli O157:H7

Non-pathogenic *Escherichia coli* is a normal inhabitant of the intestines of all animals, including humans (FDA, 2001). Most *E. coli* strains are harmless; however, some strains are pathogenic, which cause illness. There are currently six classes of diarrheagenic (cause diarrheal illness) *E. coli*: enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E .coli* (EIEC); diffusely adhering *E. coli* (DAEC); enteroaggregative *E. coli* (EAEC); and enterohemorrhagic *E. coli* (EHEC) (Matthews, T. J. M. a. K. R., 2005). The class of strains causing the most severe illnesses causing strains is the EHEC class. The *E. coli* serotype O157:H7 falls under this class and is responsible for many of the EHEC-associated disease around the world (Matthews, T. J. M. a. K. R., 2005).

E. coli O157:H7 is a facultative anaerobic, Gram-negative bacillus which emerged as a food-borne pathogen in 1982 (Barrera, O., Rodriguez-Calleja, J.M., Santos, J.A., Otero, A. and Garcia-Lopez, M.L., 2007, Besser, R. E., Griffin, P.M. and Slutsker, L., 1999). The bacterium is classified based upon two types of surface structures, which are the O and H antigens. The O antigen refers to the LPS carbohydrate moieties and the H antigen is the flagellar antigen (Besser, R. E., Griffin, P.M. and Slutsker, L., 1999). *E. coli* O157:H7 is different from other strains of *E. coli* because of its inability to ferment sorbitol rapidly as well as inability to produce the enzyme β-glucuronidase (Besser, R. E., Griffin, P.M. and Slutsker, L., 1999). This strain is also unusually tolerant of acidic

environments and is capable of growing at a minimum pH of 4.0 to 4.5 (Matthews, T. J. M. a. K. R., 2005). This pathogen is also capable of growing at 8 ºC, a temperature below which ready-to-eat meals and lightly processed salad vegetables may be exposed for several hours during marketing, transportation or on restaurant buffet counters (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993).

E. coli O157:H7 infection most commonly presents with symptoms such as abdominal pain, watery diarrhea, bloody diarrhea (hemorrhagic colitis), vomiting and a mild fever (FDA, 2001). It is also possible for people to become infected but show no signs of the illness, which is referred to as asymptomatic infection (Matthews, T. J. M. a. K. R., 2005). Following ingestion of the organism, there is typically an incubation period of about 3 to 4 days before patients develop diarrhea and for about 25 – 75% of patients, the illness remains relatively mild (Besser, R. E., Griffin, P.M. and Slutsker, L., 1999). During this incubation period is when colonization of the large intestine occurs and the progression of illness from watery diarrhea to bloody diarrhea lasts for 4 to 10 days (Matthews, T. J. M. a. K. R., 2005). The exact infectious dose of *E. coli* O157:H7 is not known, but it is thought to be extremely low and may be similar to that of *Shigella* spp. which can be as few as 10 organisms (FDA, 2001, Matthews, T. J. M. a. K. R., 2005). This estimation of a low infectious dose is based on evidence of minute amounts (0.3 to 0.4 colony forming units) of *E. coli* O157:H7 per g being detected in meat implicated in various food borne outbreaks across the United States (Matthews, T. J. M. a. K. R., 2005).

The virulence of *E. coli* O157:H7 is primarily due to production of verocytotoxins, which are most often correlated to a family of bacterial cytotoxins

produced by *Shigella dysenteriae* (Sungsu Park, W., Randy W., and Durst, Richard A., 1999). The production of these shiga toxins by EHEC strains cause an infection which is linked to the hemolytic-uremic syndrome (HUS) (Sungsu Park, W., Randy W., and Durst, Richard A., 1999). This severe condition is the most common cause of acute renal failure in children and has a mortality rate of approximately 5% (Sungsu Park, W., Randy W., and Durst, Richard A., 1999). Central nervous system manifestations, including lethargy, seizures, coma, hemiparesis, or decerebrate posturing, occur in 30% of patients with HUS (Besser, R. E., Griffin, P.M. and Slutsker, L., 1999). Shiga toxins produced by *E. coli* O157:H7 and other EHEC *E. coli* are also capable of causing thrombotic thrombocytopenic purpura (TTP). This disease occurs mainly in adults and is characterized by hemolysis, thrombocytopenia, renal failure, neurological problems, and a fluctuating fever (Sungsu Park, W., Randy W., and Durst, Richard A., 1999).

***E. coli* O157:H7 Outbreaks Associated with Meat Products:**

E. coli O157:H7is commonly part of the normal microbial Number in the intestinal tract of many warm-blooded animals including dogs, birds, sheep, deer goats and cattle (Matthews, T. J. M. a. K. R., 2005). Cattle, however, are the most frequently implicated reservoir of *E. coli* O157:H7 (Besser, R. E., Griffin, P.M. and Slutsker, L., 1999). Historically, food-borne outbreaks of *E. coli* O157:H7 are most commonly associated with beef and studies have shown that this pathogen is often isolated from the feces of healthy cattle (Sungsu Park, W., Randy W., and Durst, Richard A., 1999). A 2002 study by the USDA showed that 38.5% of dairy farms had at least one cow that was positive for *E. coli* O157:H7 and 4.3% of individual cows were *E. coli* O157:H7 positive (Meng, J., Doyle, M.P., Lhao, T. and Zhao, S. , 2007). The pathogen is frequently found

in water troughs on farms and can consequently survive for weeks or months in bovine feces and water (Matthews, T. J. M. a. K. R., 2005). Beef may become contaminated during slaughter, and the process of grinding beef may transfer pathogens from the surface of the meat to the interior (Besser, R. E., Griffin, P.M. and Slutsker, L., 1999).

Another multi-state outbreak associated with *E. coli* O157:H7 occurred in July 2008. Michigan state health officials tested ground beef purchased by ill persons associated with the outbreak from Kroger retail stores in both Michigan and Ohio (CDC, 2008). The number of cases in each state is as follows: Georgia (4), Indiana (1), Kentucky (1), Michigan (20), New York (1), Ohio (21), and Utah (1). It was confirmed from the ground beef tested that the isolates of *E. coli* O157:H7 that were found were the strain involved in the outbreak. There have been 49 confirmed cases linked to this outbreak of which 27 people were hospitalized and one person developed HUS (CDC, 2008).

Food-borne Disease Outbreaks Associated with Produce:

In recent years, fresh fruits and vegetables have become implicated in more *E. coli* O157:H7 outbreaks than beef. Fresh and fresh-cut produce can become contaminated during pre-harvest, through contact with soil, water, animals, and harvest equipment (Warriner, K., Ibrahim, F., Dickinson, M., Wright, C. and Waites, W.M., 2003). Contamination can occur before or during the harvest and processing (Cooley, M. B., Chao, D. and Mandrell, R.E., 2006). *E. coli* O157:H7 is likely to contaminate fresh produce through contact with feces, sewage, untreated irrigation water, or surface water (Takeuchi, K., Matute, C.M., Hassan, A.N. and Frank, J.F., 2000). This pathogen is capable of persisting over prolonged periods of time in soils and compost. Fresh produce

can also become contaminated by beef and improperly composted cow manure or other animal waste (Besser, R. E., Griffin, P.M. and Slutsker, L., 1999). These pathogens may also be present in water used for irrigation of produce (Beuchat, L. R., 1995). Contamination of produce is a significant issue because it is often consumed raw.

The increase in food-borne illness outbreaks associated with produce may be explained by many factors including: increased consumption; and increased globalization of fresh and fresh cut fruits and vegetables.

Fresh and fresh-cut produce can pose a food safety hazard since these products only require a minimum amount of processing. There is less chance for the elimination of pathogens that may otherwise destroyed in effective microbial inactivation steps (FDA, 2001). Due to the low infectious dose of many of these pathogens, even a small amount of contamination can result in illness. It is therefore necessary to understand and control the various points during production, harvest, processing and transporting in which contamination of produce can occur (FDA, 2001).

From 1973 through 1997, 32 states reported 190 produce-associated outbreaks, these resulted in: 16,058 reported illnesses, 598 hospitalizations, and eight deaths. Among the 85 outbreaks in which a single produce item was implicated; lettuce comprised 25 of those food borne outbreaks (Sivapalasingam, S., Friedman, C.R., Cohen, L. and Tauxe, R.V., 2004). *E. coli* O157:H7 alone was associated with 13 of the 85 outbreaks and lettuce was involved in 5 of those outbreaks (Sivapalasingam, S., Friedman, C.R., Cohen, L. and Tauxe, R.V., 2004).

***E. coli* O157:H7 Outbreaks linked to produce consumption:**

In 1996, state health department personnel in Connecticut and Illinois reported a noticeable increase in the number of *E. coli* O157:H7 infections. A thorough investigation confirmed that a multi state outbreak of *E. coli* O157:H7 had occurred associated with the consumption of mesclun lettuce from a single producer (Hilborn, E. D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K. and Slutsker, L., 1999). This outbreak of mesclun lettuce in Connecticut and Illinois resulted in infection and illness of at least 61 persons, with 21 hospitalizations and 3 cases of HUS (Hilborn, E. D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K. and Slutsker, L., 1999). The outbreak was thought to be caused by a small grower-producer of mesclun lettuce. According to the field investigations, there were many possible sources of contamination including: a neighboring cattle ranch, free-range chickens with access to areas where lettuce was being grown and recirculation of wash water through a used filter (Hilborn, E. D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K. and Slutsker, L., 1999). There was also a lack of hand-washing facilities for workers and no hygienic controls during processing (Hilborn, E. D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K. and Slutsker, L., 1999). One or a combination of these factors is likely where *E. coli* O157:H7 contamination occurred. Since lettuce does not go through further processing or any other steps to eliminate pathogens once it is purchased by consumers, it is

important for producers to ensure all possible safety measures in points where contamination could occur.

In 1997, there was an outbreak of *E. coli* O157:H7 in Michigan and Virginia associated with alfalfa sprouts (CDC, 1997). A total of 60 people were reported to be affected in Michigan and of those 44 were reported to have bloody diarrhea, 25 were hospitalized, two people developed HUS and 1 person had thrombotic thrombocytopenic purpura (CDC, 1997). In Virginia, 48 cases of infection were also diagnosed (CDC, 1997). The alfalfa seeds that were implicated in this outbreak were from a single grower which then distributed the contaminated seeds to two farms, one in Michigan and one in Virginia, which produced the contaminated sprouts (CDC, 1997).

A large and very well publicized *E. coli* O157:H7 outbreak involving the consumption of bagged spinach occurred in September 2006 from a plant in California where the contaminated products had been processed (FDA, 2001). The FDA along with California's department of health services conducted an investigation on the cause of the outbreak and found that *E. coli* O157:H7 was associated with Dole baby spinach (FDA, 2001). The direct cause of *E. coli* O157:H7 contamination of the spinach is not clear, but is thought to be at or near the field which included the presence of wild pigs, irrigation wells used to irrigate produce for ready-to-eat packaging, and surface waterways exposed to feces from cattle and wildlife (FDA, 2001). All of these risk factors were associated with the field that happened to contain the strain of *E. coli* O157:H7 that matched the strain that caused the outbreak. Therefore, it is likely that one of these potential sources of contamination was linked to the outbreak of bagged spinach containing *E. coli* O157:H7 which resulted in 205 confirmed illnesses and 3 deaths (FDA, 2001).

Spinach:

Spinach (*Spinacia oleracea*) is a leafy green and most often used in salads. Spinach is a hardy cool season crop and is capable of surviving severe frosts in the winter. It also has a deep, thick root system in which branching roots can extend several feet. Spinach grows in a rosette and is a fleshy green annual that has broad, tender leaves (Mills, H. A., 2001). This crop prefers temperatures between 50 and 60 ºC and will not grow if soil temperature rises above 85 ºC (Mills, H. A., 2001).

Spinach is commonly consumed raw and for this reason harvest, processing and general handling becomes important because of potential contamination occurring. California is responsible for over one half of the spinach production in the United States. California is also the leader in the processing of spinach, producing over one third of the total U.S. production (Mills, H. A., 2001). When spinach is harvested and processed it is cut about an inch above the soil surface and is then put into bulk trucks or trailers for transport to retail markets (Mills, H. A., 2001).

Once spinach reaches the fresh market it should be held at 32ºF and 95-100% relative humidity. Spinach quality will deteriorate quickly and should only be stored for about 10-14 days (Mills, H. A., 2001). Removal of excess water is very important once taken from the field. This is done by a vacuum system in order to maintain and preserve freshness. Spinach can then either be sold loose, in bulk with cartons and bunches or, most commonly, in polyethylene bags (Mills, H. A., 2001). Spinach that is sold in retail stores is prepackaged in transparent plastic film bags which allow gas exchange, but maintain high humidity by reducing evaporation of water (Morelock, T. E. a. C., James C., 2008). Health concerns regarding spinach are primarily determined by the consumer

acceptance of the prepackaged triple washed baby leaf product (Morelock, T. E. a. C., James C., 2008).

Lettuce:

Lettuce, *Lactuca saliva*, is also a cool-season shallow rooted crop which is sensitive to high temperatures (Davis, U., 2006). There are several types of lettuce including: Butterhead (Boston), Cos (Romaine), Crisphead (iceberg) and loose leaf. Iceberg lettuce is the most common and available as a fresh market variety (Davis, U., 2006). Incidentally, it is this type of lettuce that is most often implicated in food-borne illness outbreaks (A.D. King Jr., J. A. M., T. Torok, and N. Goodman, 1991). Iceberg lettuce is primarily sold as head lettuce and is cut or partially processed for use in salad mixes.

Leaf lettuce is quite different from iceberg, and has considerable variation in leaf size, and forms a rosette of leaves that may have elongated shape resembling oak leaves with yellow or green colors (Mou, B., 2008). This cut lettuce is sold in bags which is convenient for customers to use as a ready to eat product. Lettuce is normally harvested when immature, which leads to an accelerated rate of respiration (A.D. King Jr., J. A. M., T. Torok, and N. Goodman, 1991). Due to the increased respiration rate, lettuce needs to be stored at low temperatures (0-2 ºC) in order to maintain quality and prolong shelf life. The shelf life of head lettuce is usually about 2-3 weeks at preferred storage temperature, but as the temperature increases, the length of shelf life decreases (Takeuchi, K. and J. F. Frank, 2000).

Modified Atmosphere Packaging:

Modified atmosphere packaging (MAP) involves controlling or modifying the atmosphere surrounding the product within a package (FDA, 2001). The package is made of one or a combination of films (FDA, 2001). The purpose of MAP for fresh-cut produce is to maintain quality by extending the shelf life. When using this method to enhance the quality of fruits and vegetables, oxygen concentrations within the package are decreased and carbon dioxide concentrations are increased within the package (Ji Gang Kim, Y. L., Yang Tao, Robert A Saftner and Kenneth C Gross, 2005). The technique in which fresh produce is packaged is extremely important because factors such as respiration rates, optimal storage temperatures, water absorption and byproducts must be taken into consideration in order to maintain the quality of the food.(FDA, 2001).

The modified atmosphere in MAP alters the normal concentration of air (78% nitrogen, 21% oxygen, 0.03% carbon dioxide and traces of noble gases) to a desirable composition of gases in order to prolong the storage length of the product (FDA, 2001). MAP is typically used on smaller quantities of produce and the atmosphere is only initially modified instead of controlling the atmosphere inside the package throughout the length of storage (CAS) (FDA, 2001). There are two methods of creating modified atmospheres, active and passive. The process of active modification occurs through removing the gases within the package which are then replaced by a different combination of gases. These are typically packaged in a barrier package that does not allow gas exchange. Passive modification involves just packaging the product with a selected porous film type and then naturally allowing an atmosphere to develop as a

result of the products respiration and the transmission of gases through the film (FDA, 2001).

The gases that are used in MAP are oxygen, carbon dioxide and nitrogen. O_2 is typically kept at a concentration of around 1-5% in order to decrease the respiration rate of the produce (FDA, 2001). This also attempts to create conditions less favorable for aerobic spoilage microorganisms. The passive changes in package atmosphere are due to an interaction between respiration rate of the product inside the package and the permeability characteristics of the film (Ji Gang Kim, Y. L., Yang Tao, Robert A Saftner and Kenneth C Gross, 2005). Therefore, it is imperative to keep the O_2 concentration low in order to prolong the shelf life of produce. If the O_2 concentration becomes too low (<1%), however, anaerobic respiration may occur, which results in deterioration of quality as well as opportunity for the growth of food borne pathogens. For these reasons it is necessary to keep the percentage of O_2 between 1 and 5% (FDA, 2001). CO_2 is usually kept at a concentration of 10-20% in MAP. These concentrations are considered moderate carbon dioxide levels because this percentage of CO_2 can create injury in lettuces. This gas has direct antimicrobial properties which can inhibit the growth of certain microorganisms (FDA, 2001). Carbon dioxide's antimicrobial effect results in an increased lag phase and generation time during the logarithmic phase of growth of the organisms involved (FDA, 2001). The typical MAP concentrations for leafy greens are 1-5% oxygen and 0-2.5% carbon dioxide within packages. The third gas used in MAP, nitrogen, is used as a filler to balance out the other gases in order to maintain the integrity of the package (FDA, 2001).

One concern regarding MAP is that certain food-borne pathogens such as *E. coli* O157:H7 can survive at refrigeration temperatures and grow at 10 ºC or greater as well as survive in modified atmospheres (Gunes, G. G. a. H., J.H., 2002). By extending a product's shelf life, MAP may allow time for *E. coli* O157:H7 to multiply without adversely affecting the organoleptic quality of produce and therefore could increase the risk of food-borne illness (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993, Gunes, G. G. a. H., J.H., 2002).

Takeuchi et al. (Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001) found that altering the atmosphere within a package at different temperature combinations affected the attachment and penetration of *E. coli* O157:H7 cells to leaf lettuce at 37 ºC. This study found that changing the temperature and oxygen concentrations modified the respiration rate of lettuce. This in turn affected attachment and penetration of *E. coli* O157:H7. These results suggest that modified atmosphere storage along with temperature control may decrease bacterial penetration and therefore allow for increased removal by subsequent washing (Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001).

A study on the survival and growth of *E. coli* O157:H7 on salad vegetables done by Abdul-Raouf et al. (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993) investigated the effects of modified atmosphere packaging, storage temperature, and storage time on *E. coli* O157:H7 on lettuce leaves. Their results showed that numbers of *E. coli* O157:H7 on shredded lettuce stored at 5 ºC decreased during the 14 day storage period, while the pathogen grew on lettuce stored at 12 and 21 ºC. It was also found, however, that the combination of varied storage temperatures, storage times, and

modified atmospheres within the package did not have an effect on the *E. coli* O157:H7 number on the lettuce (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993).

In 2001, a study done on the effects of storage temperature and package atmosphere on growth and survival of *E. coli* O157:H7 on iceberg lettuce by Francis et al. (Francis, G. A. a. O. B., D., 2001) found that temperature effected *E. coli* O157:H7 while packaging under modified atmospheres did not. The lettuce used in this study was packaged in bags composed of polypropylene packaging film which were later heat sealed (permeability to O_2 of 1200 ml/m^2/day/atm and CO_2 of 4000 ml/m^2/day/atm). The lettuce was sealed in these packages initially enclosing and air and during storage the gas atmospheres within packages were modified mainly as a result of the respiration of the iceberg lettuce (Francis, G. A. a. O. B., D., 2001). Numbers of viable *E. coli* O157:H7 decreased on lettuce stored at 5 ºC and then increased during storage at 12 and 21 ºC. When gas atmospheres were modified to CO_2 levels of 9-12% and O_2 levels of 2-4% within the package, there was no inhibitory effect on the growth of *E. coli* O157:H7 on shredded lettuce. Francis et al. also concluded that packaging vegetables under an atmosphere containing 3% O_2 and 97% N_2 had no effect on the growth of *E. coli* O157:H7 (Francis, G. A. a. O. B., D., 2001).

Attachment on Cut vs. Whole Surfaces:

Cutting produce is routinely done in the food industry and is often part of the minimal processing fruits and vegetables are subjected to (Beuchat, L. R., 1995). Throughout the length of storage of produce in retail food markets there are many opportunities for the product to be cut or damaged. *E. coli* O157:H7 has been shown to preferentially attach to cut surfaces of lettuce leaves (Seo, K. H. and J. F. Frank, 1999,

Takeuchi, K. and J. F. Frank, 2000, Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001). Takeuchi and Frank observed an increased number of *E. coli* O157:H7 cells at the cut and damaged edges of lettuce compared to those at uncut surfaces (Takeuchi, K. and J. F. Frank, 2000). These observations were also concluded by Seo and Frank as well as Takeuchi et al. under modified atmospheres at 4 and 22ºC as a result of the respiration rate of the lettuce (Seo, K. H. and J. F. Frank, 1999, Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001).

E. coli O157:H7 has been shown to attach to surfaces of produce. It is generally believed that bacterial attachment is the primary step in which produce initially becomes contaminated. Takeuchi et. al found that penetration of *E. coli* O157:H7 into leaf lettuce was affected by temperature under modified atmospheric conditions (Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001). Their results indicated that great numbers of *E. coli* O157:H7 attached to lettuce at 22ºC compared to 4, 10 and 37ºC (Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001). Previous research proves that this pathogen preferentially attaches to cut edges of lettuce leaves than to intact surfaces (Seo, K. H. and J. F. Frank, 1999, Takeuchi, K. and J. F. Frank, 2000, Takeuchi, K. and J. F. Frank, 2001). It is not well understood how bacteria are able to attach to surfaces of produce.

Green Fluorescent Protein:

The green fluorescent protein (GFP) was discovered from a jellyfish, *Aequorea victoria*, and has the ability to generate a visible internal fluorophore. This protein has proven to be a valuable tool for studying gene expression as well as tagging cells in culture (M. Vialette, A.-M. J.-R., C. Guyard, O. Legeay, A. Pinon and M. Lange, 2004). The chromophore of GFP is a *p*-hydroxybenzylideneimidazolinone and acquires visible

absorbance and fluorescence when oxygen dehydrogenates the α-β on residue 66. In order for this fluorescent mechanism to occur, atmospheric oxygen is required and fluorescence of anaerobically performed GFP develops with simple exponential time (Tsien, R. Y., 1998). GFP is expressed by introducing a plasmid containing the gene into the microorganism of interest (Noah, C. W., Shaw, C.I., Ikeda, J.S., Kreuzer, K.S. and Sofos, J.N., 2005). The gene encoding GFP is fused with the gene encoding the endogenous protein; the result is a fusion protein that has its normal functions and localizations of the host protein and can fluoresce (Tsien, R. Y., 1998). GFP has a major excitation peak at 395 nm, a minor peak at 475 nm, and an emission peak at 509 nm (Takeuchi, K. and J. F. Frank, 2001). GFP is able to be used as a marker in different organisms because no cofactors are needed for the folded protein to develop fluorescence (Nico Stuurman, C. P. B., Helmi R. M. Schlaman, Andre H. M. Wijifjes, Guido Bloember, and Herman P. Spaink, 2000). GFP is an ideal biological marker to use in protein tagging because it is highly stable to heat, alkaline pH, detergents, and many proteases (Takeuchi, K. and J. F. Frank, 2001).

Studies that have been done to show expression of GFP in *E. coli* cells have confirmed it's stability and effectiveness as a useful marker on produce (Noah, C. W., Shaw, C.I., Ikeda, J.S., Kreuzer, K.S. and Sofos, J.N., 2005). In 2000, a study done by Takeuchi et. al. was done on the use of GFP to detect and determine the attachment of *E. coli* O157:H7 to green leaf lettuce (Takeuchi, K. and J. F. Frank, 2001). Their results indicated that *E. coli* O157:H7 cells preferentially attach to lettuce at cut edges, which is consistent with previous research done with iceberg lettuce. The attachment profiles of *E. coli* cells were identified by observing the green fluorescence of EGFP-expressing *E.*

coli O157:H7 (Takeuchi, K. and J. F. Frank, 2001). The use of GFP transformation of *E. coli* O157:H7 is an ideal method to follow the growth and survival of these cells (M. Vialette, A.-M. J.-R., C. Guyard, O. Legeay, A. Pinon and M. Lange, 2004).

Franz et. al used a GFP-expressing *E. coli* O157:H7 strain in a study in order to determine the possibility of the pathogen's internalization in lettuce (Eelco Franz, A. A. V., Anee D. Van Diepeningen, Michel M. Klerks, Aad J. Termorshuizen, Ariena H.C. van Bruggen, 2006). They found that *E. coli* O157:H7 is able to be present in high levels at internal locations despite these areas being protected against sterilization. Their results also indicated that the growth circumstances of lettuce have an effect on the occurrence of contamination by *E. coli* O157:H7 as the pathogen was present in plants grown in potting soil. This study effectively incorporated the use of GFP to monitor the activity of *E. coli* O157:H7 through lettuce leaves (Eelco Franz, A. A. V., Anee D. Van Diepeningen, Michel M. Klerks, Aad J. Termorshuizen, Ariena H.C. van Bruggen, 2006).

References

A.D. King Jr., J. A. M., T. Torok, and N. Goodman. 1991. Microbial Flora and Storage Quality of Partially Processed Lettuce. *Journal of Food Science*. 56:459-461.

Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S. 1993. Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Applied and Environmental Microbiology*. 59:1999-2006.

Ana Allende, Y. L., James L. McEvoy, Francisco Artes, Chien Y. Wang. 2004. Microbial and quality changes in minimally processed baby spinach leaves stored under super atmospheric oxygen and modified atmosphere conditions. *Postharvest Biology and Technology*. 33:51-59.

Barrera, O., Rodriguez-Calleja, J.M., Santos, J.A., Otero, A. and Garcia-Lopez, M.L. 2007. Effect of different storage conditions on *E. coli* O157:H7 and the indigenous bacterial microflora on lamb meat. *International Journal of Food Microbiology*. 115:244-51.

Besser, R. E., Griffin, P.M. and Slutsker, L. 1999. *Escherichia coli* O157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. *Annual Review of Medicine*. 50:355-67.

Beuchat, L. R. 1995. Pathogenic Microorganisms Associated with Fresh Produce. *Journal of Food Protection*. 59:204-216.

CDC. 1997. Outbreak of *Escherichia coli* O157:H7 Infection Associated with Eating Alfalfa Sprouts – Michigan and Virginia, June – July 1997. *MMWR*. 46(32):741-744.

CDC. Date, 2008, Multistate Outbreak of *E. coli* O157:H7 infections – Indiana, Kentucky, Michigan, New York, and Ohio. Available at: http://www.cdc.gov/ecoli/june2008outbreak/. Accessed 2008.

Chua, D., Goh, K., Saftner, R.A. and Bhagwat, A.A. . 2008. Fresh-cut lettuce in modified atmosphere packages stored at improper temperatures supports enterohemorrhagic *E. coli* Isolates to survive gastric acid challenge. *Journal of Food Science*. 00:M1-M6.

Cooley, M. B., Chao, D. and Mandrell, R.E. 2006. *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *Journal of Food Protection*. 69:2329-35.

Davis, U. Date, 2006, Lettuce. Available at: http://vric.ucdavis.edu/veginfo/commodity/garden/crops/lettuce.pdf. Accessed, 2007.

Delaquis, S., Stewart, S., Cazaux, S. and Toivonen, P. 2002. Survival and growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in ready-to-eat iceberg lettuce washed in warm chlorinated water. *Journal of Food Protection*. 65:459-64.

Eelco Franz, A. A. V., Anee D. Van Diepeningen, Michel M. Klerks, Aad J. Termorshuizen, Ariena H.C. van Bruggen. 2006. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar *Typhimurium*. *Food Microbiology*. 24:106-112.

FDA. Date, 2001, *Escherichia coli* O157:H7. Available at: http://www.cfsan.fda.gov/~mow/chap15.html. Accessed, 2007.

FDA. 2001. Microbiological Safety of Controlled and Modified Atmosphere Packaging of Fresh and Fresh-Cut Produce. *In* C.f.F.S.a.A. Nutrition (ed.).

FDA. Date, 2001, Outbreaks Associated with Fresh and Fresh-Cut Produce. Incidence, Growth, and Survival of Pathogens in Fresh and Fresh-Cut Produce. Available at: http://www.cfsan.fda.gov/~comm/ift3-4a.html. Accessed, 2007.

FDA. Date, October 2004, From Production to Consumption: 2004 Action Plan to Minimize Foodborne Illness Associated with Fresh Produce Consumption. Available at: http://www.cfsan.fda.gov/~dms/prodpla2.html. Accessed, 2007.

Francis, G. A. a. O. B., D. 2001. Effects of vegetable type, package atmosphere and storage temperature on growth and survival of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Journal of Industrial Microbiology and Biotechnology*. 27:111-6.

Gunes, G. G. a. H., J.H. 2002. Growth and survival of *Escherichia coli* O157:H7 on fresh-cut apples in modified atmospheres at abusive temperatures. *Journal of Food Protection*. 65:1641-5.

Hao, Y. a. B., RE. 1993. Growth of Escherichia coli O157:H7 in modified atmosphere. *Journal of Food Protection*. 56:330.

Hereford, M. 2003. Effects of Apple Development and Damage on the Internalization of *Escherichia coli* O157:H7 as Observed Under Field and Laboratory Conditions. p. 48. *In*, Food Science and Technology, vol. Master of Science. Virginia Tech, Blacksburg.

Hilborn, E. D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K. and Slutsker, L. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Archives of Internal Medicine*. 159:1758-64.

Ji Gang Kim, Y. L., Yang Tao, Robert A Saftner and Kenneth C Gross. 2005. Effect of initial oxygen concentration and film oxygen transmission rate on the quality of fresh-cut romaine lettuce. *Journal of the Science of Food and Agriculture*. 85:1622-1630.

Lang, M. M., Harris, L.J. and Beuchat, L.R. 2004. Survival and recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on lettuce and parsley as affected by method of inoculation, time between inoculation and analysis, and treatment with chlorinated water. *Journal of Food Protection*. 67:1092-103.

Li, Y., Brackett, R.E., Chen, J. and Beuchat, L.R. 2001. Survival and growth of *Escherichia coli* O157:H7 inoculated onto cut lettuce before or after heating in chlorinated water, followed by storage at 5 or 15 degrees C. *Journal of Food Protection*. 64:305-9.

Liesbeth Jacxsens, D., Frank, Van der Steen, Caroline and Debevere, Johan 29 June 2001. Effect of high oxygen modified atmosphere packaging on microbial growth and sensorial qualities of fresh-cut produce. *International Journal of Food Microbiology*. 71:197-210.

M. Vialette, A.-M. J.-R., C. Guyard, O. Legeay, A. Pinon and M. Lange. 2004. Validating the use of green fluorescent-marked *Escherichia coli* O157:H7 for assessing the organism behaviour in foods. *Journal of Applied Microbiology*. 96:1097-1104.

M.A Del Nobile, B., A., Benedetto, E. and Massignan, L. . 2005. Respiration rate of minimall processed lettuce as affected by packaging
Journal of Food Engineering. 74:60-69.

Matthews, T. J. M. a. K. R. 2005. *Food Microbiology: An Introduction.* ASM Press, Washington, DC.

Meng, J., Doyle, M.P., Lhao, T. and Zhao, S. (ed.). 2007. Food Microbiology Fundamentals and Frontiers. ASM Press, Washington D.C.

Mills, H. A. Date, 2001, Vegetable Crops. Available at: http://www.uga.edu/vegetable/home.html. Accessed, 2007.

Morelock, T. E. a. C., James C. 2008. Spinach. p. 189-219. *In* J.a.N. Prophens, Fernanado (ed.), Handbook of Plant Breeding, vol. 1. Springer, New York.

Mou, B. 2008. Lettuce. p. 75-119. *In* J.P.a.F. Nuez (ed.), Handbook of Plant Breeding, vol. 1. Springer, New York.

Nico Stuurman, C. P. B., Helmi R. M. Schlaman, Andre H. M. Wijifjes, Guido Bloember, and Herman P. Spaink. 2000. Use of Green Fluorescent Protein Color Variants Expressed on Stable Broad-Host Range Vectors to Visualize Rhizobia Interacting with Plants. *The American Phytopathological Society*. 13:1163-1169.

Noah, C. W., Shaw, C.I., Ikeda, J.S., Kreuzer, K.S. and Sofos, J.N. 2005. Development of green fluorescent protein-expressing bacterial strains and evaluation for potential use as positive controls in sample analyses. *Journal of Food Protection*. 68:680-6.

Paton, J. C., and A. W. Paton. 1998. Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infections. . *Clinical Microbiology Reviews*. 11:450-479.

Seo, K. H., and J. F. Frank. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *Journal of Food Protection*. 62:3-9.

Sivapalasingam, S., Friedman, C.R., Cohen, L. and Tauxe, R.V. 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection*. 67:2342-53.

Sungsu Park, W., Randy W., and Durst, Richard A. 1999. *Escherichia coli* O157:H7 As An Emerging Foodborne Pathogen. *Critical Reviews in Food Science and Nutrition*. 39:481-502.

Takeuchi, K., and J. F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. *Journal of Food Protection*. 63:434-40.

Takeuchi, K., and J. F. Frank. 2001. Expression of red-shifted green fluorescent protein by *Escherichia coli* O157:H7 as a marker for the detection of cells on fresh produce. *Journal of Food Protection*. 64:298-304.

Takeuchi, K., and J. F. Frank. 2001. Quantitative determination of the role of lettuce leaf structures in protecting *Escherichia coli* O157:H7 from chlorine disinfection. *Journal of Food Protection*. 64:147-51.

Takeuchi, K., Hassan, A.N. and Frank, J.F. 2001. Penetration of *Escherichia coli* O157:H7 into lettuce as influenced by modified atmosphere and temperature. *Journal of Food Protection*. 64:1820-3.

Takeuchi, K., Matute, C.M., Hassan, A.N. and Frank, J.F. 2000. Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Pseudomonas fluorescens* to lettuce leaves. *Journal of Food Protection*. 63:1433-7.

Tsien, R. Y. 1998. The green fluorescent protein. *Annual Review of Biochemistry*. 67:509-44.

Warriner, K., Ibrahim, F., Dickinson, M., Wright, C. and Waites, W.M. 2003. Interaction of *Escherichia coli* with growing salad spinach plants. *Journal of Food Protection*. 66:1790-7.

Yoonseok Song, N. V., and Kit L. Yam. 2001. Modeling respiration-transpiration in a modified atmosphere packaging system containing blueberry. *Journal of Food Engineering*. 53:103-109.

Chapter 3: Effect of storage temperature and atmosphere on the survival of *Escherichia coli* O157:H7 on cut and whole leaf lettuce and spinach

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ABSTRACT

Numerous food-borne outbreaks of *Escherichia coli* O157:H7 have been linked to leafy greens in recent years. An overwhelming amount of lettuce and spinach on the market is sold in modified atmosphere packaging as ready to eat salad mixes. The objectives of this study were to determine the effects of modified atmosphere, storage temperature, and inoculum size on survival of *E. coli* O157:H7 on cut and whole leaf lettuce and spinach. *E. coli* O157:H7 H1730 was inoculated onto cut and whole leaves of leaf lettuce and spinach. Samples were held under normal atmospheric conditions or in a modified atmosphere package at either 4ºC or 10ºC to simulate display and abuse temperatures. Leaves were sampled at Days 0, 1 and every other day until noticeable spoilage occurred (7 days for lettuce, 9 days for spinach). *E. coli* O157:H7 was able to survive at 4° and 10°C regardless of atmosphere and inoculum size for 7 days on cut and whole lettuce and 9 days on cut and whole spinach. Overall, Numbers of *E. coli* O157:H7 increased (1 log) throughout the storage period on spinach, and decreased on lettuce (1-1.5 log). Significantly higher ($P \leq 0.05$) numbers of *E. coli* O157:H7 were found on lettuce and spinach stored at 10ºC than when stored at 4ºC. There were no significant differences ($P \geq 0.05$) in Numbers of *E. coli* O157:H7 with respect to atmosphere, leaf type or inoculum size. If contamination of lettuce or spinach with *E. coli* O157:H7 occurred, the pathogen may survive well under typical packaging and storage conditions.

INTRODUCTION

An increase in fresh produce consumption in recent years has coincided with an increase in the number of outbreaks caused by food-borne pathogens associated with produce (Sivapalasingam, S., Friedman, C.R., Cohen, L. and Tauxe, R.V., 2004). Since fresh-cut produce is often consumed raw and is only subjected to minimal processing, contamination with pathogens is of major concern in regards to incidences of food-borne illness (FDA, October 2004). Lettuce and spinach have been implicated as the vehicles responsible for many food-borne outbreaks and has been linked to *E. coli* O157:H7 (Beuchat, L. R., 1995, Sivapalasingam, S., Friedman, C.R., Cohen, L. and Tauxe, R.V., 2004). The infectious dose of *E. coli* O157:H7 is suspected to be very low (ranging from 1 to 100 colony-forming units) which increases the likelihood of food-borne illness if contamination with fresh produce occurs (Paton, J. C. and A. W. Paton, 1998).

Modified atmosphere packaging (MAP) is commonly used by the food industry to package lettuce and other fresh cut fruits and vegetables in order to extend their shelf life by delaying product respiration (FDA, 2001). MAP has been shown to be most effective with low storage temperature in order to prolong the shelf life of produce (Liesbeth Jacxsens, D., Frank, Van der Steen, Caroline and Debevere, Johan 29 June 2001). By increasing the shelf life of produce, pathogens which may be present, will have more time to grow as well as inhibit the growth of spoilage aerobes. Previous research has shown that *E. coli* O157:H7 can survive and/or grow in modified atmosphere packaging at low storage temperatures on produce (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993, Francis, G. A. a. O. B., D., 2001, Gunes, G. G. a. H., J.H., 2002, Hao, Y. a. B., RE, 1993). Temperature abuse during storage can further enhance the growth and survival of

E. coli O157:H7 and this pathogen has been reported to grow at 8ºC and above (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993, Francis, G. A. a. O. B., D., 2001). Since *E. coli* O157:H7 has been shown to preferentially attach to cut edges of lettuce, it may additionally attach to cut spinach as well.

The majority of fresh produce items maintain ideal quality and freshness at temperatures near 0º C, but are usually kept in retail food displays and sold to consumers at around 4ºC (FDA, 2001). *E. coli* O157:H7 is able to survive on a variety of produce commodities at low temperatures ($\leq$5ºC) (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993, Beuchat, L. R., 1995, Hao, Y. a. B., RE, 1993, Li, Y., Brackett, R.E., Chen, J. and Beuchat, L.R., 2001). Li et al. studied the influence of temperature and modified atmosphere on growth of *E. coli* O157:H7 and observed that the pathogen was able to survive at 5ºC on shredded lettuce over 18 days (Li, Y., Brackett, R.E., Chen, J. and Beuchat, L.R., 2001). In their study, PD-961 EZ film bags (OTR 7,000 cc/m^2/24 h and CO_2 21,000 cc/m^2/24 h) were used and bags were sealed under ambient conditions (Li, Y., Brackett, R.E., Chen, J. and Beuchat, L.R., 2001). Similar observations were found by Abdul-Raouf et al., which showed that numbers *E. coli* O157:H7 were detected on packaged shredded lettuce in polyolefin L-bags (OTR 3,000 cm^3/m^2/24 h and CO_2 9,800 cm^3/m^2/24 h) stored at 5ºC survived throughout a 14 day storage period (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993).

E. coli O157:H7 also preferentially attaches to cut surfaces of lettuce leaves (Seo, K. H. and J. F. Frank, 1999, Takeuchi, K. and J. F. Frank, 2000, Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001). Takeuchi and Frank observed an increased number of *E. coli* O157:H7 cells at the cut and damaged edges of lettuce compared to those at uncut

surfaces (Takeuchi, K. and J. F. Frank, 2000). These observations were also concluded by Seo and Frank as well as Takeuchi et al. under modified atmospheres at 4 and 22ºC as a result of the respiration rate of the lettuce (Seo, K. H. and J. F. Frank, 1999, Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001). Since most bagged lettuces are cut or chopped prior to packaging, this may create another advantage for *E. coli* O157:H7 to persist in bagged lettuces.

The objectives of this study were to determine the survival of low (3 log CFU/g) and high Numbers (6 log CFU/g) of *E. coli* O157:H7 on whole and fresh cut leaf lettuce and spinach surfaces when packaged under passively modified atmosphere, and stored at 4 and 10ºC until noticeable spoilage occurs.

MATERIALS AND METHODS

GFP Transformation

E. coli O157:H7 strain H1730, a clinical isolate associated with a lettuce outbreak (obtained from Dr. Larry Beuchat, University of Georgia) was stored at -80 ºC. The culture was activated through a series of three overnight transfers in tryptic soy broth (TSB; Difco, Detroit, MI) at 37ºC. Cells were streaked onto Sorbitol Mackonkey agar (SMAC; Difco, Detroit, MI) to identify *E. coli* O157:H7. Further serological confirmation of cells was completed using the RIM latex agglutination test (Remel, Lenexa, KS) to confirm the presence of the O:157 and H7 antigens.

The Clontech Plasmid (Clontech, Palo Alto, CA) PW250 (Invitrogen, Carlsbad, CA) coding for GFP expression and kanamycin resistance was inserted into

electrocompetent *E. coli* O157:H7 strain H1730 using electroporation method per manufacturers instructions (Bio-Rad GenePulser Xcell, Bio-Rad Laboratories, Hercules, CA). In the presence of isopropyl-beta-D-thiogalactoside (IPTG), the lac promoter is "turned on", inducing the expression of GFP, which fluoresces green under long wave UV light (Hereford, M., 2003).

Preparation of Inoculum

Transformed cells were frozen and maintained as stock cultures at -80 ºC in a 30% glycerol/ TSB. Prior to each experiment, the culture was activated through three overnight transfers in TSB supplemented with 100 µg/ml of kanamycin (Fisher Scientific, Fair Lawn, NJ) and 10 µg/ml of IPTG (Acros organics, NJ) at 37ºC. Cells were then streaked onto Tryptic Soy Agar (TSA; Difco, Detroit, MI) containing 100 µg/ml of kanamycin and 10 µg/ml of IPTG (TSA-K). Typical colonies were confirmed by fluorescence under long wave UV light.

The activated culture was centrifuged (4000xg, 15 min) and, the cells were washed twice and resuspended in sterile distilled water to achieve desired concentration (6 log or 3 log CFU/ml).

Two Inoculation levels of *E. coli* O157:H7 inoculum on lettuce and spinach were studied. A high inoculum (6 log CFU/ml) of *E. coli* O157:H7 was prepared as previously described; a lower inoculum (3 log CFU/ml) was prepared by serially diluting the high inoculum in 0.1% sterile peptone water.

Lettuce, Leaf inoculation procedures

Green leaf lettuce was purchased at a local supermarket in Blacksburg, VA on the day of inoculation. Lettuce was stored at 4ºC for no longer than 6 hours prior to initiation of experiments. Prior to inoculation, outer leaves of lettuce were removed, and lettuce was washed with a 100 ppm sodium hypochlorite solution. Solution used was prepared by adding 0.17 ml commercial solution of sodium hypochlorite to 1 liter of distilled water. Lettuce was then submerged in chlorine for one minute and dried in a salad spinner for approximately 2.5 minutes (Chua, D., Goh, K., Saftner, R.A. and Bhagwat, A.A. , 2008).

E. coli O157:H7 H1730 was inoculated onto cut and whole leaves of green leaf lettuce via spot inoculation methods described by Lang et. al. (Lang, M. M., Harris, L.J. and Beuchat, L.R., 2004). Single whole leaves (10g per sample) were placed in a single layer on a sterile cutting board in a laminar flow bio-safety cabinet to facilitate drying. Inoculum (50 µl) was deposited at three points onto the surface of each intact lettuce leaf (150 µl total), to yield initial *E. coli* O157:H7 concentrations of approximately 3 log CFU/g (low inoculum) or 6 log CFU/g (high inoculum). Cut and intact tissues were evaluated. To prepare cut samples, the cuticle in the middle of the leaf was disrupted by making a shallow incision with a sterile scalpel careful not to cut through the entire leaf. For cut samples, inoculum (150 µl) was deposited at the incision. After lettuce pieces were inoculated, they were held in the bio-safety cabinet to facilitate drying of the inoculum for one hour at 22 $\pm$ 2ºC.

Spinach Inoculation Procedures (Cut and Whole)

Fresh Express bagged spinach was purchased at a local supermarket in Blacksburg, VA. Spinach was stored at 4ºC for no longer than 6 hours prior to initiation of experiments.

E. coli O157:H7 H1730 was inoculated onto cut and whole leaves of spinach leaves via spot inoculation methods described by Lang et. al. (Lang, M. M., Harris, L.J. and Beuchat, L.R., 2004). Single whole spinach leaves were placed in a single layer on a sterile cutting board in a laminar flow bio-safety cabinet to facilitate drying. Inoculum (50 µl) was deposited at three points onto the surface of each leaf (150 µl total), to yield initial *E. coli* O157:H7 concentrations of 3 log CFU/g (low inoculum) or 6 log CFU/g (high inoculum). Cut and intact tissues were evaluated. In order to prepare cut samples, the cuticle in the middle of the leaf was disrupted by making a shallow incision with a sterile scalpel. For cut samples, inocula was deposited at the incision. Following inoculation, samples were held in the laminar flow hood for one hour at 22 $\pm$ 2ºC to facilitate drying

Packaging of Lettuce

Un-inoculated (control) and inoculated cut and whole leaf lettuce samples (10 grams per sample) were transferred aseptically into polyethylene PD 900 bags (O_2 transmission rate = 200 cc/100 sq. in. and a CO_2 transmission = 632 cc/100 sq. in.) (Cryovac, Duncan, SC). In order to simulate retail packaging conditions, the bags were flushed completely (100%) with Nitrogen before sealing the bags using a Koch vacuum packaging machine (Koch, San Mateo, CA, model # UV250).

Packages of lettuce were then held at 4ºC and 10ºC until noticeable spoilage occurred. Spoilage of lettuce was defined as described by Abdul-Raouf, et. al as a loss of typical color, loss of turgidity, development of sliminess and weeping of tissue fluid (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993). Each sample bag was destroyed upon analysis and a fresh, unopened bag was used for each sampling time. Bags were never resealed to continue the test. An equal number of bags were sealed under normal atmospheric gas conditions, this was done by putting 6 holes in each bag using a standard hole puncher.

Packaging of Spinach

Un-inoculated (control) and inoculated cut and spinach leaf samples were transferred aseptically into polyethylene PD 961 bags (O_2 transmission rate = 450 cc/100 sq. in. and a CO_2 transmission = 1355 cc/100 sq. in.) to achieve 10 grams in each bag (Cryovac, Duncan, SC). In order to simulate retail packaging conditions, spinach leaves were sealed in packages air using a Koch vacuum packaging machine under a 30% vacuum. During storage, the respiration of the spinach modified the atmosphere within the packaged. Packages of spinach were then held at either 4ºC or 10ºC and sampled until noticeable spoilage occurred. Spoilage of lettuce was defined as described by Abdul-Raouf, et. al as a loss of typical color, loss of turgidity, development of sliminess and weeping of tissue fluid (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993).

Each bag was destroyed at sampling time, bags were not resealed to continue the experiment. An equal number of bags were sealed under atmospheric gas conditions by altering bags with 6 perforations using a standard hole puncher.

Analysis of the gaseous atmospheres inside the packages

On each sampling day (0, 1, 3, 5, 7), headspace gas within each package was withdrawn and analyzed to determine the percentage of O_2 and CO_2 in the packages over time using an oxygen and carbon dioxide gas analyzer (PBI-Dansensor, PBI Development, Denmark, Model 58042610).

Enumeration of *E. coli* O157:H7

Samples (10g each treatment and un-inoculated control) were aseptically transferred from packaging material into a 400 ml stomacher bag with 90 ml sterile peptone water and pummeled for 3 minutes. Serial dilutions of each homogenized sample were made in 0.1% peptone water and were surface plated (0.1 ml per plate) in duplicate onto TSA–K agar and incubated at 37ºC for 24 hours. Typical GFP-expressing *E. coli* O157:H7 colonies were identified by fluorescence under long wave UV light. Random colonies were selected for further confirmatory testing by streaking cells onto Sorbitol Mackonkey Agar (SMAC) and using the RIM latex agglutination test for confirmation of O:157 and H7 antigens.

Statistical Analysis:

All experiments were repeated three times. Data were analyzed using the Statistical Analysis System (SAS Institute, Cary, NC) for analysis of variance. Significant

differences between means were determined by using Duncan's multiple range tests. A significance level of 0.05 was used for all analyses.

Results and Discussion

The ability of high and low levels of *E. coli* O157:H7 to survive on cut and whole green leaf lettuce and spinach stored at 4 and 10ºC under passively modified atmospheric conditions for 7 days (lettuce) and 9 days (spinach) of storage was investigated. *E. coli* O157:H7 was not detected in un-inoculated control samples of lettuce and spinach. Researchers took care to re-create conditions similar to those used in industry practices over the course of this study. *E. coli* O157:H7 was not detected in un-inoculated control samples of packaged lettuce and spinach.

The concentrations of oxygen (O_2) and carbon dioxide (CO_2) achieved within the packages are shown in Tables 3.1 and 3.2. Lettuce was sealed under modified atmosphere with initial concentrations of O_2 and CO_2, between 0.5% - 0.7% (Table 3.1). On each sampling day (0, 1, 3, 5, 7), gases within each packaged were analyzed. The packaged lettuce inoculated with the high inoculum showed that the levels of oxygen increased to 12% and the carbon dioxide levels increased to 0.9% during the 7 day storage period. The concentrations of gases in packaged lettuce when inoculated at low levels achieved an atmosphere of 14 % O_2 and 0.5% CO_2 during the storage period of 7 days. The O_2 and CO_2 concentration was not significantly affected by storage temperature.

Spinach leaves were sealed in bags to achieve initial concentrations of O_2 between 14% - 16% and CO_2 of 0.6% - 1.3% (Table 3.2). On each sampling day (0, 1, 3, 5, 7, 9), gases within each package were analyzed. The concentrations of oxygen (O_2) and carbon dioxide (CO_2) achieved within the packages are shown in Table 3.2. The packaged spinach inoculated with the high inoculum showed that the levels of oxygen increased to 15.9% and the carbon dioxide levels increased to 1.4% during the 9 day storage period. The concentrations of gases in packaged spinach when inoculated at low levels achieved an atmosphere of 16.1 % O_2 and 1.2% CO_2 during the storage period of 9 days. The difference in gas concentration between the two temperatures was not significant.

Effect of modified atmospheric packaging on survival of *E. coli* O157:H7

The modified atmosphere packaging did not significantly affect the survival of *E. coli* O157:H7. Lettuce stored under modified atmospheric conditions remained acceptable for consumption as long as lettuce packaged under atmospheric conditions. Regardless of temperature, leaf type and inoculum size, Numbers of *E. coli* O157:H7 consistently declined from throughout the 7 day storage period on lettuce packaged under modified and normal atmospheric conditions.

Within each inoculum size, temperature and leaf type there was no significant difference in bacterial counts of *E. coli* O157:H7 on bagged lettuce stored under modified atmospheric conditions than those stored under normal atmospheric conditions ($P \geq 0.05$) (Figures 3.1 and 3.2). This observation suggests that the gas composition inside the bags did not directly effect the survival or reduction of *E. coli* O157:H7 on the lettuce.

E. coli O157:H7 decreased significantly by day 1 with a total reduction of 2 log CFU/g by day 7 on lettuce stored under modified atmospheric conditions at 4ºC when inoculated at both low and high levels (Fig. 3.1). Under normal atmospheric conditions an overall reduction in numbers of *E. coli* O157:H7 was significant by day 1 with a total reduction of 1.5 logs observed by day 7.

Numbers of *E. coli* O157:H7 slightly increased throughout the 9 day storage period on spinach packaged in both modified and atmospheric conditions. Within each inoculum, temperature and leaf type there was no significant difference in bacterial counts of *E. coli* O157:H7 on bagged spinach stored under modified atmospheric conditions than those stored under normal atmospheric conditions ($P \geq 0.05$). This observation suggests that the gas composition inside the bags did not directly effect the survival of *E. coli* O157:H7. Spinach stored under modified atmospheric conditions remained acceptable for consumption as long as spinach packaged under normal atmospheric conditions.

E. coli O157:H7 increased 0.5 log CFU/g within 9 days on cut spinach leaves stored under modified atmospheric conditions at 4ºC when inoculated at both low and high levels (Fig. 3.3). There was no change, however, at 4ºC on cut spinach inoculated at low and high levels under normal atmospheric conditions. A significant increase ($P \leq .05$) of 0.5 logs was observed on cut spinach inoculated at low levels at 10ºC under both modified and typical atmospheric conditions throughout the 9 day storage period (Figure 2). At 10ºC when inoculated at high levels a significant increase ($P \leq .05$) of approximately 1 log CFU/g was observed on cut spinach at both modified and atmospheric atmospheres. Whole spinach packaged under both modified and normal

atmospheric conditions did not have any effect on the number of *E. coli* O157:H7 cells when inoculated at both high and low inoculum levels at 4 and 10ºC over 9 days.

Other studies have shown significant changes in microbial Numbers on fresh produce under modified atmospheres (Gunes, G. G. a. H., J.H., 2002, Hao, Y. a. B., RE, 1993). Gunes et. al studied survival of *E. coli* O157:H7 on fresh cut apples in modified atmosphere packaging and found that at a storage of 21% O_2 and 30% CO_2 at 20ºC significantly inhibited growth of *E. coli* O157:H7 (Gunes, G. G. a. H., J.H., 2002). However, inhibition was most likely due to the high concentration of CO_2 present. CO_2 at levels as low as 10% can be bactericidal (M.A Del Nobile, B., A., Benedetto, E. and Massignan, L. , 2005).

In this study, gas atmospheres within packages that were modified had no significant effect to the growth or survival of *E. coli* O157:H7. Similar observations have been reported that indicate that modified atmospheres have little to no effect on the survival of *E. coli* O157:H7 (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993). Abdul-Raouf et al. concluded that there was no significant effect on numbers of *E. coli* O157:H7 under modified atmospheres containing 3% O_2 and 97%N_2 stored at 5 and 12ºC (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993). The concentration of CO_2 in their study was not determined.

Survival on cut vs. whole leaf

There were no significant differences found ($P \geq 0.05$) between numbers of *E. coli* O157:H7 on cut and whole (intact) leaves irrespective of storage temperature, inoculum size, or atmosphere within packages of leaf lettuce. These results suggest that under these conditions, leaf type (cut or whole) may have little effect on the survival of *E.*

coli O157:H7. There was, however, a significant difference in *E. coli* O157:H7 numbers between cut and whole leaf lettuce on day 7 when inoculated at low levels at a storage temperature of 4ºC irrespective of atmospheric conditions. This may have occurred for several reasons, one possibility being competition with indigenous micro flora in the cut lettuce resulting in fewer numbers of *E. coli* O157:H7.

There were no significant differences found ($P \geq 0.05$) between cut and whole (intact) leaves on numbers of *E. coli* O157:H7 irrespective of storage temperature, inoculum size, or atmosphere within packages of spinach leaves. These results suggest that leaf type (cut or whole) has no effect on the survival of *E. coli* O157:H7.

There are no studies that have explored the survival of *E. coli* O157:H7 on cut versus whole spinach leaves. Studies have been conducted on attachment to various lettuce surfaces. Greater numbers of *E. coli* O157:H7 attach to cut edges of lettuce than to undamaged whole leaves (Seo, K. H. and J. F. Frank, 1999, Takeuchi, K. and J. F. Frank, 2000, Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001, Takeuchi, K., Matute, C.M., Hassan, A.N. and Frank, J.F., 2000). Takeuchi and Frank discovered that penetration of *E. coli* O157:H7 cells was greater into the cut edges than uncut surfaces of lettuce compared to higher temperatures (Takeuchi, K. and J. F. Frank, 2000). In another study, Takeuchi et. al discovered an increased penetration of cells at cut edges at 21% O_2 at low temperatures (4ºC) (Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001). They also found that attachment and penetration were reduced at 2.7% O_2 as opposed to 21% O_2 (Takeuchi, K., Hassan, A.N. and Frank, J.F., 2001). They speculated that modifying the O_2 content may affect the degree of penetration of *E. coli* O157:H7 into lettuce leaves. This may explain why results from the current study do not follow previous literature on

the effect of cut and damaged lettuce and spinach leaves on numbers of *E. coli* O157:H7. In the current study, O_2 concentrations did not exceed 17% in any given bag of inoculated spinach and 14% in any give bag of inoculated lettuce. These results suggest that survival of *E. coli* O157:H7 on cut and whole leaf lettuce and spinach may be affected by O_2 concentrations within modified atmospheric packaging.

Effect of inoculum level on survival of *E. coli* O157:H7

Survival of high and low levels of *E. coli* O157:H7 at 4 and 10ºC on packaged lettuce during storage is shown in Figures 3.1 and 3.2, respectively. Inoculum size did not have significant effect on the survival of *E. coli* O157:H7. The high inoculum (~6 log CFU/ml) had a 1.5 log reduction in numbers of *E. coli* O157:H7 at 4ºC after 7 days while the low inoculum (~3 log CFU/ml) had a 2 log reduction after 7 days. These observations were observed consistent among both cut and whole leaf type. No significant differences were found between leaf type or atmosphere ($P \geq .05$).

A reduction in numbers of *E. coli* O157:H7 by approximately 1 log CFU/g was also observed at 10ºC on packaged lettuce when inoculated at high levels within each type of atmosphere (Fig. 2a). When cut lettuce, inoculated at low levels, was observed at 10ºC during the 7 day storage period numbers of *E. coli* O157:H7 decreased by approximately 2 log CFU/g (Fig. 2b). For packaged whole leaves inoculated at low levels, at both atmospheres, *E. coli* O157:H7 on lettuce did not change significantly throughout the 7 day storage period (Fig. 3.2b). Takeuchi and Frank found that at lower inoculum levels of *E. coli* O157:H7 more readily attached to cut and damaged edges of lettuce leaves (Takeuchi, K. and J. F. Frank, 2000). The results of their study indicated

that *E. coli* O157:H7 attaches to less favorable attachment sites once the preferred initial attachment sites are occupied (Takeuchi, K. and J. F. Frank, 2000).

Survival of *E. coli* O157:H7 at 4 and 10ºC on packaged spinach during storage is shown in Figures 3.3 and 3.4, respectively. Inoculum size had no significant effect on the survival of *E. coli* O157:H7. Both the high (~ 6 log CFU/ml) and low inoculum (~ 3 log CFU/ml) had a 0.5 log increase in numbers of *E. coli* O157:H7 at 4ºC after 9 days under modified atmospheric conditions. This was observed with both cut and whole leaf type. No significant differences were found between leaf type or atmosphere ($P \geq 0.05$). There was no significant change in *E. coli* O157:H7 numbers when packaged under normal atmospheric conditions at 4ºC over the 9 day storage period irrespective of leaf type or size of inoculum.

At 10ºC, a 1 log increase in numbers of *E. coli* O157:H7 was observed on packaged cut spinach when inoculated at high levels within each type of atmosphere (Fig. 3.4a). An increase of 0.5 log CFU/g in number of *E. coli* O157:H7 was observed at 10ºC when both cut and whole leaf spinach was inoculated at low levels (Fig. 3.4b). For packaged whole leaves inoculated at low levels, under normal atmospheric conditions, *E. coli* O157:H7 on spinach did not change significantly throughout the 9 day storage period (Fig. 2b). These results are in agreement with previous research. Abdul-Raouf et. al demonstrated that at both high (5 log) and low (2 log) inoculum levels there was a significant increase in numbers of *E. coli* O157:H7 on shredded lettuce within 7 and 3 days of storage at 12 and 21ºC under modified atmospheric conditions (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993).

Effect of storage temperature on survival of *E. coli* O157:H7

Bacterial counts were significantly affected by the temperature at which the bags were held ($P \leq 0.05$). Greater numbers of *E. coli* O157:H7 survived at a storage temperature of 10 ºC than at 4ºC within each inoculum, atmosphere, and leaf type for both lettuce and spinach (Figures 3.1-3.4).

When inoculated at high and low levels, both cut and whole lettuce leaves store at 4ºC showed the most pronounced reduction in *E. coli* O157:H7 numbers when compared to those stored at 10ºC. Similar findings have been described for lettuce stored at less than 5ºC (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993, Delaquis, S., Stewart, S., Cazaux, S. and Toivonen, P., 2002, Francis, G. A. a. O. B., D., 2001). Abdul-Raouf et al. found that numbers of *E. coli* O157:H7 stored at 5ºC significantly decreased during a 14 day storage period (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993). In this study, growth of *E. coli* O157:H7 was not observed on packaged lettuce stored at 10ºC, irrespective of atmosphere, leaf type or inoculum size. Instead, numbers *E. coli* O157:H7 cells actually decreased, although at a smaller rate than at a storage temperature of 4ºC. This decline in growth at 10ºC was unexpected, but could have occurred for a variety of reasons. Lettuce may have been unsuitable for growth due to competition from the indigenous microbial populations. This study, did not analyzed background micro flora, however, many studies have shown that background micro flora may affect the growth of *E. coli* O157:H7 (Hao, Y. a. B., RE, 1993).

At 4ºC there was no significant change in numbers of *E. coli* O157:H7 on spinach throughout the 9 day storage temperature at both inoculum levels irrespective of

atmospheric conditions or leaf type. Similar findings have been described for lettuce stored at less than 5ºC (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993, Delaquis, S., Stewart, S., Cazaux, S. and Toivonen, P., 2002, Francis, G. A. a. O. B., D., 2001). Growth of *E. coli* O157:H7 on lettuce has been demonstrated at a storage temperature of 12ºC (Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S., 1993, Francis, G. A. a. O. B., D., 2001). In this study, modest growth of *E. coli* O157:H7 was observed on packaged spinach stored at 10ºC, irrespective of atmosphere, leaf type or inoculum size.

Effect of storage time on survival of *E. coli* O157:H7

Storage time resulted in significantly different numbers of *E. coli* O157:H7. Plate count results indicated that the *E. coli* O157:H7 numbers did not change significantly ($P \geq 0.05$) on lettuce held at 4 and 10ºC between days 1 and 5, but were significantly different on Day 0 and Day 7.

Overall, numbers of *E. coli* O157:H7 declined throughout the 7 day storage period regardless of atmosphere, leaf type, inoculum size, or temperature. This reduction in numbers of *E. coli* O157:H7 may have occurred due to many different factors. In this study, cut and whole lettuce leaves were inoculated via the spot inoculation method as described by Lang et. al (Lang, M. M., Harris, L.J. and Beuchat, L.R., 2004) . Their study demonstrated that method of inoculation may have an affect on growth and survival of *E. coli* O157:H7 (Lang, M. M., Harris, L.J. and Beuchat, L.R., 2004) . Lang et. al. found that inoculation method can effect the growth and survival of *E. coli* O157:H7 (Lang, M. M., Harris, L.J. and Beuchat, L.R., 2004). Higher numbers of *E. coli* O157:H7

were recovered from dip-inoculated lettuce than from spot-inoculated lettuce (Lang, M. M., Harris, L.J. and Beuchat, L.R., 2004). It was concluded that the spot-inoculation method minimizes the distribution of microbial cells thereby resulting in fewer numbers of attached *E. coli* O157:H7 on the cut edges of the plant (Lang, M. M., Harris, L.J. and Beuchat, L.R., 2004). The results obtained by Lang et. al are similar to the results found in the current study and may explain why *E. coli* O157:H7 numbers decreased on lettuce at storage temperatures of both 4 and 10ºC over for a period of 7 days.

Storage time also resulted in significantly different numbers of *E. coli* O157:H7 on spinach. Plate count results indicated that the *E. coli* O157:H7 numbers did not change significantly ($P \geq 0.05$) on whole spinach held at 4ºC under modified atmospheric conditions inoculated at both high and low levels. At 10ºC cut and whole leaf spinach moderately increased when packaged at both high and low inoculums irrespective of atmospheric conditions. When cut spinach was inoculated at high levels at a storage temperature of 10ºC there was an overall increase of 1 log CFU/g in the 9 day storage period at both normal and modified atmospheric conditions.

Overall, numbers of *E. coli* O157:H7 increased slightly throughout the 9 day storage period regardless of atmosphere, leaf type, inoculum size, or temperature on spinach. This insignificant change in numbers of *E. coli* O157:H7 may have occurred due to many different factors. In this study, cut and whole spinach leaves were inoculated via the spot inoculation method as described by Lang et. al (Lang, M. M., Harris, L.J. and Beuchat, L.R., 2004) . The results obtained by Lang et. al are similar to the results found in the current study and may explain why *E. coli* O157:H7 numbers did not greatly

increase on spinach at storage temperatures of both 4 and 10ºC over for a period of 9 days.

E. coli O157:H7 was able to survive on cut and whole leaf lettuce and spinach leaves at storage temperatures of 4 and 10ºC under modified atmospheric conditions without causing visual defects in the packaged produce. Results from this study indicate that spinach was visually acceptable for up to 9 days under modified as well as atmospheric conditions while containing high numbers of *E. coli* O157:H7. While the pathogen did not significantly grow on spinach, it was able to survive throughout the 9 day storage period. *E. coli* O157:H7 grew to higher numbers on spinach stored at 10ºC compared to the product at 4ºC. Numbers of *E. coli* O157:H7 in spinach stored at 4ºC did not change significantly over time. The pathogen was, however, able to survive on bagged spinach at both 4 and 10ºC. Results from this study indicate that lettuce was visually acceptable for up to 7 days under modified atmospheric and normal atmospheric conditions while containing high numbers of *E. coli* O157:H7. While the pathogen did not grow on lettuce, it was able to survive throughout the 7 day storage period. These results show that temperature control during storage of spinach and lettuce is necessary to restrict the growth and survival of *E. coli* O157:H7.

References

Abdul-Raouf, U. M., Beuchat, L.R. and Ammar, M.S. 1993. Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Applied and Environmental Microbiology*. 59:1999-2006.

Beuchat, L. R. 1995. Pathogenic Microorganisms Associated with Fresh Produce. *Journal of Food Protection*. 59:204-216.

Chua, D., Goh, K., Saftner, R.A. and Bhagwat, A.A. . 2008. Fresh-cut lettuce in modified atmosphere packages stored at improper temperatures supports enterohemorrhagic *E. coli* Isolates to survive gastric acid challenge. *Journal of Food Science*. 00:M1-M6.

Delaquis, S., Stewart, S., Cazaux, S. and Toivonen, P. 2002. Survival and growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in ready-to-eat iceberg lettuce washed in warm chlorinated water. *Journal of Food Protection*. 65:459-64.

FDA. 2001. Microbiological Safety of Controlled and Modified Atmosphere Packaging of Fresh and Fresh-Cut Produce. *In* C.f.F.S.a.A. Nutrition (ed.).

FDA. Date, 2001, Outbreaks Associated with Fresh and Fresh-Cut Produce. Incidence, Growth, and Survival of Pathogens in Fresh and Fresh-Cut Produce. Available at: http://www.cfsan.fda.gov/~comm/ift3-4a.html. Accessed, 2007.

FDA. Date, October 2004, From Production to Consumption: 2004 Action Plan to Minimize Foodborne Illness Associated with Fresh Produce Consumption. Available at: http://www.cfsan.fda.gov/~dms/prodpla2.html. Accessed, 2007.

Francis, G. A. a. O. B., D. 2001. Effects of vegetable type, package atmosphere and storage temperature on growth and survival of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Journal of Industrial Microbiology and Biotechnology*. 27:111-6.

Gunes, G. G. a. H., J.H. 2002. Growth and survival of *Escherichia coli* O157:H7 on fresh-cut apples in modified atmospheres at abusive temperatures. *Journal of Food Protection*. 65:1641-5.

Hao, Y. a. B., RE. 1993. Growth of Escherichia coli O157:H7 in modified atmosphere. *Journal of Food Protection*. 56:330.

Hereford, M. 2003. Effects of Apple Development and Damage on the Internalization of *Escherichia coli* O157:H7 as Observed Under Field and Laboratory Conditions. p. 48. *In*, Food Science and Technology, vol. Master of Science. Virginia Tech, Blacksburg.

Lang, M. M., Harris, L.J. and Beuchat, L.R. 2004. Survival and recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on lettuce and parsley as affected by method of inoculation, time between inoculation and analysis, and treatment with chlorinated water. *Journal of Food Protection*. 67:1092-103.

Li, Y., Brackett, R.E., Chen, J. and Beuchat, L.R. 2001. Survival and growth of *Escherichia coli* O157:H7 inoculated onto cut lettuce before or after heating in chlorinated water, followed by storage at 5 or 15 degrees C. *Journal of Food Protection*. 64:305-9.

Liesbeth Jacxsens, D., Frank, Van der Steen, Caroline and Debevere, Johan 29 June 2001. Effect of high oxygen modified atmosphere packaging on microbial growth and sensorial qualities of fresh-cut produce. *International Journal of Food Microbiology*. 71:197-210.

M.A Del Nobile, B., A., Benedetto, E. and Massignan, L. . 2005. Respiration rate of minimall processed lettuce as affected by packaging
Journal of Food Engineering. 74:60-69.

Paton, J. C., and A. W. Paton. 1998. Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infections. . *Clinical Microbiology Reviews*. 11:450-479.

Seo, K. H., and J. F. Frank. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *Journal of Food Protection*. 62:3-9.

Sivapalasingam, S., Friedman, C.R., Cohen, L. and Tauxe, R.V. 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection*. 67:2342-53.

Takeuchi, K., and J. F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. *Journal of Food Protection*. 63:434-40.

Takeuchi, K., Hassan, A.N. and Frank, J.F. 2001. Penetration of *Escherichia coli* O157:H7 into lettuce as influenced by modified atmosphere and temperature. *Journal of Food Protection*. 64:1820-3.

Takeuchi, K., Matute, C.M., Hassan, A.N. and Frank, J.F. 2000. Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Pseudomonas fluorescens* to lettuce leaves. *Journal of Food Protection*. 63:1433-7.

Table 3.1. Headspace O_2 and CO_2 levels in packaged cut and whole leaf lettuce* stored at 4ºC or 10ºC for 7 days initially flushed with 100% nitrogen when inoculated with high (6 log CFU/ml) and low (3 log CFU/ml) levels of *E. coli* O157:H7

Inoculum Level	Storage Temperature (ºC)	Day	$\%O_2$	$\%CO_2$
High	4	0	0.77	0.73
		1	6.50	0.53
		3	8.70	0.77
		5	10.5	0.73
		7	12.4	0.83
	10	0	0.77	0.73
		1	4.80	0.60
		3	9.90	0.60
		5	10.9	0.70
		7	12.0	0.90
Low	4	0	0.46	0.50
		1	4.50	0.60
		3	8.40	0.60
		5	11.2	0.40
		7	13.7	0.50
	10	0	0.46	0.50
		1	4.80	0.60
		3	9.60	0.60
		5	11.8	0.40
		7	14.5	0.40

* No significant difference gases was found between leaf type (cut and whole)

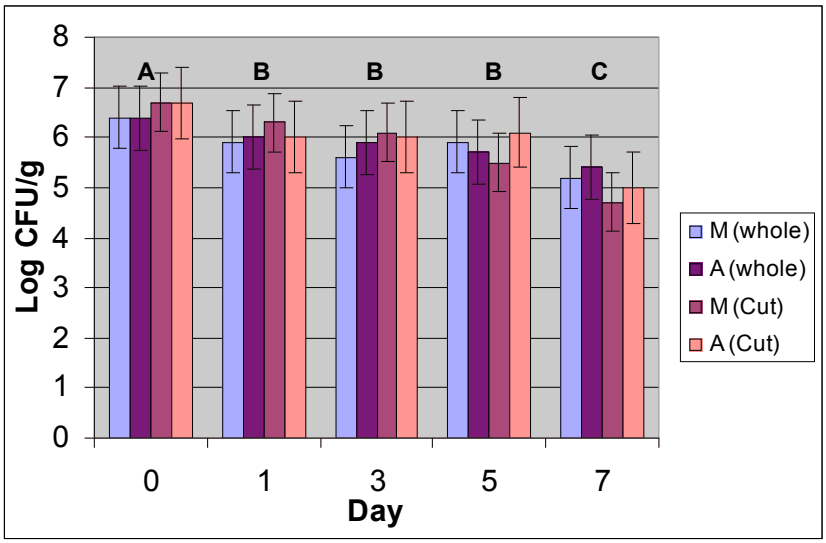
Table 3.2. Headspace O_2 and CO_2 levels in packaged cut and whole spinach leaves* stored at 4ºC or 10ºC for 9 days when inoculated with high (6 log CFU/ml) and low (3 log CFU/ml) levels of *E. coli* O157:H7.

Inoculum Level	Storage Temperature (ºC)	Day	%O2	%CO2
High	4	0	16.0	0.6
		1	16.6	0.6
		3	16.4	1.2
		5	16.3	1.1
		7	16.2	1.1
		9	15.9	1.4
	10	0	16.0	0.6
		1	16.7	1.5
		3	16.4	1.4
		5	16.0	1.3
		7	15.4	1.3
		9	15.7	1.3
Low	4	0	14.8	1.3
		1	15.9	1.6
		3	16.4	1.2
		5	16.1	1.0
		7	16.5	0.9
		9	16.1	1.2
	10	0	14.8	1.3
		1	16.3	1.3
		3	16.1	1.3
		5	15.4	1.3
		7	15.5	1.3
		9	16.0	1.1

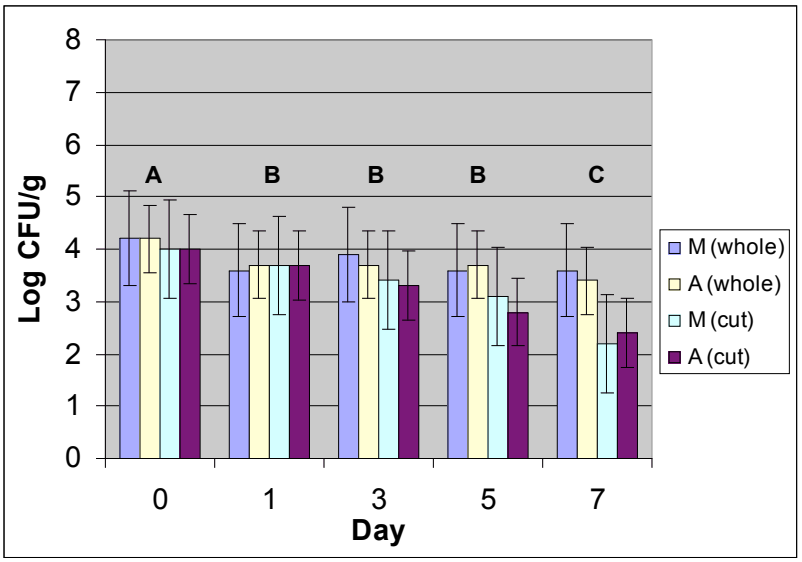
* No significant difference in gases was found between leaf type (cut and whole)

Figure 3.1 Survival of *E. coli* O157:H7 on cut and whole leaf lettuce stored under modified* (M) and atmospheric (A) conditions at 4 ºC over 7 days when inoculated at high levels (a) and low levels (b). Mean values that are not preceded by the same letter are significantly different (α = .05). N = 3 (batch size replication)

a) High Inoculum



b) Low Inoculum

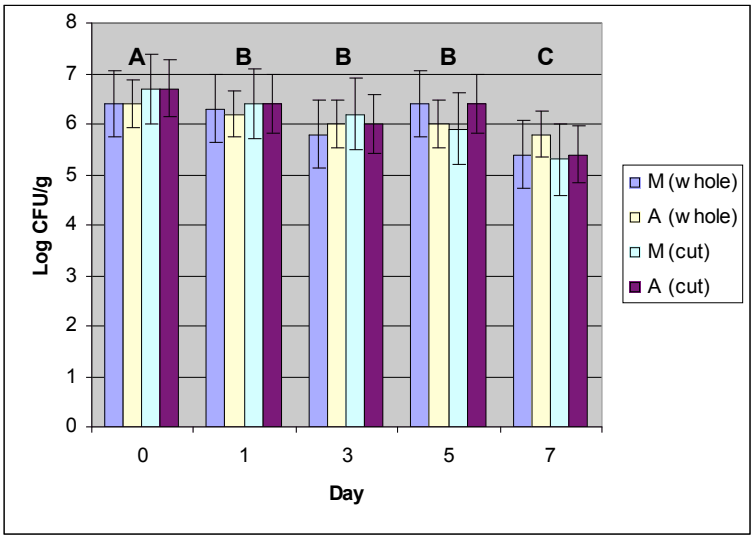


Different letters on different days, indicate significant difference in overall Number of *E. coli* O157:H7 irrespective of atmosphere or inoculation site.

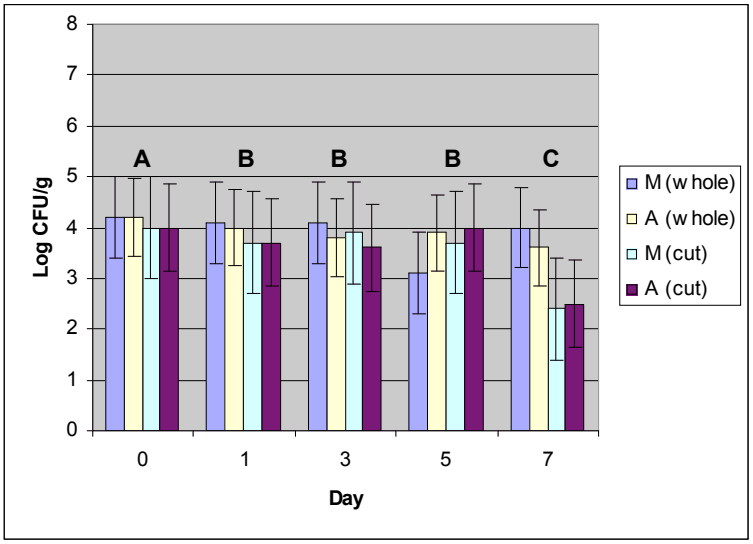
* Atmospheres within bags were modified by flushing PD 900 bags (OTR of 200 cc/100 sq. in. and CO_2 of 632 cc/sq. in.) with 100% nitrogen.

Figure 3.2 Survival of *E. coli* O157:H7 on cut and whole leaf lettuce stored under modified* (M) and atmospheric (A) conditions at 10 ºC over 7 days when inoculated at high levels (a) and low levels (b). Reported Mean values that are not preceded by the same letter are significantly different ($\alpha = .05$). N = 3 (batch size replication)

a) High Inoculum



b) Low Inoculum

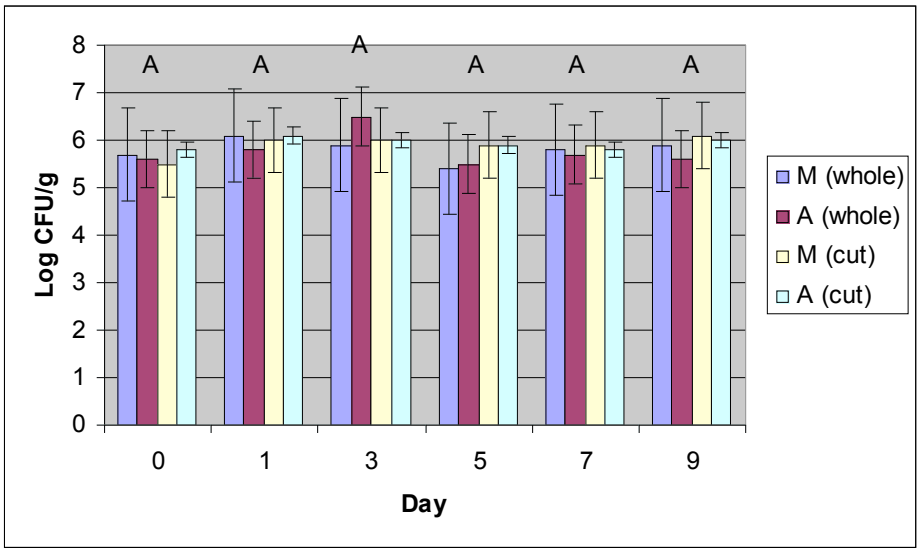


Different letters on different days, indicate significant difference in overall number of *E. coli* O157:H7 irrespective of atmosphere or inoculation site.

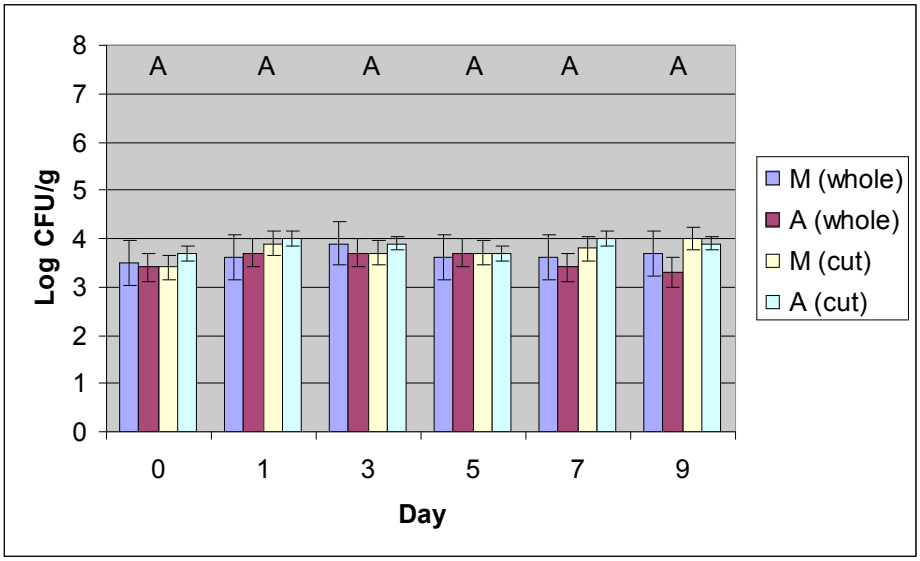
* Atmospheres within bags were modified by flushing PD 900 bags (OTR of 200 cc/100 sq. in. and CO_2 of 632 cc/sq. in.) with 100% nitrogen.

Figure 3.3. Survival of *E. coli* O157:H7 on cut and spinach leaves stored under modified* (M) and atmospheric (A) conditions at 4 ºC over 9 days when inoculated at high levels (a) and low levels (b). Mean values with the same letter are not significantly different ($\alpha = .05$). N = 3 (batch size replication)

a) High Inoculum



b) Low Inoculum

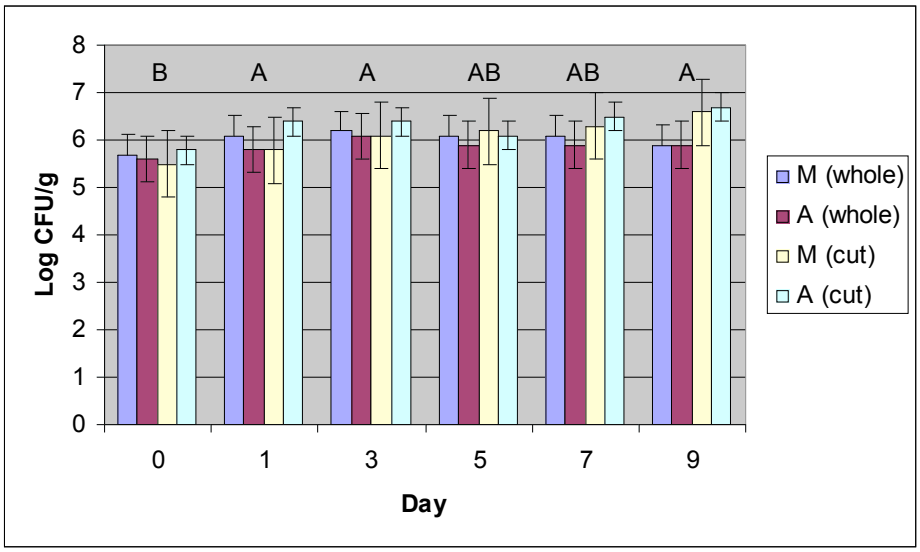


Different letters on different days, indicate significant difference in overall number of *E. coli* O157:H7 irrespective of atmosphere or inoculation site.

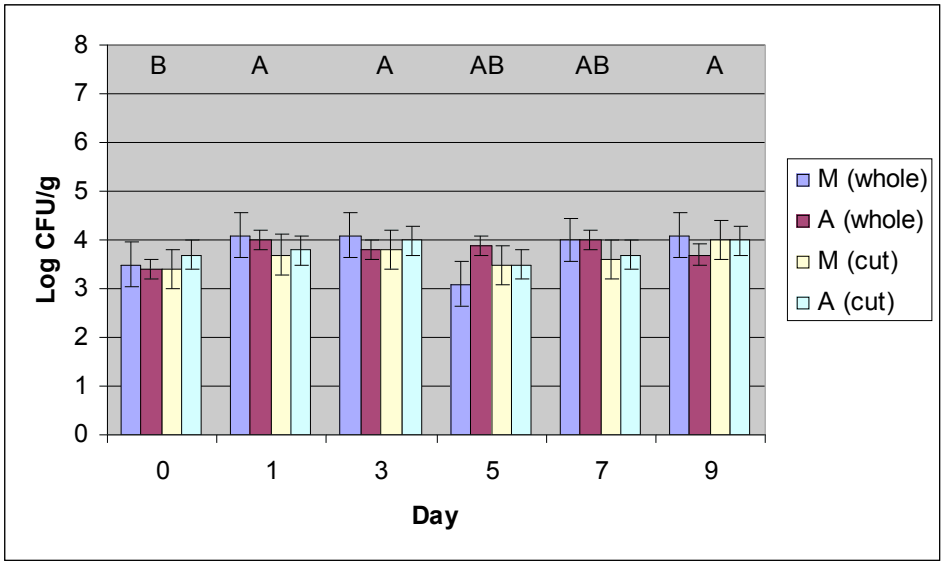
* Atmospheres within bags were modified by heat sealing PD 961 bags (OTR of 450 cc/100 sq. in. and CO_2 of 1355 cc/sq. in.).

Figure 3.4. Survival of *E. coli* O157:H7 on cut and whole spinach leaves stored under modified * (M) and atmospheric (A) conditions at 10 ºC over 9 days when inoculated at high levels (a) and low levels (b). Reported Mean values with the same letter are not significantly different ($\alpha = .05$). N = 3 (batch size replication)

a) High Inoculum



b) Low Inoculum



Different letters on different days, indicate significant difference in overall number of *E. coli* O157:H7 irrespective of atmosphere or inoculation site.

* Atmospheres within bags were modified by heat sealing PD 961 bags (OTR of 450 cc/100 sq. in. and CO_2 of 1355 cc/sq. in.).

Chapter 4: Conclusion

General Conclusions:

This work addresses issues involving the safety of fresh produce packaged in modified atmosphere packaging (MAP) and stored under typical retail refrigeration (4°C) and abusive temperatures (10°C). Specifically, this work addressed the potential for survival and/or growth of high (6 log CFU/ml) and low (3 log CFU/ml) levels of *Escherichia coli* O157:H7 on whole and fresh cut lettuce and spinach stored under modified and atmospheric conditions. Overall, survival of *E. coli* O157:H7 was dependant on produce type and storage temperature. Bagged lettuce was stored for 7 days, while bagged spinach lasted for 9 days until visual spoilage occurred in each respective product. *E .coli* O157:H7 generally survived better on cut and whole leaf lettuce (5.4 log CFU/g for both) and spinach (6.7 log CFU/g, 5.9 log CFU/g) when stored at 10ºC than at 4ºC (5 log CFU/g, 5.4 log CFU/g) for lettuce and 6 log CFU/g, 5.6 CFU/g for spinach) respectively, regardless of atmospheric conditions or inoculum size.

Storage at 4ºC allowed survival of *E. coli* O157:H7 on both lettuce and spinach throughout the respective storage periods. This study demonstrated the importance of strict temperature control during storage. It is essential that produce is maintained at refrigerated temperatures (4°C) throughout storage as well as throughout the transportation and distribution chain.

The potential for MAP packaging to encourage the growth of facultatively anaerobic pathogens such as *E. coli* O157:H7 has been questioned. While MAP has shown to extend the shelf life of various produce commodities, certain gas concentrations may have an adverse effect on leafy greens and allow survival of *E. coli* O157:H7. When

the concentration of oxygen falls below 10% respiration begins to slow. By maintaining produce in an atmosphere with less than 10% oxygen, the product's shelf life can be extended. However, a lower oxygen concentration can also encourage growth of facultative anaerobes, which will have more time to grow with an extended shelf life due to MAP of produce.

The results obtained from this study suggest that *E. coli* O157:H7 is able to survive, but not grow in MAP at low storage temperatures. Overall, E. coli O157:H7 significantly decreased throughout the 7 day storage period on leaf lettuce, whereas, the pathogen significantly increased on spinach at abusive temperatures during the 9 day storage period.

At each sampling interval, bags of lettuce and spinach were analyzed for oxygen and carbon dioxide concentrations. The modified atmospheric conditions inside packages reached levels of 16% O_2 and 1.4% CO_2 in spinach and 12-14% O_2 and 0.9% CO_2 in packaged lettuce, which follow industry specifications. These gas concentrations within MAP leaf lettuce and spinach did not prove to have an affect on survival of *E. coli* O157:H7.

Limitations and Pitfalls:

The researchers consulted with Cryovac throughout the experiment to ensure that care was taken to adequately follow industry specifications and practices during the packaging of the lettuce and spinach. Ideally, for a research project like this, the spinach and lettuce would be freshly harvested from the field, washed according to industry standards and then packaged by out lab. In the case of this research, that was not possible. However, there may have been discrepancies associated with the freshness of

the products that we used since they were already on the store shelf. The time that fresh produce requires for transportation and distribution before it reaches retail markets is not clearly known but could be as long as a week. The spoilage of the product may have occurred earlier than expected. Analysis of total microbial load on lettuce and spinach was not done in this experiment, however, it would be useful to understand the relationship between *E. coli* O157:H7 and microorganisms naturally present on leafy greens.

Future Research:

This study solely investigated the affect of modified atmospheric conditions and storage temperature on the survival of *E. coli* O157:H7 on cut and whole leaf lettuce and spinach. As previously discussed within the limitations of this project, it is necessary for the researcher to grow leafy greens to ensure freshness of the product in order to overcome these obstacles. There may be other factors involved in the growth and survival of *E. coli* O157:H7 on fresh produce. One such variable includes the indigenous background microbial population present on the product. When produce begins to spoil, cellular fluid may leak from the leafy greens, which may enhance growth of *E. coli* O157:H7, since this fluid contains high amounts of nutrients that can be utilized by the pathogen. Temperature may also have an effect on the number of microorganisms naturally present on these produce commodities. Future work is needed to determine the effect of competition between *E. coli* O157:H7 and background micro flora on spinach and lettuce.

Appendix 1: Use of GFP fluorescence assay to quantify *E. coli* O157:H7 numbers from lettuce samples

Methods:

Methods were followed as described in chapter 2 with the following exceptions:

After samples (10g per bag) were placed into 400 ml stomacher bags with 100-ml sterile 0.1% peptone water and pummeled for 4 minutes, homogenates were stored in 1 ml aliquots in cryogenic vials. The vials containing lettuce homogenates were then stored at -80ºC for no longer than 7 days before sampling. Duplicate samples were then analyzed on a Victor 3V multilabel plate reader (Perkin-Elmer, Turku, Finland)using a 405 nm filter in order to detect *E. coli* O157:H7 H1730 transformed with GFP. A standard curve was generated with known concentrations of *E. coli* O157:H7 and the correlated fluorescence readings from lettuce homogenates were taken using the micro plate reader. This was compared to conventional plating techniques shown in chapter 2 Figures 2.1 and 2.2.

Conclusion: As shown by the low R^2 values for each inoculum size and temperature combination, detection of *E. coli* O157:H7 using a fluorescent micro plate reader is not a suitable comparison to convention plating techniques. Sporadic data points were most likely due to the fact that after homogenates were stored in vials, these samples were frozen for up to one week. This may have caused the *E. coli* O157:H7 cells to lose the GFP plasmid, therefore, no fluorescence would be detected in samples.

Figure AI.1. Standard curve with known concentrations of *E. coli* O157:H7 and the correlated fluorescence readings from the plate reader. N=3

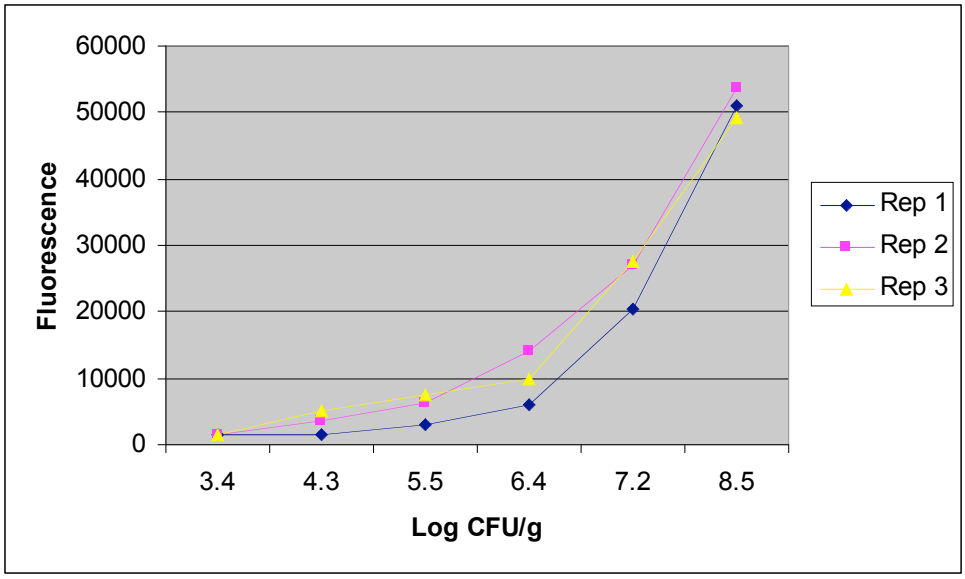
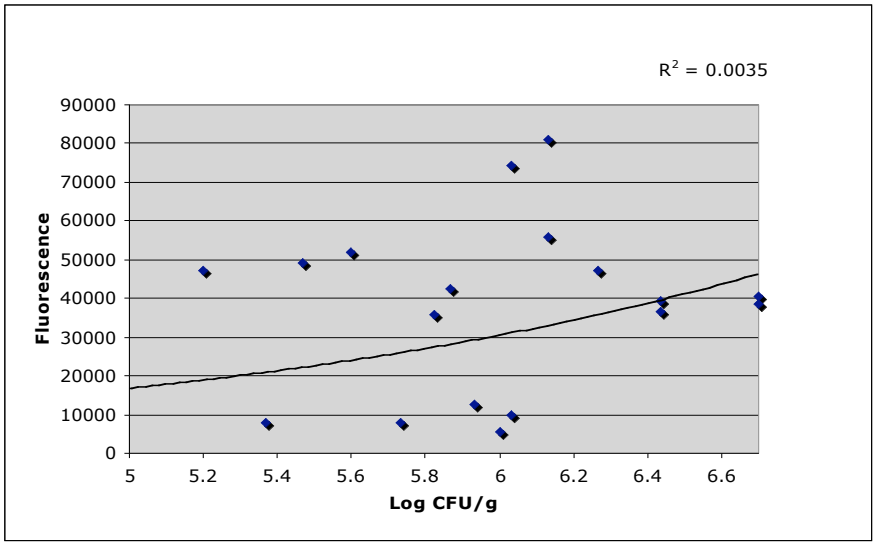


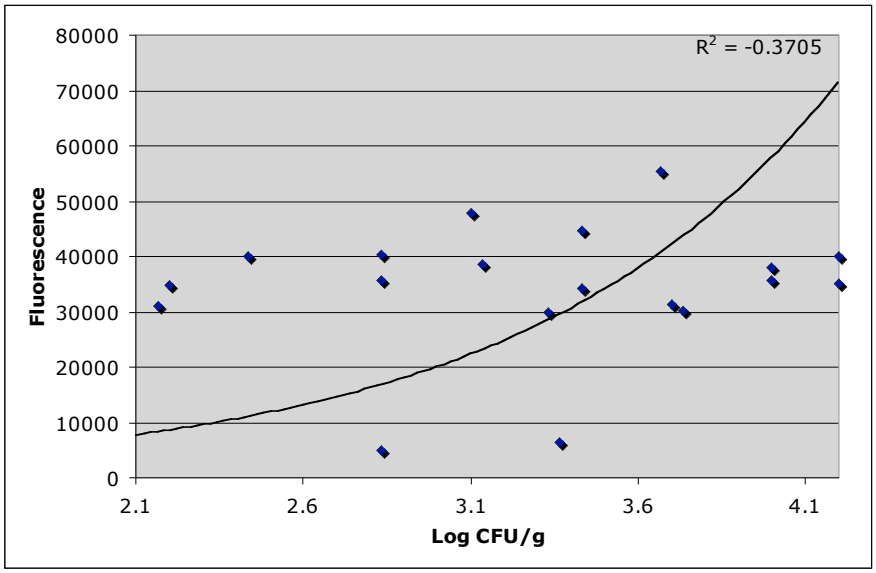
Figure AI.2. Use of a known standard curve to evaluate discrepancy between fluorescence of *E. coli* O157:H7 and standard plate counts on cut and whole leaf lettuce stored under modified and atmospheric conditions at 4ºC over 7 days when inoculated at high levels (a) and low levels (b). N=3

a) High Inoculum



$R^2 = 0.0035$

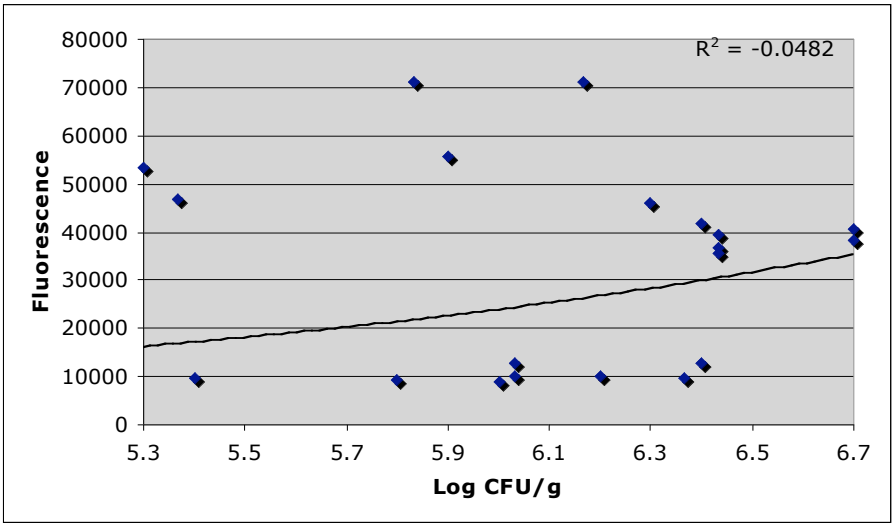
b) Low Inoculum



$R^2 = -.3705$

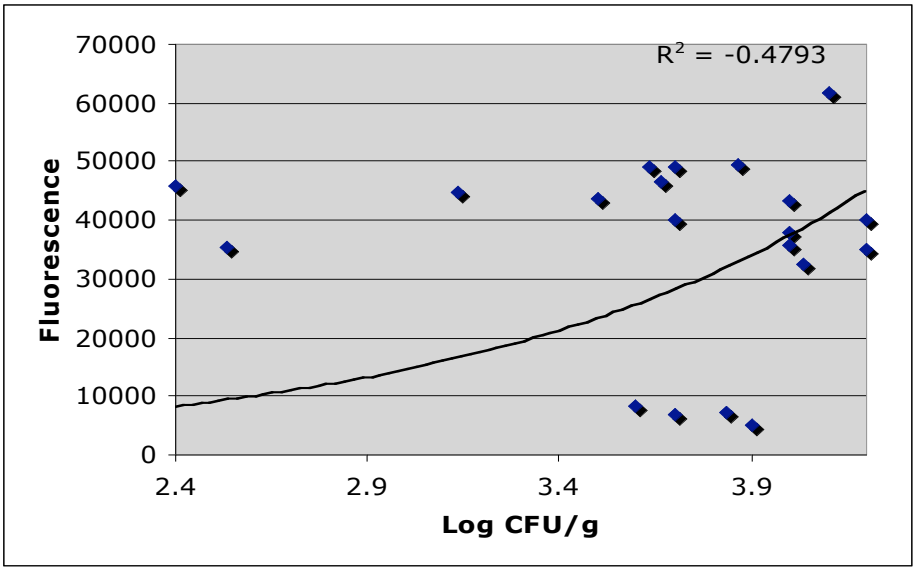
Figure AI.3. Use of a known standard curve to evaluate discrepancy between fluorescence of *E. coli* O157:H7 and standard plate counts on cut and whole leaf lettuce stored under modified and atmospheric conditions at 10ºC over 7 days when inoculated at high levels (a) and low levels (b). N=3

a) High Inoculum



R^2 = -0.0482

b) Low Inoculum



R^2 = -0.4793

Appendix 2: Use of GFP fluorescence assay to quantify *E. coli* O157:H7 numbers from spinach samples

Methods: Methods were followed as described in chapter 3 with the following exceptions:

After samples (10g per bag) were placed into 400 ml stomacher bags with 100-ml sterile 0.1% peptone water and pummeled for 4 minutes, homogenates were stored in 1 ml aliquots in cryogenic vials. The vials containing spinach homogenates were then stored at -80ºC for no longer than 9 days. Duplicate samples were then analyzed on a Victor 3V multilabel plate reader (Perkin-Elmer, Turku, Finland) using a 405 nm filter in order to detect *E. coli* O157:H7 H1730 transformed with GFP. A standard curve was generated with known concentrations of *E. coli* O157:H7 and the correlated fluorescence readings from the micro plate reader. This was compared to conventional plating techniques shown in Figures 1 and 2.

Conclusion: As shown by the low R^2 values for each inoculum size and temperature combination, detection of *E. coli* O157:H7 using a fluorescent micro plate reader is not a suitable comparison to convention plating techniques. Sporadic data points were most likely due to the fact that after homogenates were stored in vials, these samples were frozen for up to one week. This may have caused the *E. coli* O157:H7 cells to lose the GFP plasmid, therefore, no fluorescence would be detected in samples.

Figure AII.1. Standard curve with known concentrations of *E. coli* O157:H7 and the correlated fluorescence readings from the plate reader. N=3

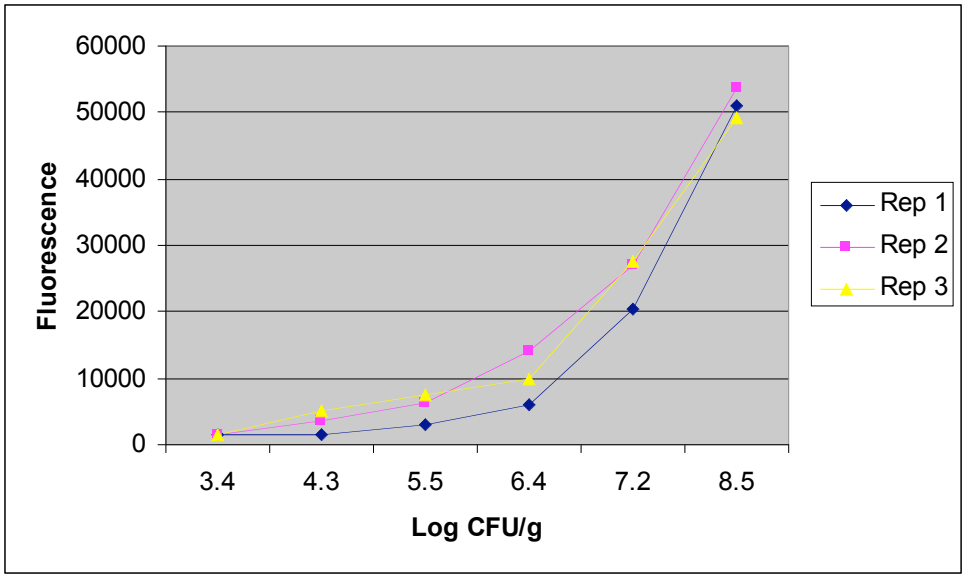
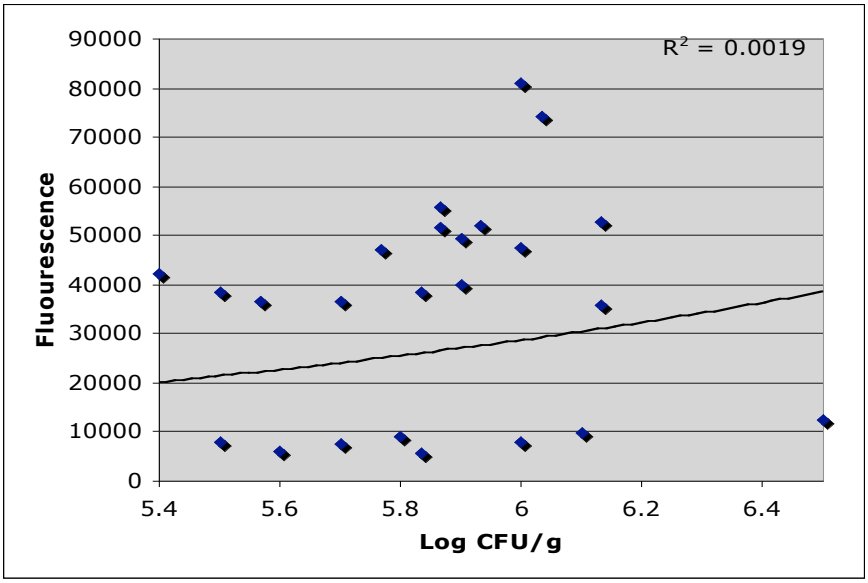


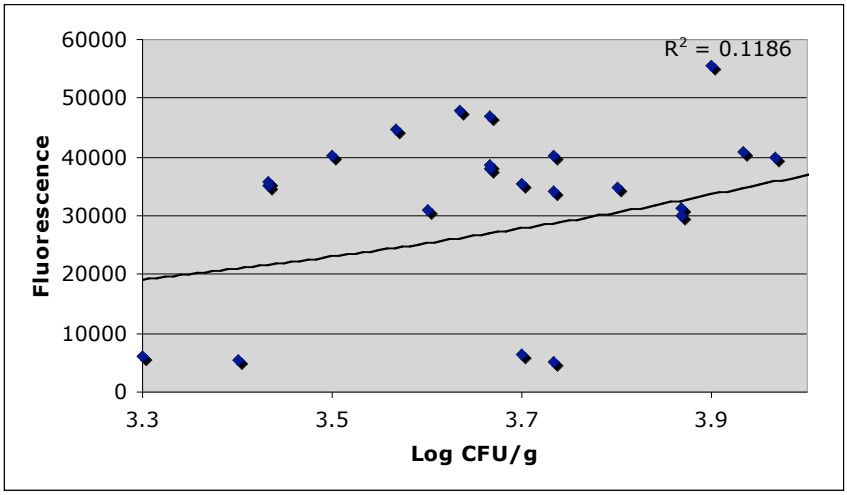
Figure AII.2. Use of a known standard curve to evaluate discrepancy between fluorescence of *E. coli* O157:H7 and standard plate counts on cut and whole spinach stored under modified and atmospheric conditions at 4ºC over 9 days when inoculated at high levels (a) and low levels (b). N=3

a) High Inoculum



$R^2 = 0.0019$

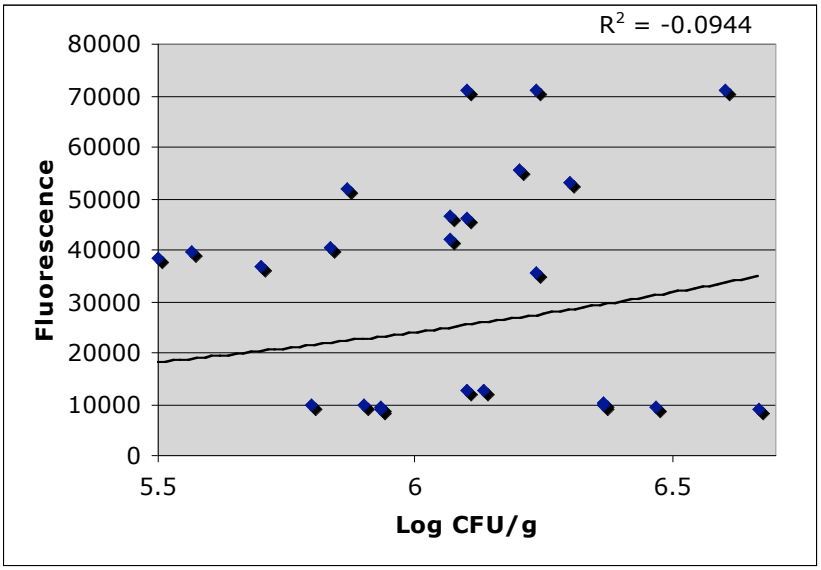
b) Low Inoculum



$R^2 = 0.1186$

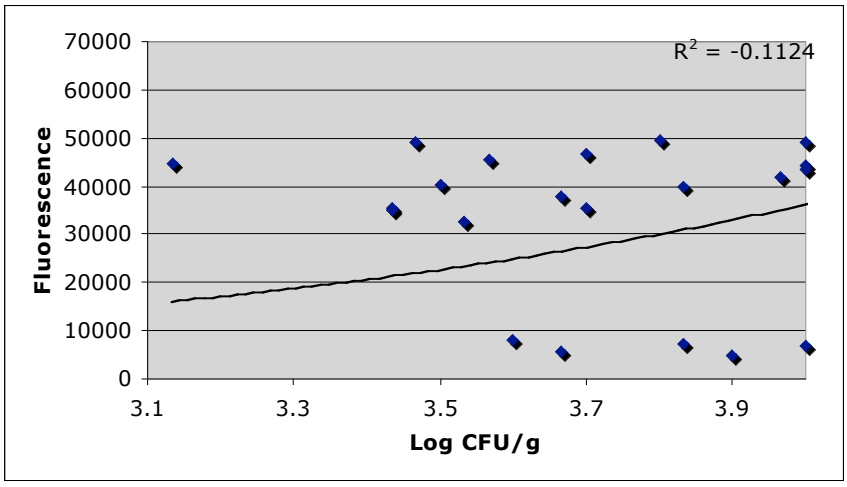
Figure AII.3. Use of a known standard curve to evaluate discrepancy between fluorescence of *E. coli* O157:H7 and standard plate counts on cut and whole leaf lettuce stored under modified and atmospheric conditions at 10ºC over 9 days when inoculated at high levels (a) and low levels (b). N=3

a) High Inoculum



$R^2 = -0.0944$

b) Low Inoculum



$R^2 = -0.1124$