THE USE OF LACTIC ACID BACTERIA AS A POST–HARVEST INTERVENTION TO CONTROL *ESCHERICHIA COLI* O157:H7 IN FRESH SPINACH

by

SARA ELIZABETH GRAGG, B.S.

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Approved

Mindy M. Brashears
Chairperson of the Committee

J. Chance Brooks

Markus F. Miller

Leslie D. Thompson

Fred Hartmeister
Dean of the Graduate School

December, 2008

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ABSTRACT

In recent years, fresh spinach has been identified as a vehicle for *Escherichia coli* (*E. coli*) O157:H7 transmission. Multiple studies have demonstrated the ability of lactic acid producing bacteria (LAB) to reduce the presence of *Escherichia coli* O157:H7 in food products and the efficacy of LAB cultures as a post–harvest intervention in fresh spinach production should be evaluated. To determine the effect of spinach inoculated with *Escherichia coli* O157:H7, spinach samples were rinsed with sterile distilled water and a four–strain LAB cocktail at a target concentration of $2.0x10^8$ CFU/mL. Both treatments were compared to an inoculated control over a 24–hour time period at 7^oC. According to composite LS means data obtained for each treatment, water and LAB resulted in significant reductions of 0.88 logs ($p<0.0001$) and 1.03 logs ($p<0.0001$) in comparison to the control, respectively. The improved reduction of LAB was significantly better than that of water ($p=0.0363$), making it the most effective treatment.

A triangle test was conducted to determine if a statistically significant difference in sensory characteristics exists when LAB is applied to fresh spinach. Two samples were rinsed with tap water and considered to be identical. The remaining sample was rinsed with LAB at a concentration of $2.0x10^8$ CFU/mL. 40 panelists participated in the test and 16 correctly identified the LAB–treated spinach as being the one unique sample. These results indicate that a statistically significant difference does not exist ($\alpha=0.05, 0.01$) when LAB is

applied to fresh spinach and that the use of LAB may be acceptable from a consumer acceptance standpoint.

The ability of LAB to control *E. coli* O157:H7 populations in combination with the industry standard chlorine rinse was determined in a 12 day shelf–life study at 7^{o}C. The multi–hurdle intervention was evaluated in comparison to water, LAB and chlorine rinses. LAB cultures were applied at a concentration of $2.0x10^{8}$ CFU/mL, while chlorine was utilized at the 200 ppm level. As indicated by composite LS means data, significant reductions in comparison to control populations were achieved by the LAB ($p=0.0215$), chlorine ($p=0.0002$) and multi–hurdle treatments ($p<0.0001$). However, the multi–hurdle treatment produced the greatest reductions with 1.35 logs. This reduction was significantly improved upon LAB ($p=0.0012$) and chlorine ($p=0.0815$), indicating that the application of chlorine and LAB is most effective as a combination treatment.

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CHAPTER I

INTRODUCTION

Consumption of fresh fruits and vegetables has increased in the U.S. in recent years (Cooley et al., 2007; Meakin and Dickinson, 2006; Lin et al., 2002). This increase in consumption is believed to be a result of the increased public awareness about the health benefits associated with produce consumption (Meakin and Dickinson, 2006). As consumption of produce increased, the number of foodborne disease outbreaks originating from produce also rose. Takeuchi et al. (2001) suggests that increases in produce–related foodborne disease may be the result of changes in processing, distribution, consumption patterns and agricultural processes.

Over the last decade an increase in per capita consumption has led to a significant increase in sales for the produce industry. Between the years of 1987 and 1997, produce sales in foodservice and retail rose from $34.6 billion to $70.8 billion (Lang et al., 2004). Sales of fresh, bagged salads began in the mid–1990s and have rapidly increased each year. Their convenience and year–round availability improve consumer demand for such products (Meakin and Dickinson, 2006). This high demand has aided in increasing the international trade market, allowing for the constant supply of fresh leafy greens during off–season months (Johnston et al., 2005).

The incidence of *E. coli* O157:H7 infections in the United States has been estimated to be as high as 73,000 (Cooley et al., 2007; Islam et al., 2004)

illnesses and 61 deaths each year (Islam et al., 2004). Estimates generated by the Centers for Disease Control (CDC) have been as high as 74,000 illnesses and 250 deaths each year as a result of *E. coli* O157:H7 infections (Brashears et al., 2003). Multiple avenues exist by which the CDC obtains information in order to generate these estimates. FoodNet was established in 1996 and is the United States Foodborne Diseases Active Surveillance Network (Flint et al., 2005). This government entity is charged with the responsibility of surveillance of over more than 450 laboratories (Flint et al., 2005). Clinical laboratories and physicians are also administered surveys about diagnostic practices (Flint et al., 2005). Additionally, FoodNet conducts telephone surveys of the general population (Flint et al., 2005). All estimates provided by the CDC are calculated based on the data obtained by FoodNet surveillance efforts (Flint et al., 2005).

Since 1995, there have been 22 *E. coli* O157:H7 outbreaks that have been traced to fresh spinach or lettuce, 9 of which were associated with products that originated in or near the Salinas Valley of California (Cooley et al., 2007). A total of 18,000 confirmed foodborne illnesses were linked to the consumption of fresh produce between 1990 and 2002 (Warriner et al., 2005). This number is compared to 9,195 cases associated with beef, 9,612 with poultry, 9,249 with eggs and 6,781 with seafood (Warriner et al., 2005). Cattle are the primary reservoir for *E. coli* O157:H7 (Brashears et al., 2003; Jiang et al., 2003; Delaquis et al., 2007; Wang et al., 1996) and infections caused by *E. coli* O157:H7 are often associated with bovine food products, particularly ground beef (Hilborn et al., 1999; Ackers et al., 1998; Beuchat, 1999). However, since a string of

outbreaks related to various raw or minimally processed fruits and vegetables occurred in the mid–1990s, fresh produce has been recognized as a potential mode of *E. coli* O157:H7 transmission (Delaquis et al., 2007).

Contamination of fresh produce can occur during both pre–harvest and post–harvest production of fresh spinach. Pre–harvest contamination may be the result of contaminated irrigation water, fertilization with contaminated or improperly composted manure, fecal contamination from wild animals and employees. Contamination post–harvest can occur via a variety of sources, including contaminated wash water, infected equipment or trucks and cross–contamination from other produce. Because there are multiple opportunities for microbial contamination throughout the entire production process, an emphasis must be placed on controlling pathogenic contamination at each step from farm to table. (Tauxe et al., 1997)

Currently, the primary post–harvest intervention utilized in the production of fresh spinach is a sodium hypochlorite (chlorine) wash (Behrsing et al., 2000), with up to 90% of processors implementing such an intervention (Pirovani et al., 2000). While chlorine has proven to be a powerful bactericidal compound, the effectiveness against microorganisms on spinach and other leafy greens is dependant upon numerous factors, including contact time, pH and water temperature (Pirovani et al., 2004). Additionally, the location of bacterial attachment to the surface of the leaf may play a role in the effectiveness of chlorine washes (Takeuchi and Frank, 2000). Bacterial internalization into leaf tissues or through the root system has also been proven, thus reducing the

effectiveness of post–harvest chlorine washes (Hora et al., 2005). It has been generally accepted that the use of chlorine as a post–harvest intervention in fresh spinach and leafy green production is effective at reducing the microbial population by only <2 logs (Warriner et al., 2003).

Identification of a more efficacious post–harvest intervention is required in order to improve the safety of fresh spinach. Lactic acid bacteria (LAB) have been proven to be effective antimicrobial agents in other food industry applications. Brashears et al. (1998) observed reductions in *E. coli* O157:H7 in broth and raw chicken meat held at 7^{o}C by using *Lactobacillus lactis* as an intervention. Smith et al. (2005) utilized a four–strain combination of LAB cultures in ground beef. After three days and five days of refrigerated storage, *E. coli* O157 counts declined by 2 and 2.5 log cycles, respectively. Similarly, *Salmonella* in ground beef was decreased by 3 logs and to non–detectable levels after three and five days of refrigerated storage, respectively (Smith et al., 2005).

Lactic acid bacteria can also be utilized as direct–fed microbials to control *E. coli* O157 in cattle. Younts–Dahl et al. (2005) fed NP51 at concentrations of 10^7, 10^8 and 10^9 cells per head. When averaged across time, they discovered that the highest concentration produced the largest reduction, which was calculated to be a 77% less likelihood of recovering *E. coli* O157 in comparison to control–fed cattle.

Lactic acid bacteria are antagonistic towards *E. coli* O157:H7 because of their excellent ability to outcompete for nutrients. Additionally, their production of antimicrobial metabolites that are lethal to other microorganisms is another way

in which LAB are bactericidal. Such metabolites include hydrogen peroxide, lactic acid and other organic acids, bacteriocins and diacetyl (Amézquita and Brashears, 2002). The proven success of LAB in other industry applications makes these beneficial microorganisms a feasible option as a post–harvest intervention in the production of fresh spinach.

The primary objective of this project was to determine if the use of Bovamine® Meat Culture (a commercially available LAB culture product) as a post–harvest intervention in the processing of fresh spinach was effective at controlling *Escherichia coli* O157:H7. The completion of this research was multi–faceted and required the evaluation of numerous components. As a result, many variables were taken into consideration while developing the experimental design.

A total of three individual protocols were completed to meet project objectives. Initial research focused on the comparison of Bovamine® Meat Culture and sterile distilled water as post–harvest wash interventions. Furthermore, to ensure that the addition of Bovamine® Meat Culture did not have an adverse affect on the sensory characteristics of fresh spinach, a triangle test was performed. Because some level of success was achieved with the Bovamine® Meat Culture treatment, the final objective was the completion of a 12–day study to evaluate the effectiveness of multiple interventions throughout the shelf–life of fresh spinach.

The hypothesis for this project was that a hurdle intervention consisting of both sodium hypochlorite and Bovamine® Meat Cultures will result in the most

significant reductions when applied as a rinse to fresh spinach. Additionally, it is hypothesized that the application of Bovamine® Meat Cultures will not adversely affect the sensory characteristics of fresh spinach.

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CHAPTER II

REVIEW OF THE LITERATURE

Escherichia coli O157:H7

History and Background

Escherichia coli O157:H7 was first recognized as a human pathogen in 1982 (Hilborn et al., 1999; Meng et al., 2001; Meng et al., 2005; Meng and Doyle, 1998; Mead and Griffin, 1998). Since then, this microorganism has been the causative agent in numerous food and non-food related outbreaks, including water, ground beef, apple cider and produce (Meng et al., 2001; Meng et al., 2005). Theodor Escherich first isolated *Escherichia coli* (*E. coli*) as a cause of diarrhea in 1885 (Robins–Browne and Hartland, 2002) and his discovery led to the microorganism being named in his honor. Since then, a sophisticated method for classifying and serotyping specific strains of *Escherichia coli* has been developed that differentiates pathogenic from non–pathogenic *E. coli* and also identifies the various types of pathogenic *E. coli* in existence.

The genus *Escherichia* is comprised of enteric bacteria that are natural inhabitants of the intestinal tract of humans and warm-blooded animals (Madigan et al., 2003). While these microorganisms are prominent in their environment they are not dominant (Madigan et al., 2003). Non-pathogenic strains are beneficial to the host by aiding in digestion and the synthesis of vitamins (Madigan et al., 2003). *Escherichia coli* is one species within this genus that encompasses both commensal and pathogenic strains (Welch, 2006).

Classification

Members of the *E. coli* family are gram-negative rods and are classified using the antigens present on the surface of the cell. The somatic antigen (O), flagellar antigen (H), and the capsule antigen (K) allow for serotyping of specific strains (Meng et al., 2001; Meng et al., 2005). The serogroup of a strain is identified by the O antigen while the H antigen indicates the serotype of the microorganism (Meng et al., 2001; Meng et al., 2005). *E. coli* that are associated with diarrheal disease in humans need only be identified using their O and H antigens and are further classified according to their virulence, methods of pathogenicity and the clinical disease they are associated with (Meng et al., 2001; Meng et al., 2005). The categories of diarrheagenic *E. coli* include enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAggEC), diffuse-adhering (DAEC), and enterohemmorrhagic (EHEC) (Meng et al., 2001; Meng et al., 2005). *E. coli* O157:H7 belongs to the EHEC category and will be the focus of this thesis.

Mechanisms of Pathogenicity

E. coli O157:H7 is the primary strain associated with EHEC-related illnesses in North America (Meng et al., 2005). All EHEC strains produce Shiga toxins (Stx) that can lead to bloody diarrhea (hemorrhagic colitis), and a potentially life-threatening condition known as hemolytic uremic syndrome (HUS). Shiga toxins are comprised of one A and five B subunits (A_1B_5) that are encoded

for on a bacteriophage inserted into the O157 chromosome (Mead and Griffin, 1998). The membranes of eukaryotic cells possess a glycolipid, known as globotriaosylceramide (Gb_3) that the B subunits bind to. The Shiga toxin then enters the cell by means of endocytosis where it alters the 60S ribosomal subunit by enzymatic inactivation (Mead and Griffin, 1998). This alteration in the rRNA results in the inhibition of protein synthesis and cell death (Mead and Griffin, 1998; Meng et al., 2001, Meng et al., 2005).

The name Shiga toxin was adapted for these EHEC toxins because of their similarity to the toxins produced by *Shigella dysenteriae*. Production of one or more Shiga toxins by *E. coli* O157:H7 is a virulence mechanism that has been acquired throughout the evolution of this microorganism. The genes for Shiga toxin production are encoded within the *Shigella dysenteriae* chromosome but are easily spread to *E. coli* O157:H7 through a horizontal gene transfer by toxin-encoding bacteriophages (Robins–Browne and Hartland, 2002). Shiga toxin 1 is identical to the Shiga toxin produced by *Shigella dysenteriae* type 1 (Mead and Griffin, 1998). Conversely, Shiga toxin 2 is only about 56% homologous to the amino acid structure of Shiga toxin 1 (Mead and Griffin, 1998). While most *E. coli* O157:H7 strains produce Shiga toxin 2, a wide range can produce both toxins. A series of isolates from Europe indicated that less than 25% produced both toxins, while greater than 80% of isolates in a series from Japan and North America produced both Shiga toxins 1 and 2 (Mead and Griffin, 1998). The discrepancy in Shiga toxin production between varying strains of *E. coli* O157:H7 is evidence that this microorganism has undergone a great deal of evolution.

E. coli O157:H7 does not invade the intestinal epithelium cells to cause illness. This microorganism is believed to be pathogenic through its ability to adhere to the host cell membrane. (Robins–Browne and Hartland, 2002; Meng et al., 2001; Meng et al., 2005). The locus of enterocyte effacement (LEE) is located on the EHEC chromosome and genetically encodes for the attachment and effacement of *E. coli* O157:H7 to the intestinal cells. This attachment and effacement creates an AE lesion on the surface of the intestinal cells and is facilitated by the production of the intimin protein. The *eae* gene is located on the LEE and encodes for intimin production. Adherence is attained after the Tir protein is injected from the bacterial cell into the host cell to act as the intimin receptor. Translocation of Tir into the host cell is achieved by the type III secretion system, which is responsible for the exportation of virulence mechanisms. After adherence is achieved, a pedestal is formed (Meng et al., 2001; Meng et al., 2005) where the *E. coli* O157:H7 cells are attached. Pedestal formation occurs as a result of the accumulation of cytoskeletal components (Paton and Paton, 1998).

The LEE is not encoded in non-pathogenic strains of *E. coli* but comprises a large region on the chromosome of EHEC strains that create the AE lesion. Large blocks of DNA that encode numerous virulence mechanisms and are not present in non-pathogenic strains of the same species are referred to as pathogenicity islands (Meng et al., 2001). The locus of enterocyte effacement meets the requirements to be considered a pathogenicity island and is commonly referred to in short as the LEE island.

Throughout the evolution of *E. coli* O157:H7, this microorganism obtained a large plasmid that is believed to contribute to its virulence. The plasmid, known as pO157, encodes for the production of an EHEC-hemolysin and catalase-peroxidase (Meng et al., 2001; Meng et al., 2005). While the exact virulence associated with the plasmid is not known, it is believed that the EHEC-hemolysin allows *E. coli* O157:H7 to utilize the blood released into the intestine as a source of iron (Mead and Griffin, 1998). The production of catalase-peroxidase protects the *E. coli* cell from the oxidative stress caused by the mammalian cell as a defense mechanism in response to bacterial infection (Meng et al., 2001; Meng et al., 2005). The pathogenicity properties of *E. coli* O157:H7 not only cause disease in humans, but also protect the cell against immune responses from the host to facilitate the pathogens survival.

Genetic Evolution

Analysis of DNA by Whittam et al. (1993) using multi-locus enzyme electrophoresis illustrated that *E. coli* O157:H7 is most genetically similar to a non-Shiga toxin-producing enteropathogenic (EPEC) strain of serotype O55:H7. Therefore, *E. coli* O157:H7 is believed to have evolved from this EPEC organism. Feng et al. (1998) proposed a model for the evolution of *E. coli* O157 from the EPEC ancestor. Before obtaining the O55 somatic antigen and H7 flagellar antigen, the EPEC ancestor expressed β-glucuronidase, fermented sorbitol, possessed the LEE island and produced the γ-form of intimin. There is strong evidence to support the early presence of the LEE, as all strains of the O55 and

O157 serogroups possess an identical insertion of the LEE on the chromosome, as well as the production of an indistinguishable intimin protein (Law, 2000).

It is believed that the O55:H7 strain obtained the ability to produce Shiga toxin 2 by acquiring a phage that carried Shiga toxin 2 and was capable of converting toxins (Law, 2000; Whittam, 1998). Because of the variation among the Shiga toxin 2 group, it is thought that the organism acquired Shiga toxin 2 far earlier than Shiga toxin 1 (Law, 2000). Additionally, because of the vast difference between Shiga toxins 1 and 2, it is implied that these two toxins experienced most of their evolution outside of the *E. coli* organism and, instead, were fairly recently acquired by means of a horizontal gene transfer (Law, 2000).

The next step in evolution was for the O55:H7 microorganism to mutate in the β–glucuronidase gene, obtain the large pO157 plasmid and the ability to produce the O157 antigen (Whittam, 1998). While the pO157 plasmid that exists in *E. coli* O157:H7 is not transferable, it is believed that it was initially transferable and obtained through a direct transfer (Law, 2000). The plasmid became non-transferable when the gene for ToxB, a putative cytotoxin, was inserted into the genes responsible for plasmid transfer (Burland et al., 1998).

This primal *E. coli* O157:H7 strain is believed to be the origin of two divergent lineages that have evolved. The first lineage lost motility and gave rise to the sorbitol–fermenting and β–glucuronidase–producing *E. coli* O157:H- strains that tend to be found in parts of East Europe and Germany. The other lineage lost the ability to ferment sorbitol and obtained a phage that encoded for Shiga toxin 1. Therefore, the resultant pathogen was a sorbitol non-fermenting,

β–glucuronidase–producing *E. coli* O157:H7 with the ability to produce both Shiga toxins 1 and 2 (Law, 2000).

Throughout further evolution the ability of *E. coli* O157:H7 to produce β–glucuronidase and, subsequently, hydrolyze 4-methlyumbelliferyl-D-gluconoride (MUG) was lost (Law, 2000; Meng et al., 2001), giving rise to the direct ancestor of the pathogen that has spread throughout the world. Some common variants of this ancestor have recently lost the Shiga toxin 1 and motility genes, accounting for the variation of isolates that is seen today (Whittam, 1998). While the exact timeline for the evolution of *E. coli* O157:H7 is unknown, there is evidence to indicate that the evolutionary period from the EPEC O55:H7 ancestor was relatively rapid (Law, 2000).

Escherichia coli O157:H7 Related Illnesses

The pathogenicity in combination with the low infectious dose of *E. coli* O157:H7 make infection with this microorganism a potentially severe and even deadly disease. It is estimated that only 1 to 100 colony forming units (CFU) are sufficient to cause disease in humans (Paton and Paton, 1998). A multi-state outbreak that occurred in 1993 in the United States from contaminated frozen ground beef patties reported an enumeration level as low as 0.3 to 15 CFU per gram (Meng et al., 2001; Meng et al., 2005), indicating that the infectious dose may have been below 1 CFU. Disease associated with *E. coli* O157:H7 infection can range from asymptomatic carriage to non-bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome, thrombotic thrombocytopenic purpura

and even death (Mead and Griffin, 1998; Meng et al., 2001; Meng et al., 2005; Pickering et al., 1994). Incubation period for this microorganism is typically around three days. However, reports of incubation have been as short as 1 day and as long as 8 days (Mead and Griffin, 1998) up to 12 days (Meng et al., 2001; Meng et al., 2005). While humans of all ages are at risk for developing disease from an *E. coli* O157:H7 infection, individuals that are young, elderly or immunocompromised are most at risk.

Hemorrhagic colitis is characterized by a progression from non-bloody diarrhea accompanied by abdominal cramps to bloody diarrhea within 1-2 days. Over 70% (Mead and Griffin, 1998) up to approximately 90% (Meng et al., 2001; Meng et al., 2005) of patients report the occurrence of bloody diarrhea, ranging from a few bloody streaks to bowel movements consisting of almost entirely blood. Roughly 30-60% of individuals who develop HC experience vomiting and only 30% develop a fever (Mead and Griffin, 1998). Most individuals with HC recover on their own within a week (Meng et al., 2001; Meng et al., 2005) but the length of disease can range between 4 and 10 days (Mead and Griffin, 1998; Meng and Doyle, 1998).

Hemolytic uremic syndrome is the more advanced and far more serious disease that can progress from HC. Hemolytic uremic syndrome has been reported to develop in as few as 3-7% and up to 20% of cases in some outbreaks (Mead and Griffin, 1998) and is one of the primary causes of acute renal failure in the western regions of the world (Delaquis et al., 2007). The onset of HUS is typically around 6 days after the beginning of diarrhea (Mead and Griffin, 1998).

The damage that is caused by Shiga toxins to the endothelial cells results in platelet aggregation at the injured site (Rose and Chant, 1998). Hemolytic uremic syndrome occurs when the platelets form blood clots that enter into the blood stream and settle in the kidneys, resulting in acute renal failure (Kaplan et al., 1998).

This disease tends to manifest itself most frequently in children aged 5 and under (Pickering et al., 1994). While HUS can develop in older children and adults, it is rare and much less likely to occur (Ruggenenti et al., 2001). Though uncommon, adults are more prone to developing thrombocytopenic purpura (TTP) as a result of infection with *E. coli* O157:H7. While this illness has many similarities to HUS, TTP patients also experience neurological problems and a fluctuating fever (Rose and Chant, 1998).

Implementing the appropriate treatment for *E. coli* O157:H7 patients is difficult and usually involves the use of antibiotics, anti-motility agents, anti-diarrheal agents, and other measures to restore health, including hydration. Because *E. coli* O157:H7 is typically sensitive to antibiotics that are commonly prescribed, antibiotic treatments seem to be a great option for preventing complications and transmission, as well as decreasing a patient's symptoms. However, there is a great deal of conflicting evidence in terms of antibiotic use. Investigation into the use of antibiotics in various outbreaks revealed varying levels of success. (Neill, 1998)

In two completely unassociated outbreaks, a total of 43 individuals became ill from *E. coli* O157:H7 exposure (Neill, 1998). Of these patients, 23

were known to have received antibiotics (Neill, 1998). Those who received the antimicrobial treatments did not have a shorter illness duration than patients who did not (Neill, 1998). Additionally, there were no cases of HUS or development of complications in any of the 43 patients (Neill, 1998). The information obtained from this outbreak suggests that antimicrobial agents have little to no effect on *E. coli* O157:H7–related illnesses.

Shortly after the previous outbreak, 55 residents and 18 staff members of a nursing home contracted *E. coli* O157:H7 in Canada. It was assumed because of the elderly population, there were numerous complications associated with this outbreak. Additionally, HUS developed in 12 (22%) of the 55 nursing home residents. The result was a 31% fatality rate. Patients administered antibiotics before the onset of illness still became primary cases and were at an increased risk of becoming a secondary case. Additionally, antibiotics administered after illness onset were associated with an increased fatality rate. This outbreak suggests that administering antibiotics for *E. coli* O157:H7 patients can actually be more harmful than beneficial. (Neill, 1998)

There is other evidence available that supports the suggestion that antibiotics may actually be harmful if administered to *E. coli* O157:H7 patients. Research conducted in the laboratory has revealed that antibiotic exposure to *E. coli* O157:H7 may increase Shiga toxin production. Furthermore, administering antibiotics at concentrations sublethal were known to increase the release of Shiga toxins. However, a discrepancy on the degree of increase in Shiga toxins caused by antimicrobial treatment was also observed. (Neill, 1998)

An outbreak in the state of Washington was associated with a fast–food chain and became the focus of large investigations. The analysis was retrospective and consisted of 278 affected children that were less than 16 years of age. It was determined that antibiotic treatment was not beneficial, nor did it prevent the onset of HUS. However, Japanese investigators took a different approach on analyzing outbreak and sporadic cases of *E. coli* O157:H7 in Japan in 1996. They conducted a retrospective questionnaire to evaluate the effects of antimicrobial treatment. Questionnaires were sent to 3,908 hospitals all across Japan. Head physicians completed the questionnaire and returned them through April 30, 1997. The collection of answers obtained suggested that the antibiotic fosfomycin decreased the risk of developing HUS when administered by day 3 of onset, in comparison to treatment after the illness had further progressed. (Neill, 1998)

The compilation of evidence from the outbreaks discussed provides little to no guidance on antibiotic use for *E. coli* O157:H7 infections. There is evidence that antibiotics have no effect, while other information suggests the use of antibiotics is harmful (Neill, 1998). Additional information provides hope that antibiotics are beneficial if administered at the appropriate time over the course of the illness (Neill, 1998). Analysis of these outbreaks is further complicated by the fact that different antibiotics were administered to patients and at different times over the course of their illness (Neill, 1998). The lack of consistency makes it difficult to draw a definitive answer.

Similar to antibiotic use, antimotility agents do not provide much promise for battling *E. coli* O157:H7 infections. In fact, these compounds may be associated with the development of a more severe disease. A Canadian study consisting of 118 *E. coli* O157:H7 patients examined the use of an antidiarrheal said to have antimotility properties. A total of 25% of patients had HUS and the cause was associated with the administration of such antidiarrheal agents for 24 hours. Also, the retrospective study of the Washington state outbreak previously discussed revealed that administration of antimotility agents during the diarrhea phase of the illness increased the chances for HUS development. Additionally, it was noted that those given antimotility agents without progressing to HUS experienced a longer bout with bloody diarrhea. These outbreaks provide evidence that antimotility agents are not beneficial for battling *E. coli* O157:H7 infections and should not be implemented as treatment plans. (Neill, 1998)

The contraction of an *E. coli* O157:H7 infection may follow a seasonal pattern. While there is the potential for contamination with this pathogen year–round, the prevalence of *E. coli* O157:H7 in feedlot cattle is highest during the warmer months (Barkocy–Gallagher et al., 2003; LeJeune et al., 2004; Loneragan and Brashears, 2005). A study conducted on the prevalence and shedding concentrations of *E. coli* O157 in beef cattle in Scotland reported that high shedding beef cattle shed the pathogen in greater concentrations during the warmer months (Ogden et al., 2006). The number of clinical cases in humans has been proven to spike during the warmer months, as well (Barkocy–Gallagher et al., 2003). Therefore, it is perhaps during this time that the risk may increase

for human exposure to *E. coli* O157:H7. Additionally, it could be hypothesized that the warmer months increase the risk for spinach to become infected by means of contaminated manure, which is capable of contaminating the product either directly or indirectly.

Spinach and *E. coli* O157:H7

Spinach Overview

Minimally processed vegetable products are those that have undergone various production practices to improve food safety and consumer demand without altering freshness or nutritional value of the product (da Cruz et al., 2006). While the product may undergo such practices as washing, shredding or slicing; there is no preservation step incorporated into the production of these vegetable products. Typically, minimally processed vegetables are produced with the convenience of the target consumer in mind and fresh, bagged spinach is an excellent example of such a product (Phillips and Harrison, 2005).

The lack of a thermal processing step in the production of fresh spinach increases the risk of producing and distributing a product that is contaminated with pathogens, including *E. coli* O157:H7. Microbial contamination of spinach can occur at any point during both pre–harvest and post–harvest production practices. It is for this reason that each step in the production process must be carefully controlled in order to prevent pathogenic contamination. (Schuenzel and Harrison, 2002)

Modes of Transmission

The environment in which spinach is produced is a key factor in the microbiological quality of the product. It has been well documented that *E. coli* O157:H7, as well as other microorganisms and pathogens, is capable of surviving and proliferating in soil and manure for extended periods of time. Additionally, cattle have been identified as the main reservoir for *E. coli* O157:H7 (Brashears et al., 2003b; Jiang et al., 2003; Delaquis et al., 2007; Wang et al., 1996). For these reasons manure is believed to be the leading mode of transmission for *E. coli* O157:H7 contamination in fresh spinach (Islam et al., 2005). However, *E. coli* O157:H7, and other enteric pathogens, could also be distributed by wild animals, contaminated irrigation water, humans or air (Delaquis et al., 2007; Beuchat, 2002; Pirovani et al., 2000; Aruscavage et al., 2006; Solomon et al., 2002a; Solomon et al., 2002b). Additionally, inadequate employee hygiene and poor sanitation of processing equipment may play a role in spinach contamination (Johnston et al., 2005).

The use of manure in production agriculture is a common practice due to the numerous benefits obtained through manure application. Nutrients found in animal manure help to build and maintain fertility of the soil. Manure has also been attributed with increasing the soil's water-holding capacity and tilth. Additionally, spreading manure across the land is beneficial for reducing water and wind erosion, as well as improving soil aeration and promotion of beneficial microorganisms. (Islam et al., 2005)

Despite the advantages provided by manure application, the potential for manure to contain *E. coli* O157:H7 and other pathogens is very real and is something that must be addressed before use in the production of fresh spinach. As previously stated, cattle have been identified as the main reservoir for *E. coli* O157:H7 (Brashears et al., 2003b; Jiang et al., 2003; Delaquis et al., 2007; Wang et al., 1996). The gastrointestinal (GI) tract harbors this microorganism while the animal remains asymptomatic (Delaquis et al., 2007). The bacterial cells are excreted with the feces and contaminate the surrounding environment. Contaminated manure becomes a food safety concern on many levels in production agriculture, as it can contaminate produce in multiple ways. Feedlot animals have been surveyed to determine the frequency of *E. coli* O157:H7, with the prevalence found to be as high as 36.8% (Chapman et al., 1997; Islam et al., 2005; Solomon et al., 2002b; Solomon et al., 2003). Brashears et al. (2003b) stated that up to 30% of feedlot cattle may shed *E. coli* O157:H7 and that as many as 70% of feedlots harbor at least one animal that is positive for the pathogen. One study determined that 100% of the 73 feedlots tested contained *E. coli* O157 (Younts et al., 2004; United States Department of Agriculture, 2001). A study conducted by Doane et al. (2007) tested rectal and environmental swabs of dairy cattle from four dairy farms and discovered that the isolation rate of the pathogen was similar to that of beef cattle farms at a rate of 3.4%. They believed their findings were in accordance with other studies that found a rate of occurrence anywhere from 1.5 to 15.7%. According to Islam et al. (2004), cattle in the northern U.S. carry *E. coli* O157:H7 at a prevalence of 6 to

9%. Additionally, they reported that dairy cattle may have a prevalence of up to 8.3%. Elder et al. (2000) evaluated 29 lots of cattle for the presence of *E. coli* O157 and found that 72% had at least one fecal sample and 38% had hide samples that tested positive for the pathogen. Another survey revealed that, at a minimum, 1 to 5% of cattle shed *E. coli* O157:H7 in their feces at levels ranging from $<10^2$ CFU/g up to 10^5 CFU/g (Jiang et al., 2003). The same study reported the prevalence of *E. coli* O157:H7 in fecal shedding to be notably increased throughout the summer months and as high as >25% (Jiang et al., 2003).

Composting manure is commonly practiced on many farms as a means to eliminate the presence of pathogens and the risk for foodborne disease. During the composting process, microorganisms produce heat as a result of their normal metabolic activities. This heat builds and becomes warm enough to kill many microorganisms, including *E. coli* O157:H7 and other pathogens. Theoretically, the end product of the composting process should be free of pathogens and safe for application on farm land and crops. However, the composting process does not always produce a consistent, pathogen free product. Variability of environmental conditions and temperatures within the compost may result in a lack of uniform lethality among microorganisms. Unless the heat is evenly distributed throughout the entire mound of compost there is potential for pathogens to survive and contaminate the spinach during growth. Even if the composting process is effective, during storage on the farm there is potential for raw manure contaminated with *Escherichia coli* O157:H7, or other pathogens, to

mix with the composted materials and re-infect the entire batch. (Islam et al., 2005)

A study conducted by Droffner and Brinton (1995) revealed that *E. coli* O157:H7 was present for at least 59 days in manure that had been composted and held at 60^{o}C. Similar data was obtained by Wang et al. (1996) who reported that *E. coli* O157:H7 is capable of surviving in bovine feces for 70 days. Islam et al. (2005) identified the ability of *E. coli* O157:H7 to not only survive, but also to produce deadly verotoxins in bovine feces up to 70 days. Several other studies have determined that *E. coli* O157:H7 can survive in mounds of manure for periods ranging from a few months up to 21 months (Islam et al., 2005). The results from these studies indicate that regulations requiring the composting of cattle manure for 60 days before use as a fertilizer may be less than adequate.

Manure from wild and feral animals located within the vicinity of growing spinach plants can also result in contamination with *E. coli* O157:H7. Direct contact of manure with growing spinach plants, as well as manure particles carried by wind and air, are of great concern in the spinach industry. While cattle are considered to be the main reservoir for this microorganism, *E. coli* O157:H7 has also been isolated from sheep, pigs, deer and goats (Islam et al. 2005); all of which have been found to be feral in different parts of the United States and the world. Preventing contamination from wild, free roaming animals is very difficult and the lack of control can result in a considerable source for *E. coli* O157:H7 transmission in fresh spinach.

Contaminated manure can also indirectly contaminate fresh spinach by infiltrating water sources (Beuchat, 2002) used for irrigation or post–harvest washing of the product. With a water rinse serving as the main barrier against contamination, foodborne disease is virtually imminent in the event that the wash water is contaminated with *E. coli* O157:H7. Considering the lack of a thermal processing step during production, fresh spinach is particularly at risk for this form of *E. coli* O157:H7 transmission. For this reason, it is necessary that the wash water be high quality and free of microbial contamination. In 1996 an *E. coli* O157:H7 outbreak occurred in fresh lettuce as a result of contaminated wash water use in a processing shed on–site at the farm, sickening 61 individuals (Wachtel et al., 2002). On–site sampling did not recover the pathogen. However, the access of free–roaming chickens to lettuce fields, as well as the cow pasture neighboring the farm, was believed to be contributing factors (Wachtel et al., 2002).

Numerous cases and outbreaks of infection with *E. coli* O157:H7 have been traced to food or water that had been either indirectly or directly contaminated with animal manure (Jiang et al., 2003; Jiang et al., 2002). One *E. coli* O157:H7 outbreak affected the members of four families and was connected to food grown on the farm that had been fertilized with cattle manure (Jiang et al., 2002; Islam et al., 2004). In another case of *E. coli* O157:H7, a woman became ill after consuming poorly washed vegetables grown in a garden that had been fertilized with cattle manure (Jiang et al., 2002; Islam et al., 2004). A large *E. coli* O157:H7 outbreak occurred in Canada in May of 2000 as a result of

contaminated water (Islam et al., 2004). This outbreak resulted in seven deaths and greater than 2,000 ill people in Walkerton, Ontario. It was determined that the town's water supply had become contaminated after application of manure to the land on a nearby farm (Islam et al., 2004).

The quality of irrigation water is one of the main safety concerns associated with the production of minimally processed vegetables. Irrigation water has frequently been proven to possess high levels of viruses and enteric bacteria. Oftentimes the highest level of irrigation–associated contamination is found in leafy vegetables, such as spinach. This susceptibility to microbial contamination is believed to be as a result of their large surface area and physiological characteristics that promote the adhesion of microorganisms (da Cruz et al., 2006). The danger is dramatically increased if contamination of the edible parts of the plant occurs close to harvest (Coetzer, 2006).

Irrigation method is believed to play a role in the level of microbial contamination on vegetable surfaces. Spray irrigation results in direct contact of contaminated water, increasing the probability of product contamination in comparison to deep irrigation methods. This idea is confirmed in a study conducted by Solomon et al. (2002b) that was designed to compare the contamination of *E. coli* O157:H7 in lettuce plants following surface or spray irrigation. A total of 64 plants were sampled, with 32 exposed to each irrigation method. Spray irrigation resulted in a total of 29 (91%) plants positive for the pathogen while surface irrigation merely had 6 (19%) positive plants. Ideally irrigation water would not be of questionable nature and irrigation method would,

therefore, not be as important. However, given that *E. coli* O157:H7 has proven its ability to persist in deionized and lake water for many weeks (Islam et al., 2004), both irrigation method and water quality must be examined for safety.

In addition to environmental sources, inadequate employee hygiene poses a threat to the safety of fresh spinach, both in the field and in processing facilities. Outbreaks of foodborne disease associated with fresh produce have been linked to the exposure of the product to infected employees followed by temperature abuse, which allows for the proliferation of any pathogens that may be present. Infected individuals have the potential to inadvertently contaminate other workers, supplies and water sources as well as the produce itself. This is possible because infected employees may be asymptomatic while still shedding pathogens. These asymptomatic periods of shedding may last for only a few hours and up to a decade or longer. Due to the potential severity of this issue, all employees must be educated on the importance of personal cleanliness and proper use of bathroom facilities, both at work and home, while employed in the produce industry. Adequate hand hygiene has been identified as the utmost important method to prevent transmission of infectious disease. Additionally, because the hands are the main point of contact in the transfer of spinach from farm–to–table, an emphasis must be placed on proper hand sanitation with all employees in the produce industry. (Michaels and Todd, 2006)

Inadequate sanitation of processing equipment is also a key avenue for *E. coli* O157:H7 contamination during pre–harvest and post–harvest processing of fresh spinach. All surfaces that regularly come into contact with the spinach

leaves are potential sources of contamination (Fonseca, 2006) and must be addressed in a strict sanitation program. Effective cleaning and sanitation is of paramount importance in preventing the buildup of organic deposits that may promote microbial growth and biofilm formation (Heard, 2002). In particular, brushes used in spinach processing plants are known to harbor contaminating bacteria and must also be properly sanitized on a regular basis (Fonseca, 2006). With proper and thorough sanitation, the risk of *E. coli* O157:H7 contamination from processing equipment can be controlled.

Due to the nature of spinach production and distribution, *E. coli* O157:H7 contamination in fresh spinach is typically spread throughout a wide geographic region and is difficult to trace during an outbreak. This occurs because a single field of spinach may be grown by one grower but produced for multiple brands and companies. One particular lot of spinach could potentially be dispersed throughout the entire United States. Additionally, products produced by multiple growers are often packaged together, but shipped through different retailers and distributors. This coupled with the short shelf–life of fresh spinach and rapid product turnover makes traceability of an outbreak nearly impossible. (Tauxe et al., 1997)

The most devastating outbreak associated with fresh spinach occurred in the fall of 2006. Consumption of fresh spinach, and other spinach–associated products, processed under commercial brand names by Natural Selection Foods in San Juan Bautista, California was implicated in the outbreak. Between the dates of August 19th and September 5th, 2006, 199 individuals were sickened

across a span of 26 states. By October 6th, 2006, 31 patients had developed acute renal failure as a result of HUS or TTP. By mid–October, two elderly patients and a two–year–old child had died as a result. (Maki, 2006)

Patients from 10 states provided 13 packages of opened spinach for microbiological testing. *E. coli* O157:H7 was isolated from each package, 11 of which contained lot numbers from a single manufacturing facility and were processed on the same day. DNA Pulsed Field Gel Electrophoresis (PFGE) patterns obtained from all 13 isolates were found to match the outbreak strain that had been isolated from multiple patients. On October 12th, 2006, the same strain of *E. coli* O157:H7 was detected by authorities in manure that had originated from one of four adjacent cattle ranches that were suspected sources of contamination. (Maki, 2006)

According to the Food and Drug Administration (FDA) (2007), the outbreak ultimately resulted in 205 cases of illness and three deaths. The DNA fingerprinting obtained from product provided by consumers allowed investigators to pinpoint one field that contained *E. coli* O157:H7 environmental samples matching that of the outbreak strain. Within close proximity to the implicated field, investigators identified several environmental factors, including the presence of wild pigs and exposure of surface waterways to cattle feces, as being potential sources for contamination.

Microbial Colonization of Plant Leaves

The leaves of plants, also known as the phyllosphere, provide a unique environment for microbial colonization and growth. It is well understood that bacteria, among other microorganisms, are common inhabitants of the plant phyllosphere. Bacteria that colonize the phyllosphere and are capable of living on plant surfaces are referred to as epiphytes or epiphytic bacteria. Communities of epiphytic bacteria are highly diverse and have been shown to exceed 78 bacterial species with 37 bacterial genera represented. (Beattie and Lindow, 1999)

Colonization of the phyllosphere is not uniform across the entire surface of the leaf. Typically, leaf colonization is restricted to particular physiologic structures. The use of scanning electron microscopy (SEM) revealed the stomata, base of the trichomes, the grooves along veins and the more depressed portions of the leaf as being common sites of colonization (Delaquis et al., 2007; Beattie and Lindow, 1995; Beattie and Lindow, 1999; Romantschuk et al., 1996; Carmichael et al., 1999; Seo and Frank, 1999; Takeuchi and Frank, 2000). Seo and Frank (1999) employed CSLM to observe *E. coli* O157:H7 attachment on the leaf surface, stomata, trichomes and cut edges of lettuce leaves. They observed that most stomata contained the pathogen as well as an obvious preference for attachment at the site of damaged tissue. Takeuchi et al. (2000) also reported preferential attachment along cut edges or damaged tissues of lettuce leaves. Furthermore, Takeuchi et al. (2000) revealed that *E. coli* O157:H7 will begin

attaching to less preferred sites once the preferred sites for attachment have reached capacity.

Pseudomonas syringae (*P. syringae*) is a plant pathogen that is capable of extensive proliferation on the surface of plant leaves (Suslow, 2002). In the event that the leaf surface is moist, *E. coli* is capable of surviving on leaves to the same extent as *P. syringae*. The same study identified the survival rate of *E. coli* on leaves exposed to dry conditions to be only approximately 0.1% of the population, while *P. syringae* strains had a far superior survival rate at greater than 30% (Suslow, 2002). These results indicate the reduced ability of *E. coli* to survive on the leaf surface under conditions that are not optimum for survival and proliferation. However, given the correct environmental conditions, *E. coli* has the capacity to utilize available nutrient sources on the surface of leaves.

Suslow (2002) hypothesizes the behavior of *E. coli* on the surface of leaves to be indicative of stress sensitivity to particular environmental stresses often experienced by plants, including dry conditions. Because *E. coli* is likely found on leaves as a result of contaminated water, soil or feces; plant surfaces are not a natural habitat for this pathogen. As a result, *E. coli* may not be well equipped with the proper stress survival characteristics to withstand certain environmental conditions associated with plant life.

The leaf of a plant is a hydrophobic surface which may interfere with bacterial adhesion. While specific mechanisms are not known, adhesion may be a two–step process. Rapid adhesion is the first step in the process and is initiated by bacteria upon contact with the leaf (Beattie, 2002). The negative

surface potential found on both the surface of the leaf and the microbial cell may cause electrostatic repulsion to develop; keeping the two surfaces separated (Romantschuk et al., 1996). Because of this, bacterial adhesion may be facilitated by pili or other bacterial structures (Beattie, 2002; Romantschuk et al., 1996). The pili aid in overcoming the electrostatic repulsion by extending from the microbial cell and binding to the plant surface. To achieve contact, attraction forces are utilized by the microbe structures, including hydrophobic interactions, hydrogen bonding and/or van der Waals forces (Romantschuk et al., 1996). Proteins or carbohydrates on the bacterial cell surface may also play a role in this initial adhesion (Beattie, 2002). Early attachment appears to be dependant upon the properties of the microbe surface, including the presence of pili and other structures (Romantschuk et al., 1996).

The presence of bundle–forming pili on the surface of *E. coli* O157:H7 cells suggests that this pathogen may employ these same tactics to attach to spinach leaves and other produce (Warriner et al., 2003). Similar to intestinal epithelial cells, pili have been associated with the attachment of *E. coli* O157:H7 to alfalfa sprouts (Brandl, 2006). These findings provide understanding as to how a human pathogen is capable of taking up residence in a non–host environment.

Bacterial attachment to plant surfaces is further facilitated by the production of an exopolysaccharide or exopolymeric substance (EPS) by the microbe (Beattie, 2002; Ramey et al., 2004). Exopolysaccharide is a polysaccharide with the consistency of jelly, allowing for bacterial adherence to

leaves and other surfaces. The production of EPS has been demonstrated using scanning electron microscopy (Beattie and Lindow, 1995; Beattie and Lindow, 1999; Romantschuk et al, 1996; Morris et al., 1997). Scanning electron micrographs of leaves have illustrated strands of an unstructured substance surrounding bacterial cells (Beattie and Lindow, 1999; Beattie, 2002) and large aggregates of bacteria (Beattie and Lindow, 1999; Beattie, 2002; Morris et al., 1997). The bacteria associated with the aggregates are tightly packed and clearly fixed within a material that lacks form or shape and is assumed to be EPS produced by the bacteria themselves (Beattie and Lindow, 1999). Bacterial production of EPS is further supported by evidence that the contact of bacteria with a solid surface has been found to induce genes responsible for the production of EPS (Beattie, 2002). Once produced, the EPS aids in anchoring the bacterial cells to the surface of the leaves, completing the colonization process.

Biofilms on Plant Leaves

The production of EPS by microorganisms in the aggregated state not only aids in attachment, but also leads to the formation of a biofilm. A biofilm is a community of microorganisms that are attached to a solid surface, or one another, and encased within an exopolysaccharide matrix (Fujishige et al., 2006). Therefore, the production of EPS by microorganisms in the aggregated state not only aids in attachment, but also leads to the formation of a biofilm.

In nature, bacteria and other microorganisms exist naturally in the biofilm state (Fujishige et al., 2006; Ramey et al., 2004; Rodríguez–Navarro et al., 2007). Of the total bacterial populations that naturally reside on the surface of leaves, 30–80% of the entire populations have been found in the aggregated state. These biofilm structures have measured several hundred microns long and are housed within EPS (Morris and Monier, 2003).

The biofilm exists as a defense mechanism which allows microorganisms to withstand conditions that are less than optimum, or even deadly. Because the phyllosphere is exposed to numerous conditions considered harsh for bacteria, the development of a biofilm is highly advantageous for leaf–dwelling microbes. Fluctuations in temperature and relative humidity, ultraviolet radiation from the sun and the lack of ample moisture and nutrients are all factors that reduce cell viability on plant surfaces (Delaquis et al., 2007; Warriner et al., 2003; Brandl, 2006; Suslow, 2002; Beattie and Lindow, 1999).

The production of EPS is one key contributing factor to improved survival within a biofilm. On the plant surface, EPS is believed to prevent dehydration of bacterial cells (Romantschuk et al., 1996; Beattie and Lindow, 1995; Beattie and Lindow, 1999; Carmichael et al., 1999) and improve conditions for survival. Biofilms have been known to resist the actions of antibiotics, protect cells from predation, obtain nutrition from dilute sources by concentrating nutrients and defend against lytic enzymes and other inhibitory agents (Beattie and Lindow, 1999), including sanitizers (Carmichael et al., 1999). These biofilms are typically multi–species and consist of both gram negative and gram positive bacteria; as

well as filamentous fungi and yeasts (Carmichael et al., 1999; Morris and Monier, 2003).

A study determining the effects of low relative humidity on bacteria present on the surface of bean leaves revealed improved survival for bacteria present in aggregates, specifically large aggregates. Individual bacterial cells and cells associated with small aggregates died after exposure to recurring dehydration stress. Bacterial cells associated with larger aggregates experienced a higher survival rate. The plants were exposed to low relative humidity conditions three times, resulting in 0.2% of total live cells surviving as individual cells and over 90% of the total live cells surviving in aggregates of greater than or equal to 100 cells. (Morris and Monier, 2003)

Microorganisms associated with biofilms are symbiotic and have distinct physiological differences from the same cell in its planktonic state (Ramey et al., 2004; Beattie and Lindow, 1999). Cells housed within a biofilm are in close proximity to one another allowing for improved communication between cells (Ramey et al., 2004). This cell–cell communication is referred to as quorum sensing and is a density–dependant phenomenon. Quorum sensing has been shown to play a significant role in plant–associated microbes ability to determine the density of surrounding inhabitants and form a biofilm (Fujishige et al., 2006). It is also known that quorum sensing is responsible for inducing virulence genes, including those associated with EPS production (Beattie and Lindow, 1999). Once a biofilm is formed, associated cells alter their metabolism relative to their location within the biofilm community, respond to diffusion gradients of waste

products and nutrients and communicate with other cells (Ramey et al., 2004). Additionally, due to adaptations in physiology, biofilm cells display increased tolerance to antimicrobials in comparison to their planktonic counterparts (Ramey et al., 2004).

This highly resistant nature of biofilms presents a serious problem for the spinach industry. Due to their ability to resist the actions of antimicrobials and sanitizers, removal of biofilms from the surface of spinach leaves is highly unlikely using the typical post–harvest wash. These biofilms have been demonstrated on the surface of spinach leaves (Beuchat, 2002) and may harbor pathogenic bacteria, including *E. coli* O157:H7, posing a tremendous threat to the safety of fresh spinach. A study conducted by Seo and Frank (1999) utilized confocal scanning laser microscopy (CSLM) to illustrate the colonization of *E. coli* O157:H7 and *Pseudomonas fluorescens* (*P. fluorescens*) on lettuce leaves. The CSLM images revealed that *P. fluorescens* adhered to undamaged leaf surfaces and produced a biofilm. *E. coli* O157:H7 attached to the stomata and cut edges of the leaf but did not adhere to the *P. fluorescens* biofilm.

Inadequate sanitation in fresh produce processing plants can result in biofilm formation (James, 2006) on processing equipment as well as in environmental areas. These biofilms are most likely formed by bacteria that originate from the fresh spinach. Poor sanitation may cause the establishment of biofilms on equipment surfaces (James, 2006) that can lead to cross–contamination of fresh spinach and other produce. Whether they are produced by pathogenic or non–pathogenic bacteria, biofilms in the processing

environment typically have the ability to resist the effects of cleaners, sanitizers and heat treatment (James, 2006). For these reasons, biofilms must be considered when designing food safety programs for the spinach industry.

Internalization of Bacteria

Multiple studies have illustrated the internalization of enteric pathogens into multiple produce products through natural plant openings (Solomon et al., 2006). Some studies report the internalization of human pathogens through the vascular system of the plant during growth (Warriner et al., 2003; Hora et al., 2005). This occurs when *E. coli* O157:H7 is present in the soil and enters the plant through the root tissue (Hora et al., 2005). Once in the root tissue, pathogens can then be transported to the leaves by means of the vascular system (Hora et al., 2005). Other research has established that internalization can occur within sprouted seeds (Warriner et al., 2003). Some studies claim that *E. coli* O157:H7 internalization is limited to seedlings and is far less common in mature plants. It has been hypothesized that mature plants have defense mechanisms against the invasion of bacteria, making seedlings more susceptible than mature plants to bacterial internalization (Hora et al., 2005).

According to Takeuchi et al. (2000), a high rate or respiration in lettuce plants reduced the internalization of *E. coli* cells by producing a "counterforce." Warming the lettuce during particular stages of fresh–cut lettuce production should encourage respiration and decrease the amount of bacterial

internalization from the wash water (Bartz, 2006). Therefore, promoting tissue respiration may be one technique for controlling internalization (Bartz, 2006).

Entrance into plant tissues is commonly believed to occur at the site of plant damage or through natural plant openings. The stomata, broken trichomes, scars on stems and damage to the waxy cuticle lining the surface of the leaf have been identified as possible points of entry (Solomon et al., 2006; Warriner et al., 2003;). A study conducted by Seo and Frank (1999) identified penetration of *E. coli* O157:H7 along the cut edges of lettuce leaves after 24 hours of incubation in a suspension of bacterial cells. Little penetration was observed among bacteria adherent to the intact lettuce leaf surface. *E. coli* O157:H7 cells gathered in most stomata of the leaf, but penetration of the leaf tissue was not observed. Takeuchi et al. (2000) also reported the penetration of *E. coli* O157:H7 cells along the cut edges of the lettuce leaves. However, they did not observe the internalization of bacterial cells into cells of the lettuce leaves. They identified the plasma membrane located beneath the cell wall of plant cells as being a potential barrier against internalization of *E. coli* O157:H7.

Takeuchi et al. (2001) studied the effects of temperature and modified atmospheric conditions on the internalization of *E. coli* O157:H7 into lettuce. They discovered that both the attachment and penetration of the pathogen were decreased when inoculated under 2.7% oxygen in comparison to 21% oxygen. Additionally, inoculum temperature at both 4^{o}C and 22^{o}C resulted in greatest penetration under 21% oxygen than 2.7% oxygen, with the highest level of penetration occurring under 21% oxygen at 4^{o}C. These results indicate that

oxygen levels and temperature play a role in the attachment and internalization of *E. coli* O157:H7 into lettuce tissues.

Hora et al. (2005) investigated mechanical root disruption as a means to favor the uptake of *E. coli* O157:H7 into the edible portions of spinach plants. The seminal root and roots hairs of spinach plants were cut and placed into soil inoculated with *E. coli* O157:H7. The data obtained proved that mechanical disruption did not support internalization of the pathogen into the edible portions of the spinach plant. The investigators hypothesized these findings to be the result of antimicrobial compounds released from the damaged root tissue, interfering with the viability of *E. coli* O157:H7 cells.

In a study conducted by Solomon et al. (2002a), internalization was observed in lettuce grown in contaminated soil. Fresh cow manure was inoculated with *E. coli* O157:H7, mixed with soil and used to plant green ice lettuce seeds. Seedlings were sampled and CSLM revealed the pathogen up to 45 μm below the surface of the lettuce leaf tissue. These findings indicate that *E. coli* O157:H7 cells are capable of relocating from the soil upward to internal locations, including the edible leaf portions of the plant. A similar study contradicted the results obtained by Solomon et al. (2002a). Johannessen et al. (2005) did not identify *E. coli* O157:H7 on lettuce seedlings grown in contaminated soil. They hypothesized their results may have been associated with bacterial cell concentration in the soil and time of infection.

Internalization poses a serious threat to the produce industry as a whole. Once internalized, bacteria are inaccessible to surface sanitizing agents and are

protected from environmental stresses (Bartz, 2006; Aruscavage et al., 2006). Since 1945, it has been known that concentrations of chlorinated water exceeding 5,000 parts per million (ppm) are incapable of eradicating plant pathogens from the inoculated wounds of fresh fruits and vegetables (Bartz, 2006). This understanding may provide evidence as to the ineffectiveness of currently employed postharvest wash treatments (Solomon et al., 2002a; Hora et al., 2005; Seo and Frank, 1999; Takeuchi et al., 2000).

Current Post–Harvest Interventions

The spinach industry employs a post–harvest washing system for product disinfection. With up to 90% of processors utilizing chlorine in the wash water (Pirovani et al., 2000), chlorine is the most common sanitizing agent utilized as a wash treatment in the produce industry (Sapers, 2006; Fonseca, 2006; Heard, 2002; Behrsing et al., 2000; Pirovani et al., 2004; Takeuchi et al., 2000). Three types of chlorine have been accepted for use on fresh spinach including; sodium hypochlorite (NaOCl), chlorine gas (Cl_2) and calcium hypochlorite ($CaClO_2$) (Sapers, 2006; Fonseca, 2006; Gil and Selma, 2006). The efficacy of chlorine washes is dependant upon the level of free chlorine available in the wash water solution. The temperature, pH and level of organic material present in the wash water are also of great importance (Fonseca, 2006; Gil and Selma, 2006).

Despite the minimal antimicrobial action achieved, chlorine is mainly used because of its ease of use and low cost (Fonseca, 2006). Some reports have identified a reduction of initial microbe populations using a chlorine wash

(Fonseca, 2006). However, no significant differences were detected after a few days amongst water–washed and chlorine–washed lettuce (Beuchat and Brackett, 1990; Fonseca, 2006). Washing produce with water is typically effective at reducing initial microbial populations on the product surface by a mere 1 log (Heard, 2002; Rodgers et al., 2004). Similarly, it has been generally accepted that the implementation of post–harvest chlorine washes achieves a reduction of microbial populations by <2 logs (Warriner et al., 2003). Work conducted by Beuchat (1999) indicated that spraying with water was just as effective at reducing *E. coli* O157:H7 populations as spraying with 200 ppm chlorine. These examples suggest that water is as effective as chlorine when applied to the surface of produce as a post–harvest intervention.

Chlorine belongs to the halogen group and is a highly effective oxidizing agent. At room temperature, chlorine is a yellow–green gas with a potent odor that can cause irritation. When used as a sanitizing agent, the term chlorine refers to the existence of active, also known as oxidized, chlorine compounds that are produced in water (McDonnell, 2007). Such active compounds include chlorine gas (Cl_2), hypochlorite ion (OCl^-), and hypochlorous acid (HOCl) (Sapers, 2006; McDonnell, 2007; Varoquaux and Mazollier, 2006). The ratio in which these compounds are produced is dependant on the pH of the solution (Sapers, 2006). The antimicrobial properties associated with chlorine are attributed to the combination of these compounds and contribute to the level of free available chlorine. Free available chlorine refers to the sum of hypochlorite ion and hypochlorous acid concentrations (Sapers, 2006). However, HOCl is

recognized as being the predominantly effective agent (Sapers, 2006; McDonnell, 2007; Beuchat, 2000) and is typically referred to as free or available chlorine (Beuchat, 2000; Gil and Selma, 2006; Pirovani et al., 2000).

The extent to which chlorine is effective as an antimicrobial agent depends greatly on temperature, pH, contact time and chlorine concentration (McDonnell, 2007; Gil and Selma, 2006; Heard, 2002; Pirovani et al., 2000). Increasing temperatures and chlorine concentrations are positively correlated with efficacy (McDonnell, 2007). However, increasing water temperatures have also been known to cause increasing levels of chlorine to vaporize from the wash solution, thereby decreasing efficacy (Beuchat, 2000). A decrease in chlorine efficacy occurs at higher pH levels as a result of increased HOCl dissociation and OCl^- production (McDonnell, 2007). Hypochlorites are known to increase the pH of wash water and are, therefore, associated with decreased efficacy (Varoquaux and Mazollier, 2002). This increase in pH is caused by the formation of sodium hydroxide (NaOH) (Gil and Selma, 2006). A pH between 4 and 7 produces optimum conditions, as this creates favorable conditions for the production of HOCl as the dominant chlorine compound. Additionally, chlorine efficacy is vulnerable to the presence of reducing agents, including copper and iron ions. Such compounds increase dissociation; causing a reduction in available chlorine. Antimicrobial activity of chlorine is also degraded by ultraviolet light, organic material and proteins (McDonnell, 2007). As levels of organic matter increase, the concentration of HOCl decreases as combined chlorine is formed, thereby decreasing efficacy (Gil and Selma, 2006).

While maximum chlorine solubility is at 4^{o}C, the ideal temperature for chlorine washes used in the produce industry is no less than 10^{o}C greater than the surface temperature of the produce. This standard creates a positive temperature differential between the wash solution and the produce. Such a temperature differential reduces the potential for chlorine uptake through stems, scars, natural openings and skin of produce. (Beuchat, 2000; Gil and Selma, 2006)

Chlorine is recognized as a broad–spectrum antimicrobial that is acknowledged as being highly effective on planktonic bacterial cells, viruses, molds and yeasts (Sapers, 2006). The production of HOCl is responsible for the vast bactericidal capabilities against a wide range of microorganisms that is characteristic of chlorine (Gil and Selma, 2006). Conversely, chlorine is considerably less effective at inactivating bacterial cells that are attached to the surface of produce and those which are encased or internalized within the product (Sapers, 2006).

Chlorine lethality is believed to be the result of numerous modes of action, including the oxidation of lipids, carbohydrates and proteins (McDonnell, 2007). The main antimicrobial mechanism attributed to chlorine is attack against proteins (McDonnell, 2007; Takeuchi et al., 2000), both functional and structural. Attack against proteins on the surface of the microorganism or found internally may be the principal mechanism of action. Additional effects cause disruption of the cell wall and membrane by attacking the lipids, carbohydrates and structural proteins (McDonnell, 2007).

Chlorine bactericidal activity is also associated with the formation of N–chloro compounds produced as a result of HOCl combining with cell membrane proteins (Beuchat, 2000). These compounds interfere with the oxidation of glucose or sulfhydryl groups (Beuchat, 2000; McDonnell, 2007). The presence of chlorine also damages membrane permeability and interferes with the transport of extracellular nutrients (Beuchat, 2000).

Hypochlorous acid is responsible for the antimicrobial effects of chlorine on *E. coli*, even at low concentrations. Inhibition of growth was observed at approximately 3 ppm of free chlorine after five minutes. DNA synthesis was nearly completely inhibited with only partial inhibition of protein synthesis. Additionally, there was no obvious interruption to the cell membrane. This suggests that synthesis of intercellular DNA is especially sensitive to the effects of chlorine at inhibitory concentrations. (McDonnell, 2007)

While chlorine has a broad spectrum of antimicrobial activity and is effective at low concentrations, efficacy on spinach and leafy greens is limited. Efficacy against *E. coli* O157:H7 may be further decreased when contamination occurs by way of bovine feces, as exposure to organic matter further decreases the effectiveness of chlorine (Beuchat, 1999). A study conducted by Beuchat (1999) revealed that *E. coli* O157:H7 applied to lettuce via bovine feces at an initial population of 1.1 CFU/g remained viable on lettuce held at 4^{o}C for at least 15 days. Spray treating the lettuce with 200 ppm chlorine was not more effective at eradicating viable *E. coli* O157:H7 populations than spraying with water. Conversely, samples with initial populations of *E. coli* O157:H7 at 10^1 to 10^2

CFU/g were significantly reduced after treatment with 200 ppm chlorine in comparison with the water control. In the same study, Beuchat (1999) also conducted a three–dimensional volume reconstruction of the interior sections of lettuce leaves and illustrated that *E. coli* O157:H7 cells were housed 20 to 100 μm below the surface in cut edges and stomata of the leaves. Therefore, chlorine and other hydrophobic sanitizers can not be expected to effectively eliminate any microbial cells that become entrapped in such areas.

Seo and Frank (1999) agree that microbes surviving the effects of chlorine treatment are likely attached to protective sites on the leaf. Their study utilized CSLM to visualize the location of *E. coli* O157:H7 on lettuce leaves. They discovered that the pathogen had associated with the stomata of the leaf tissue, but did not penetrate into the interior of the leaf. However, penetration was not required for protection, as these cells were not inactivated by chlorine.

A similar study employing CSLM in lettuce leaves was conducted by Takeuchi et al. (2000). Their work supports the findings of their colleagues that association with the plant stomata and penetration into damaged tissues protect *E. coli* O157:H7 from the lethal effects of chlorine. They stressed the importance of plant structural components in cell attachment, as well as protection from inactivation.

Other sanitizers utilized in the produce industry due to their efficacy in removing pathogens include organic acids, ozone, trisodium phosphate and hydrogen peroxide. Similar to chlorine, none of these sanitizing applications have achieved greater than a 2 log CFU/g reduction in microbial populations at

concentrations not considered to adversely affect sensory characteristics. Additionally, application of most interventions listed would not be fitting in the household environment. (Beuchat et al., 1998)

Lin et al. (2002) studied the use of hydrogen peroxide (H_2O_2) and lactic acid as potential antimicrobial agents against *E. coli* O157:H7 in lettuce. A solution of 2% H_2O_2 applied to lettuce at 50^{o}C for 90 seconds was successful in reducing *E. coli* O157:H7 numbers by 4.7 $\log_{10}$ CFU per lettuce leaf. However, the combination of H_2O_2 and lactic acid was just as, or more, effective than H_2O_2 alone. *E. coli* O157:H7 populations declined by 4.7 $\log_{10}$ CFU per lettuce leaf when exposed to a combination treatment of 1.5% lactic acid and 1.5% H_2O_2 at 40^{o}C for 15 minutes. Similarly, a solution composed of 1.5% lactic acid and 2.0% H_2O_2 at 22^{o}C was applied for 5 minutes and resulted in a decline of 4.8 $\log_{10}$ CFU per lettuce leaf in *E. coli* populations.

Successfully treating spinach and other leafy greens to improve food safety proves to be a challenge. The unique nature and physiologic structure of spinach lends itself to harboring microorganisms in locations beyond the reach of chlorine and other sanitizers. While various post–harvest treatments have indicated some success in reducing *E. coli* O157:H7 in the leafy greens industry, the majority of research presented does not boast population reductions beyond 1 to 2 $\log_{10}$ CFU/g. For this reason, further research is required to develop more effective treatment programs aimed at improving population reductions.

The Lactic Acid Bacteria

Overview of the Lactic Acid Bacteria

Microorganisms associated with the lactic acid bacteria (LAB) are gram–positive rods and cocci capable of facultative anaerobic growth (Madigan et al., 2003). Members of this group are known as fermentative microorganisms that produce lactic acid as a primary or exclusive product of glucose fermentation (Madigan et al., 2003; Carr et al., 2002). Lactic acid bacteria are a diverse group, comprised of numerous different genera; all associated with hexose (six carbon sugar) fermentation to produce lactic acid (Chen, 2002). The microorganisms most commonly associated with LAB include *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella* (Chen, 2002).

Based on the end product of fermentation, these genera are further classified as either homofermenters or heterofermenters. Homofermenters produce lactic acid as the primary product of glucose fermentation while heterofermenters generate numerous products in addition to lactic acid, some of which include acetic acid, carbon dioxide, and ethanol. *Streptococcus, Lactobacillus, Lactococcus* and *Pediococcus* are the genera classified as homofermentative LAB. A subgroup of *Lactobacillus* known as the Betabacteria and members of the *Leuconostoc* genera belong to the heterofermentative group of LAB. (Carr et al., 2002; Madigan et al., 2003)

Homofermenters are capable of producing lactic acid more directly because these microorganisms possess the key glycolysis enzyme, aldolase (Carr et al., 2002; Madigan et al., 2003). Conversely, heterofermenters ferment by utilizing the alternate pentose monophosphate pathway (Madigan et al., 2003) and the enzyme phosphoketolase to convert hexoses (Carr et al., 2002; Madigan et al., 2003), such as glucose 6–phosphate, first to 6–phosphogluconate. Next, decarboxylation of 6–phosphogluconate results in the production of pentose phosphate, a five–carbon sugar. Phosphoketolase acts on pentose phosphate, breaking it down to triose phosphate. Triose phosphate is then converted to lactic acid, an end product of heterofermentative fermentation (Madigan et al., 2003).

Antimicrobial Properties of Lactic Acid Bacteria

As a result of their unique fermentative qualities, LAB have been used for centuries to produce stable foods (Bennik et al., 2000). Lactic acid bacteria fermentations may be the oldest form of food preservation and are often referred to as biopreservation. Biopreservation is defined as “the use of LAB, their metabolic products, or both to improve or ensure the safety and quality of foods that are not seen as typical fermented foods” (Montville et al., 2001; Montville et al., 2005). Because LAB have been used to a great extent in foods without the occurrence of any negative health effects they have been given the status of generally recognized as safe (GRAS) (Bennik et al., 2000). More specifically, *Lactococcus* and *Lactobacillus* are most frequently given GRAS status, while

some opportunistic pathogens are associated with *Enterococcus* and *Streptococcus*, in addition to various other genera of LAB (Salminen et al., 1998).

Biopreservation is based on the idea that LAB proliferates within the food, competing with resident microorganisms. The metabolic activities of LAB result in the production of either specific or a variety of antimicrobial compounds that are capable of suppressing or inhibiting the growth of pathogens (Sanz et al., 2007) and a variety of different microorganisms, including fungi, bacteria and yeasts (Bennik et al., 2000). Such compounds consist of hydrogen peroxide, organic acids, lytic agents, bacteriocins or antimicrobial peptides, diacetyl, defective phages and enzymes (Bennik et al., 2000; Nes and Johnsborg, 2004). Each antimicrobial compound is not necessarily produced by all LAB, nor are they equally effective. For example, organic acids, such as lactic acid, are effective against a wide range of microorganisms while bacteriocins are spectific to a more narrow range (Bennik et al., 2000).

Bacteriocins are synthesized in the ribosome of a wide variety of bacteria, including some LAB (Bennik et al., 2000; Nes and Johnsborg, 2004; Montville et al., 2001; Montville et al., 2005). They are antimicrobial peptides capable of action against both spoilage and pathogenic bacteria (Montville et al., 2001; Montville et al., 2005; Mulet–Powell et al., 1998; Amézquita and Brashears, 2002; Smith et al., 2005). Inhibition of LAB against these two groups of bacteria can occur during growth and while stored at refrigerated temperatures, both in food products and associative cultures (Amézquita and Brashears, 2002; Smith et al., 2005). While potent in nature, bacteriocins have a fairly constricted array

of inhibition (Diep and Nes, 2002). The primary genera associated with bacteriocin production include *Streptococcus, Lactococcus, Leuconostoc, Lactobacillus* and numerous *Enterococcus* (Aslim et al., 2005).

Bacteriocins are effective against bacteria similar in nature without acting on the bacteria that it was produced by. Because LAB are gram–positive, their bacteriocins are most effective against other gram–positive bacteria. Gram–negative bacteria are susceptible to the effects of bacteriocins if injured or exposed to hydrostatic pressure or chelating agents. (Montville et al., 2001; Montville et al., 2005; Schillinger et al., 1996)

Disruption of the cytoplasmic membrane of susceptible bacteria is the mechanism of action for LAB bacteriocins. Apertures are formed in the membrane (Kostrzynska and Bachand, 2006; Montville et al., 2001; Montville et al., 2005) resulting in increased permeability to small compounds. This causes a rapid efflux of amino acids, previously accumulated ions and, in some cases, molecules of adenosine triphosphate (ATP). As these compounds reach equilibrium across the membrane, the electrical and chemical gradients essential for critical cellular tasks become obliterated (Montville et al., 2001; Montville et al., 2005).

Bacteriocins have occasionally been shown to lead to resistance in specific bacteria. This sporadic resistance is likely due to intrinsic characteristics of the cell. It is believed that the cells are capable of changing the composition of lipids in their membrane, reducing the effects of the bacteriocin. Whether or not

this mechanism is the result of spontaneous mutations is not understood. (Nes and Johnsborg, 2004)

The production of lactic acid and other organic acids by LAB is also beneficial for controlling microbial growth. Organic acid mechanism of action is similar to that of bacteriocins in that a disruption of the cytoplasmic membrane occurs. This disruption interferes with the maintenance of the membrane as well as active transport. Additionally, the presence of acid lowers the pH, resulting in an acidic environment that improves acid lethality and the inhibition of pathogen growth (Kostrzynska and Bachand, 2006). For gram–negative cells, the LPS layer surrounding the cytoplasmic membrane and peptidoglycan layer is sensitive to the actions of lactic acid and its salts. Lipopolysaccharide is fixed within the outer membrane due to its bonds with membrane proteins. Lactic acid and its salts are excellent at disintegrating the outer membrane by releasing LPS. This changes membrane permeability and allows for increased activity of other antimicrobial compounds (Belifore et al., 2007).

Ultimately, inhibition of pathogenic growth occurs because LAB are highly effective microbial antagonists. These bacteria have the ability to outcompete other microorganisms for nutrients and residency. There is a complimentary effect by the production of acid and antimicrobial compounds that increases inhibition of both pathogen and spoilage bacteria. (Kostrzynska and Bachand, 2006)

Bovamine® Meat Cultures

Research for the development of Bovamine® Meat Culture, a commercially available combination of LAB product that is produced by Nutrition Physiology Corporation, was completed at the University of Nebraska (Smith et al., 2005) and Texas Tech University (Nutrition Physiology Corporation, 2005). The focus of the research was to develop a product that inhibits both *E. coli* O157:H7 and *Salmonella* in ground beef products as well as *Listeria monocytogenes* in ready–to–eat meat products without causing spoilage or unfavorably affecting sensory properties (Nutrition Physiology Corporation, 2005). A great deal of effort was focused on identifying multiple strains of LAB that work in a synergistic manner, producing inhibitory substances under refrigerated conditions without growing (Nutrition Physiology Corporation, 2005).

The product is comprised of four LAB strains, including *Lactobacillus acidophilus* (NP 51), *Lactobacillus cristpatus* (NP 35), *Pediococcus acidilactici* (NP 3) and *Lactobacillus lactic* subsp. *lactis* (NP 7). Strains NP 51 and NP 35 were originally isolated from cattle, while NP 7 was isolated from alfalfa sprouts and NP 3 from cooked hot dogs (Smith et al., 2005). The lethal effects of this four–strain combination of LAB have proven effective against both *E. coli* O157 and *Salmonella.* In ground beef, after three days and five days of refrigerated storage, *E. coli* O157 counts declined by 2 and 2.5 log cycles, respectively. Similarly, *Salmonella* in ground beef was decreased by 3 logs and to non–detectable levels after three and five days of refrigerated storage, respectively (Smith et al., 2005; Nutrition Physiology Corporation, 2005).

Studies performed on the sensory characteristics associated with the application of Bovamine® Meat Culture in ground beef revealed promising results. A triangle test, utilizing 24 non–trained panelists, indicated no significant difference between control ground beef and ground beef treated with Bovamine® Meat Culture. These results were supported by the pH values of both uncooked and cooked ground beef samples at day 3 of refrigerated storage. No significant differences in pH were detected between the control and Bovamine® Meat Culture samples, both uncooked and cooked. (Smith et al., 2005).

A similar sensory study was conducted by Amézquita and Brashears (2002). They evaluated the effects of LAB on ready–to–eat meat products. They also executed a triangle test to determine whether an isolate *Pediococcus acidilactici* resulted in a significant difference between LAB and control frankfurters. Triangle tests were conducted on the frankfurters 9 times throughout the 56–day storage period. The number of correct responses obtained during each test was less than the number required for statistical significance. Therefore, they concluded that the application of *P. acidiliactici* does not result in a significant difference between treated and control frankfurters.

Bovamine® Meat Culture was granted GRAS status for use in ground beef by the FDA in December of 2005 (Nutrition Physiology Corporation, 2005). This provides ground beef processors with an effective intervention that is capable of inhibiting *E. coli* O157 and *Salmonella* at refrigerated temperatures without adversely affecting sensory characteristics. Because of the great success with

this product in the meat industry, application as a post–harvest intervention in the spinach industry will be the focus of this thesis research.

Applications for LAB in the Food Industry

Lactic acid bacteria are perhaps most commonly known for their role in the fermentation of various dairy products. Hard and soft cheeses, yogurt, cottage cheese, buttermilk and acidophilus are examples of products manufactured using LAB starter cultures. In addition to antimicrobial compounds, LAB fermentations produce various flavor–enhancing and aromatic substances such as diacetyl and aldehyde, which are produced by heterofermentative LAB. It is for this reason that heterofermenter LAB are typically used in the dairy industry. (Carr et al., 2002)

The consumption of various strains of LAB for the health benefits they impart has become a fairly new trend. Probiotics are described as "live microorganisms that when ingested in sufficient quantities exert positive effects on the host's health" (Sanz et al., 2007). Elie Metchnikoff first recognized that LAB may play a role in improving human health in 1907. In his book, *Prolongation of Life*, Nobel laureate Metchnikoff hypothesized that consumption of yogurt by inhabitants of the Balkans was responsible for their long life spans. He proposed that "bad" intestinal bacteria were suppressed by the bacteria present in the yogurt. The term "probiotic" was coined in the 1970's in reference to the health benefits associated with feeding microbial supplements to animals (Montville et al., 2005).

The normal microflora of the human gastrointestinal tract is comprised of over 500 different bacterial species. These microorganisms colonize the gut and maintain a natural microbial balance, creating a barrier against pathogenic infection. While aiding in the digestion and absorption of nutrients, natural gut microflora are also responsible for maintaining overall gastrointestinal health. The addition of probiotics to this microbial ecosystem works to promote the growth of native microflora and assist in the inhibiton of pathogenic colonization. (Sanz et al., 2007)

Lactic acid bacteria assist in this natural defense mechanism by producing organic acids, hydrogen peroxide and, carbon dioxide. This coupled with the decrease in pH caused by acid production results in the development of conditions unfavorable for pathogen colonization and infection. Additionally, the excellent ability of LAB to outcompete for nutrients increases the levels of beneficial bacteria while reducing pathogens and other bacteria that may upset the microbial ecosystem established in the human gastrointestinal tract (Sanz et al., 2007). Essentially, LAB are believed to provide health benefits and prevent infection in the intestinal tract in much the same way they inhibit pathogenic growth in the food supply.

De Vuyst et al. (2007) conducted a study evaluating the antimicrobial potential of probiotic and potentially probiotic LAB. They determined that the production of organic acids by lactobacilli is most likely the primary inhibitory mechanism against pathogens. They believe that organic acids are most effective at lower pHs and that other antimicrobial substances produced

contribute to the lethality of LAB. Additionally, because bacteriocins have often been isolated from bacteria associated with the *Lactobacillus acidophilus* family, the primary colonizer of the human colon, De Vuyst and colleagues (2007) suggest that bacteriocins most likely serve a purpose in the microbial community of the human gastrointestinal system.

Probiotics have also been developed and implemented as direct–fed microbials in the cattle industry. Because cattle are a noteworthy reservoir for *E. coli* O157:H7, utilizing probiotics as feed supplements may decrease the percentage of cattle that carry this pathogen and shed it in their feces. As a result, probiotic supplementation in cattle feed regimens may be an appropriate strategy to advance food safety (Younts–Dahl et al., 2005).

Loneragan and Brashears (2005) reviewed some of the most effective direct–fed microbial research conducted on cattle to date. Brashears et al. (2003a) fed a *Lactobacillus*–based direct–fed microbial to feedlot cattle and noted a 49% lower recovery of *E. coli* O157 in the feces of cattle fed the LAB product in comparison to control cattle. NP51 is believed to be the most effective strain in the direct –fed microbial that was employed. A study conducted by Younts et al. (2004) also utilized NP51. They observed *E. coli* O157 reductions of 58% in the fecal samples of NP51–fed cattle. In a related study, Younts–Dahl et al. (2005) explored the effect of dosage level on the effectiveness of NP51. Dosages of 10^7, 10^8 and 10^9 cells per head were administered and the effect averaged over the extent of the study. They determined that the 10^9 dose

resulted in the greatest reductions with a 77% less chance of recovering *E. coli* O157 in comparison to control–fed cattle.

Such significant reductions of *E. coli* O157:H7 in cattle manure may mean this probiotic application could have a direct impact on the spinach industry, as well. For example, a reduction in *E. coli* O157:H7 levels in cattle feces could significantly reduce the potential for fertilization of spinach fields with contaminated manure. Infiltration of spinach irrigation water with contaminated cattle feces may also decline. Additionally, the prevalence of contaminated air and dust as a result of dehydrated and pulverized manure particles may not be as great of a threat if contamination levels decrease with direct–fed microbial supplementation.

In an attempt to make food preservation more natural and wholesome, the fermentation capabilities of LAB have been evaluated to a great extent as potential food additives. Many studies have been completed on the application of LAB to inhibit spoilage and pathogenic growth in meat products. In particular, LAB inhibition of *E. coli* O157:H7 in meats has been illustrated with numerous studies.

One study employed *Lactobacillus reuteri* at refrigerated temperatures to control *E. coli* O157:H7 over a 25–day shelf–life study in ground beef. *L. reuteri* at concentrations of 3 and 6 log CFU/g was successful in killing the pathogen at levels of 3 and 6 log CFU/g by the end of the 25–day study (Muthukumarasamy et al., 2003). Senne and Gilliland (2003) investigated *Lactobacillus delbrueckii* subsp. *lactis* as a means to reduce *E. coli* O157:H7 on freshly slaughtered beef

and pork carcasses held at refrigerated temperatures. Their findings illustrated a significant reduction in *E. coli* O157:H7 levels as a result of *L. delbrueckii* subsp. *lactis* application. Research conducted by Brashears et al. (1998) utilized *Lactobacillus lactis* to demonstrate microbial antagonism against *E. coli* O157:H7. *L. lactis* was added at a level of $5.0x10^7$ per mL to trypticase soy broth (TSB) containing three strains of *E. coli* O157:H7 and refrigerated for 7 days. Significant decreases in pathogen levels were observed after only 3 days of storage at 7^oC. In the same study, *L. lactis* was added to raw chicken breast meat inoculated with *E. coli* O157:H7 and decreases in pathogen numbers occurred during storage at 5^oC.

While far less research has been completed evaluating the use of LAB in the fresh spinach and produce industries, the promising results obtained with meat products makes LAB an excellent candidate for post–harvest interventions in fresh spinach and produce processing. To date, studies have focused on isolating various LAB strains directly from produce products and evaluating the bacteria for microbial antagonistic abilities. However, direct application of LAB, such as Bovamine® Meat Culture, to spinach and other produce products as a post–harvest intervention to control *E. coli* O157:H7 has not been researched to any great extent, if at all. If successful at controlling pathogens such as *E. coli* O157:H7, utilizing LAB in the wash water of fresh spinach processing may prove to be a viable alternative for the produce industry.

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CHAPTER III

OBJECTIVE I

REDUCTION OF *ESCHERICHIA COLI* O157:H7 IN FRESH SPINACH USING BOVAMINE® MEAT CULTURES AS A POST–HARVEST INTERVENTION AND THE IMPACT ON SENSORY PROPERTIES

Introduction

Escherichia coli (*E. coli*) O157:H7 is a virulent pathogen that has been associated with produce in 21% of the foodborne outbreaks that occurred between 1982 and 2002 (Aruscavage et al., 2006). While *E. coli* O157:H7 is often associated with raw or undercooked ground beef (Pickering et al., 1994) awareness of the potential for fresh fruit and vegetable consumption to cause illnesses from this pathogen has increased in recent years (Delaquis et al., 2007; Johannessen et al., 2005). In the mid–1990s fresh produce was recognized as a vector for foodborne illness caused by *E. coli* O157:H7 (Delaquis et al., 2007).

Due to the nature of production, spinach is vulnerable to pathogenic contamination at every step in the production process. According to Warriner et al. (2003), post–harvest handling is believed to be the primary source of contamination. However, the same study also identified soil, water and harvest equipment as factors that may lead to the contamination of spinach plants during the growing process. As a result, it is necessary that spinach safety is emphasized throughout the entire production process.

Because fresh spinach production lacks a thermal kill–step, reliance is placed on post–harvest wash interventions to control microbial populations. Up to 90% of spinach processors utilize sodium hypochlorite (chlorine) washes as the primary barrier against pathogenic contamination (Behrsing et al., 2000). While chlorine is known to be an effective antimicrobial agent, numerous factors affect the efficacy of chlorine applied to fresh spinach, including water temperature, pH and contact time (Pirovani et al., 2004). In general, it is understood that the ability of chlorine to inactivate microorganisms present on the surface of spinach leaves is not exceptional (Lin et al., 2002). Warriner et al. (2003), stated that the efficacy of chlorine is capable of reducing total microbial populations by no more than 2 logs. Beuchat (1999) discovered that 200 parts per million (ppm) chlorinated water and deionized water were equally efficacious at killing, removing or inactivating *E. coli* O157:H7 on the surface of lettuce leaves. Lang et al. (2004) observed reductions of *E. coli* O157:H7 on lettuce leaves of only 1.10 logs in comparison to the control after treatment with 200 ppm chlorine. These minimal reductions, in combination with the lack of a thermal processing step, indicate the need for a more efficacious intervention to be implemented.

The use of lactic acid bacteria (LAB) as an intervention to control microbial growth in the food industry is not a new strategy. There are multiple properties associated with bacteria belonging to the LAB family that prove to be lethal to other bacteria, including some pathogens. Metabolism of LAB results in the production of bactericidal compounds, including hydrogen peroxide, bacteriocins,

carbon dioxide and organic acids (Kostrzynska and Bachand, 2006; Sanz et al., 2007; Schillinger et al., 1996). Production of organic acids, including lactic, propionic and acetic acid, induce lethal effects by acting on the cytoplasmic membrane of the bacterial cell (Kostryzynska and Bachand, 2006). Additionally, the creation of an acidic environment that is considered unfavorable for pathogenic growth aids in the control of *E. coli* O157:H7 (Kostryzynska and Bachand, 2006). The effects of such compounds on the sensory characteristics of fresh spinach are unknown and consumer acceptance must be determined before LAB can be implemented as a post–harvest intervention in spinach production.

Lactic acid bacteria have been successfully utilized to control *E. coli* O157:H7, and other pathogens in raw meat products (Kostrzynska and Bachand, 2006), in cooked meat products (Amézquita and Brashears, 2002) and in cattle (Brashears et al., 2003; Younts et al., 2004; Younts–Dahl et al., 2005). Therefore, LAB may be an effective intervention for the spinach industry as well.

The overall objective of this study was to determine if Bovamine® Meat Cultures, a commercially produced LAB product, can be effectively implemented as a post–harvest intervention to reduce levels of *E. coli* O157:H7 in fresh spinach and to determine if the application of Bovamine® Meat Cultures to fresh spinach resulted in a statistical difference in sensory characteristics between treated and control spinach.

Materials and Methods

Bacterial Strains

A cocktail of four *E. coli* O157:H7 strains was used for this study and included A4 966, A5 528, A1 920 and 966. All strains were isolated from cattle and originally obtained from the University of Nebraska. The cocktail was prepared by making frozen concentrated cultures of each culture as described by Brashears et al. (1998). One vial from each strain was obtained from the -80^{o}C stock culture. A sterile loop was used to add the strains to separate tubes of Brain Heart Infusion Broth (BHI) (EMD, Gibbstown, NJ). The strains were incubated overnight at 37^{o}C, transferred into fresh BHI tubes and incubated an additional night at 37^{o}C. The concentration of each strain was determined to be at the appropriate level by plating on Tryptic Soy Agar (TSA) (EMD, Gibbstown, NJ) and incubating for 24 hours at 37^{o}C. All four strains were combined in equal volumes in BHI, allowed to grow at 37^{o}C overnight and then centrifuged for 10 minutes at 4,000 g. The pellet was resuspended in BHI containing 10% glycerol and stored as a frozen culture at -80^{o}C in 1–mL portions at a concentration of 1.0x10^{9} CFU/mL in the Texas Tech University inventory.

Bovamine® Meat Culture used in this study was obtained from Nutrition Physiology Corporation (Guymon, OK). This commercially available LAB product is comprised of four LAB strains, including *Lactobacillus acidophilus* (NP 51), *Lactobacillus cristpatus* (NP 35), *Pediococcus acidilactici* (NP 3) and *Lactobacillus lactis* subsp. *lactis* (NP 7) (Smith et al., 2005). Isolates NP 51 and

NP 35 were originally isolated from cattle, while NP 3 was isolated from cooked hot dogs and NP 7 from alfalfa sprouts (Smith et al., 2005). The culture was commercially prepared and packaged in 10–g portions in a freeze–dried form prior to shipping to Texas Tech University.

Pathogen Reduction Study

Sample Preparation

Fresh bagged baby spinach was obtained from a local grocery store and weighed into a poultry rinsate bag (VWR, West Chester, PA) to ensure total weight was approximately 500 g. The four–strain cocktail of *Escherichia coli* O157:H7 was diluted 1:1000 in buffered peptone water (BPW) (OXOID, Basingstoke, Hampshire, England) to obtain a final concentration of $1.0x10^6$ CFU/mL and an inoculum volume of 5 L. The pre–weighed spinach was submerged in the inoculum and allowed to soak for 20 minutes to facilitate attachment. Using a sterile tongs, the inoculated spinach was spread evenly across sterile drying racks in a biological hood (Fisher Hamilton model #54L925, Two Rivers, WI) and allowed to dry for one hour. A LAB wash with a concentration of $2.0x10^8$ CFU/mL was prepared by combining 5 g of freeze–dried Bovamine® Meat Culture with 495 mL of sterile distilled water. The concentration of LAB was determined by making serial dilutions in buffered peptone water and plating on Lactobacilli MRS Agar (MRS) (EMD, Gibbstown, NJ). The MRS agar plates were incubated at 37°C for 24 to 48 hours. A control wash consisting of 500 mL of sterile distilled water was also prepared. Upon completion of drying,

100 g of the dry, inoculated spinach were added to the LAB rinse and 100 g to the control water rinse in sterile poultry rinsate bags. The bags were agitated for 1 minute at 230 rpm on an automatic orbital shaker (KS 260 Basic, IKA, Wilmington, NC). A third set of 100 g of dry, inoculated spinach was placed directly into a sterile Whirl–Pak (Nasco, Fort Atkinson, WI) bag to serve as the background control for this experiment. Following agitation, both rinse treatments were allowed to soak during the 0, 5 and 10 minute sampling time points. After 10 minutes, each rinse was drained in a sterile colander and transferred to sterile Whirl–Pak bags using sterile tongs. All samples were stored at 7^{o}C between sampling intervals.

Microbiological Analysis

From each rinse and the background control, 10 g of spinach were collected at 0, 5 and 10 minutes, 1, 4, 8 and 24 hours. The exact sample weight was recorded and used to determine colony forming units (CFU) on a per gram basis. At each time point, the sampled spinach was stomached (Seward Model 400, Bohemia, NY) with 90 mL of buffered peptone water at 230 rpm for 2 minutes. Homogenized samples were serially diluted and quantitatively analyzed for *Escherichia coli* O157:H7 using the Neo–Grid^{TM} Method (Neogen, Lansing, MI). Neo–Grid^{TM} filters were placed on CHROMagar (CHROMagar, Paris, France) containing tellurite at a level of 2.5 mg/L. Tellurite was added to reduce the interference from other bacteria. CHROMagar plates were incubated at 37^{o}C for 24 $\pm$ 2 hours. Mauve colonies were counted as presumptive positive for

Escherichia coli O157:H7 and agglutinated at random for confirmation using a latex agglutination kit (Remel, Lenexa, KS).

Experimental Design and Analysis

This study was classified as a complete randomized block design. The Statistical Analysis System (SAS) software was used to analyze the data. All data were subjected to the PROC MIXED and PROC UNIVARIATE commands. The Least Squares (LS) means obtained from SAS were used to identify statistical significance between each individual treatment in comparison to the control. Additionally, the LS means of the water and LAB washes were compared to identify if one treatment was significantly more effective than the other. The Shapiro–Wilk value provided by the PROC UNIVARIATE procedure was used to determine normality of the data. The experimental procedure was replicated a total of three times.

Sensory Study

Sample Preparation

Fresh bagged baby spinach was obtained from a local grocery store. All bags were combined to minimize natural variability and randomize the product. The combined spinach was then divided into three samples. One sample was rinsed with Bovamine® Meat Culture at a concentration of $2.0x10^8$ CFU/mL. The remaining two samples were rinsed with tap water and considered to be identical. All 3 samples were drained in separate colanders and distributed into sample cups labeled with their respective three–digit sample number. The samples were

placed on a tray, covered with aluminum foil and held in the refrigerator at 4^{o}C for approximately 2 hours before serving to panelists.

Experimental Design and Analysis

Appendix B contains the approved proposal submitted for the use of human subjects, as well as the recruitment material utilized for the study. Forty panelists were chosen at random to participate in the sensory study. All panelists were presented with the three samples simultaneously in a triangle test. They were instructed to evaluate each sample from left to right and identify the one sample they perceived to be different. The order in which the samples were presented to the panelists was randomized in order to decrease bias. Panelists were provided with a cracker, water and expectorant cup to clear their palate between samples. An answer sheet was supplied and panelists were encouraged to include comments (Appendix B).

Statistical significance of sensory data was evaluated using the statistical tables published in the third edition of Sensory Evaluation Techniques (Meilgaard et al., 1999). These tables were utilized to determine if statistically significant differences existed in the sensory characteristics of spinach treated with lactic acid producing–bacteria by comparing the number of responses identifying the correct “odd” sample to alpha values of 0.5 and 0.1. Additionally, the number of discriminators was calculated using methods described in chapter 5 of the first edition of Sensory Evaluation of Food (Lawless and Heymann, 1999). Discriminators are defined as those individuals who saw the true difference and

selected the correct "odd" sample. It is speculated that the remainder of participants who selected the LAB sample merely guessed and were not able to perceive the true difference. All calculations are included in Appendix C.

Results & Discussion

Pathogen Reduction Study

Because there were no time by treatment interactions, Figure 3.1 represents the LS means of all data points composited for each treatment. As illustrated by this figure, both water ($p<0.0001$) and LAB ($p<0.0001$) resulted in significant reductions in comparison to the control. Water reduced *E. coli* O157:H7 levels by 0.88 logs, whereas LAB was successful at reducing it by 1.03 logs (Figure 3.1). The improved reduction of LAB was significantly different than that of water ($p=0.0363$). This indicates that LAB was significantly more effective at reducing *E. coli* O157:H7 populations on baby spinach leaves than water when comparing the composite LS means of each treatment.

While little research has been conducted evaluating the effectiveness of LAB as an intervention for fresh spinach, the use of LAB in ground beef has been investigated. Smith et al. (2005) utilized the same combined cultures included in Bovamine® Meat Cultures as an intervention to reduce the presence of *E. coli* O157:H7 in ground beef. The cultures were added to ground beef at a level of 10^9 CFU/g and stored at 5°C for 14 days. The combined cultures significantly reduced *E. coli* O157:H7 levels by 2.0 logs and 3.0 log cycles after 3 and 5 days of storage, respectively.

Given the proven effectiveness of these LAB cultures in other food products, Bovamine® Meat Cultures may have great potential for application in the spinach industry, as well. The LAB spinach was evaluated for a mere 24 hours and resulted in reductions of 1.55 logs compared to the control at the 24 hour sampling time (Table 3.1). If the LAB–treated spinach had been held at 7°C for longer than 24 hours, perhaps the population of *E. coli* O157:H7 would have continued to decline in comparison to the control spinach and ultimately achieved reductions similar to those found by Smith et al. (2005) in ground beef. Additionally, differences in the nutrient availability of the two products may play a role in the effectiveness of Bovamine® Meat Cultures. Meat is a nutrient dense environment with a high water activity (Labadie, 1999), while the surface of spinach leaves has low water availability and is rather nutrient poor in comparison to within the plant (Suslow, 2002). A high level of nutrients and available water present in the food matrix will improve the metabolic activity of LAB and the resultant production of antimicrobial compounds will also increase.

Before application to the spinach, the LAB concentration was determined to be 7.5 $\log_{10}$ CFU/mL (3.0×10^7 CFU/mL). This value is the mean concentration of all three replications and is nearly 1 log less than the target concentration of 8.3 $\log_{10}$ CFU/mL (2.0×10^8 CFU/mL). This may be the result of adding the Bovamine® Meat Cultures to sterile distilled water. Because solutes have been removed from distilled water, the osmotic pressure is greater outside the LAB cell relative to inside the cell. As a result, water will diffuse into the cell, potentially causing lysis.

Given that the LAB treatment was only applied as a rinse and at one concentration ($2.0x10^8$ CFU/mL), perhaps an improvement in performance could be achieved with a different application method or concentration level. For example, the implementation of a spray intervention may result in differing levels of success. Additionally, the Bovamine® Meat Cultures may be capable of the same degree of reduction in *E. coli* O157:H7 populations at concentrations lower than $2.0x10^8$ CFU/mL. These items must be addressed before a definitive conclusion can be drawn about the effectiveness of LAB as a post–harvest intervention in the production of fresh spinach.

The Shapiro–Wilk values calculated for all data obtained in this study indicate normality, meaning that all data was normally distributed. There were no time/treatment interactions, however both time ($p=0.0068$) and treatment ($p<0.0001$) effects were observed (data not shown). As a result, all statistical assumptions were met and the data was analyzed accordingly.

Sensory Study

Of the 40 panelists, 40% (16) correctly selected the LAB spinach as being the one “odd” sample. For a population of 40 panelists, the number of correct responses required for statistical significance at the $\alpha=0.5$ and $\alpha=0.1$ was 18 and 20, respectively. These values were determined using an equation outlined in Table T8 of the third edition of Sensory Evaluation Techniques (Meilgaard et al., 1999). These calculations can be found in Appendix C. The null hypothesis for this triangle test states that no difference exists between the control spinach and

the spinach treated with lactic acid bacteria. Therefore, because the 16 correct responses obtained is less than the required responses of 18 and 20, there was no statistical significance and the null hypothesis was accepted at the α=0.5 and α=0.1 levels. These results are summarized in Table 3.2. Calculations to determine the number of discriminators estimated that 4 (10%) panelists perceived the true difference and selected the LAB sample as a result (Appendix C). These results suggest that a mere 25% (4 out of 16) of panelists who selected the LAB spinach truly detected a difference in the sensory properties of fresh spinach treated with Bovamine® Meat Cultures.

Sensory results on the spinach were also similar to previous reports, a 2002 study conducted by Amézquita and Brashears evaluated the effects of LAB on ready–to–eat meat products. They also executed a triangle test to determine whether an isolate *Pediococcus acidilactici* resulted in a significant difference between LAB and control frankfurters. Triangle tests were conducted on the frankfurters 9 times throughout the 56–day storage period. The number of correct responses obtained during each test was less than the number required for statistical significance. Therefore, they concluded that the application of *P. acidiliactici* did not result in a significant difference between treated and control frankfurters. Their findings support the results of the current spinach study.

The lack of statistical significance obtained with this triangle test supports the use of LAB as a post–harvest intervention in the production of fresh spinach from a consumer acceptance standpoint. However, due to the preliminary nature of this study, there is great potential for future research. As a result of

metabolism and fermentative activities, LAB produce multiple by–products that have the potential to adversely affect the sensory properties of fresh spinach, particularly during shelf–life. For this reason, it is necessary to evaluate sensory changes throughout the shelf–life to determine if the production of metabolites over time will result in a statistically significant difference and decrease the consumer acceptance of product treated with Bovamine® Meat Culture.

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Table 3.1. Least Squares Means of *E. coli* O157:H7 levels (Log_{10} CFU/g) in each spinach treatment held at the target temperature of 7°C for 24 hours.

Treatment	0 Minutes[z]	5 Minutes[z]	10 Minutes[z]	1 Hour[z]	4 Hours[z]	8 Hours[z]	24 Hours[z]
Control	5.20[a]	5.34[a]	5.38[a]	5.23[a]	5.36[a]	5.23[a]	5.38[a]
Water	4.71[b]	4.61[b]	4.48[b]	4.50[b]	4.35[b]	4.17[b]	4.08[b]
LAB[1]	4.50[b]	4.46[b]	4.33[b]	4.26[b]	4.18[b]	4.36[b]	3.83[b]

[a,b] indicates treatments that differ in each column ($p<0.05$).

[z] indicates standard error for all values within column is equal to 0.3794.

[1] LAB is representative of the Bovamine® Meat Cultures lactic acid bacteria treatment.

Table 3.2. Summary of triangle test data to determine statistical significance between tap water–treated fresh spinach and fresh spinach treated with Bovamine® Meat Cultures at an α–level of 0.05 and 0.01.

α–Level	Correct Responses Required	Correct Responses	Decision	Interpretation
0.05	18[a]	16 < 18	Accept Null	No Detectable Difference
0.01	20[a]	16 < 20	Accept Null	No Detectable Difference

[a] Reject the assumption of "no difference" if the number of correct responses is greater than or equal to the tabled value.

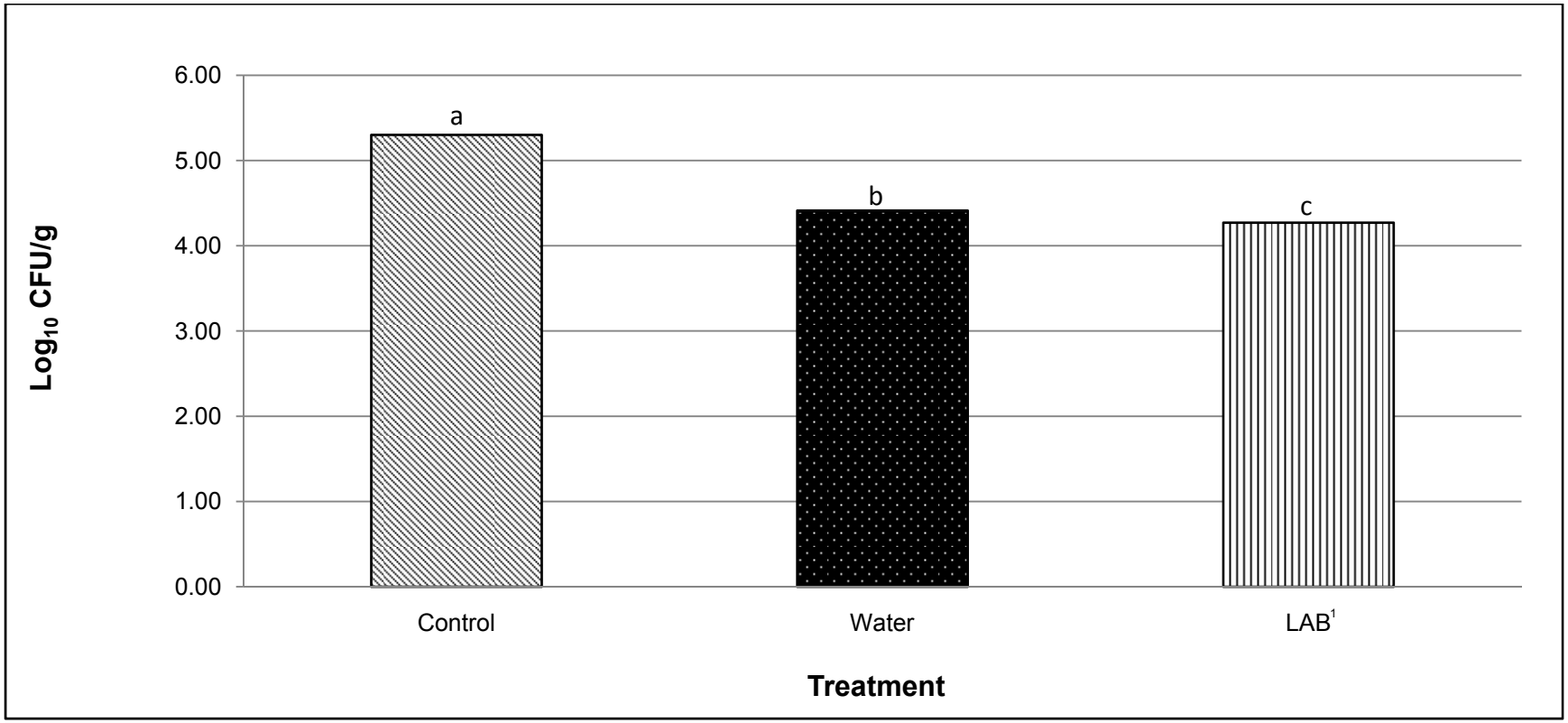

Figure 3.1. Composite Least Squares Means of *E. coli* O157:H7 levels in each spinach treatment.

^{a,b,c} indicates treatments that differ ($p<0.05$).

[1] LAB is representative of the Bovamine® Meat Cultures lactic acid bacteria treatment.

CHAPTER IV

OBJECTIVE II

REDUCTION OF *ESCHERICHIA COLI* O157:H7 IN FRESH SPINACH USING CHLORINE AND LACTIC ACID BACTERIA AS A MULTI-HURDLE INTERVENTION

Introduction

Since its discovery as a human pathogen in 1982 (Law, 2000), *Escherichia coli* O157:H7 has often been associated with raw or undercooked ground beef (Pickering et al., 1994). It wasn't until the mid–1990s, when multiple outbreaks occurred, that fresh produce was identified as a vector for the transmission of *E. coli* O157:H7 (Delaquis et al., 2007). Since that time, there has been an increased awareness about the potential for fruits and vegetables to be associated with foodborne illness (Johannessen et al., 2005).

In the fall of 2006, a large outbreak due to *Escherichia coli* O157:H7 contamination of fresh spinach sickened 205 people. The severity of illness ranged from fairly mild to severe and resulted in 103 hospitalizations and 3 deaths. This outbreak was the 20th major outbreak associated with fresh lettuce or spinach since 1995. (United States House of Representatives Committee on Government Reform, 2008)

Spinach is vulnerable to pathogenic contamination at every step in the production process. Warriner et al. (2003) reported that the majority of vegetable contamination with human pathogens was believed to be the result of

post–harvest handling, but they also identified animals, soil, water and harvest equipment as sources of spinach contamination. Therefore, spinach safety should be emphasized at every step in the production process.

Fresh spinach is classified as a minimally processed vegetable because it has undergone minimal amounts of processing, such as shredding, peeling or slicing before packaging (Manani et al., 2006). The lack of a thermal processing step results in reliance on effective post–harvest interventions to remove any associated pathogens (Schuenzel and Harrison, 2002). Currently, disinfection of produce by washing is the only processing step that is targeted towards reducing microbial levels on fresh–cut vegetables (Pirovani et al., 2004).

The use of sodium hypochlorite washes as a cold, chlorinated water rinse is the most common post–harvest intervention utilized in the fresh fruit and vegetable industry (Takeuchi and Frank, 2000; Behrsing et al., 2000). However, it is well known that the efficacy of this intervention at inactivating pathogens on the surfaces of fresh produce is not substantial depending on various conditions including the microbial load on the product and the presence of organic material (Lin et al., 2002). It has become generally accepted that implementation of post–harvest chlorine washes is only effective at reducing the total microbial population by < 2 logs (Warriner et al., 2003). Lang et al. (2004) observed reductions of *E. coli* O157:H7 on lettuce leaves of only 1.10 logs in comparison to the control after treatment with 200 ppm chlorine. An investigation conducted by Beuchat (1999) revealed that spraying 200 parts per million (ppm) chlorinated water and deionized water were equally efficacious at killing, removing or

inactivating *E. coli* O157:H7 on the surface of lettuce leaves. Therefore, development of a more effective post–harvest intervention is required to improve the safety of bagged leafy greens.

Utilizing lactic acid bacteria (LAB) to improve food safety is not a new concept. Numerous characteristics of LAB prove to be antagonistic to other bacteria, including some pathogens. The metabolic activities of LAB result in the production of antimicrobial compounds including hydrogen peroxide, bacteriocins, carbon dioxide and organic acids (Kostrzynska and Bachand, 2006; Sanz et al., 2007; Schillinger et al., 1996). In addition to creating an acidic environment that is considered unfavorable for pathogenic growth, organic acids, including acetic, lactic and propionic acid, inhibit pathogens by acting on the cytoplasmic membrane of the bacterial cell (Kostryzynska and Bachand, 2006). Success has been achieved using LAB, and more specifically Lactobacillus acidophilus NP51 in combination with other LAB, to control *E. coli* O157:H7 and other pathogens in raw meat products (Kostrzynska and Bachand, 2006; Smith et al., 2005), in cooked meat products (Amézquita and Brashears, 2002) and in cattle (Brashears et al., 2003; Younts et al., 2004; Younts–Dahl et al., 2005). Because these cultures produce inhibitory compounds under refrigerated conditions but do not grow (Nutrition Physiology Corporation, 2005), they may be able to reduce pathogens in fresh spinach.

The objective of this study was to determine if Bovamine® Meat Cultures, a commercially produced LAB product, and chlorine, alone or in combination as a multi-hurdle approach, can be effectively implemented as a post–harvest

intervention and reduce total numbers of *E. coli* O157:H7 in fresh spinach during refrigerated storage.

Materials and Methods

Bacterial Strains

A cocktail of four *E. coli* O157:H7 strains was used for this study and included A4 966, A5 528, A1 920 and 966. All strains were isolated from cattle and originally obtained from the University of Nebraska and are now maintained in the stock culture collection at Texas Tech University. The cocktail was prepared by making frozen concentrated cultures of each culture as described by Brashears et al. (1998. One vial from each strain was obtained from the -80^{o}C stock culture. A sterile loop was used to add the strains to separate tubes of Brain Heart Infusion Broth (BHI) (EMD, Gibbstown, NJ). The strains were incubated overnight at 37^{o}C, transferred into fresh BHI tubes and incubated another night at 37^{o}C. The concentration of each strain was determined to be at the appropriate level by plating on Tryptic Soy Agar and incubating at 37^{o}C overnight (TSA) (EMD, Gibbstown, NJ). All four strains were combined in equal volumes in BHI, allowed to grow at 37^{o}C overnight and then centrifuged for 10 minutes at 4,000 g. The pellet was resuspended in BHI containing 10% glycerol and stored as a frozen culture in 1 mL portions at a concentration of 1.0x10^{9} CFU/mL in the Texas Tech University inventory.

Bovamine® Meat Cultures were obtained from Nutrition Physiology Corporation (Guymon, OK) and used in this study. This commercially available

LAB product is comprised of four LAB strains, including *Lactobacillus acidophilus* (NP 51), *Lactobacillus cristpatus* (NP 35), *Pediococcus acidilactici* (NP 3) and *Lactobacillus lactis* subsp. *lactis* (NP 7) (Smith et al., 2005). Isolates NP 51 and NP 35 were originally isolated from cattle, while NP 3 was isolated from cooked hot dogs and NP 7 from alfalfa sprouts (Smith et al., 2005). The culture was prepared by a commercial manufacturer and packaged in 10 g portions in a freeze–dried form.

Treatment Preparation

A lactic acid bacteria wash (LAB) with a concentration of $2.0x10^8$ CFU/mL was prepared by combining one 10 g packet of freeze–dried Bovamine® Meat Culture with 990 mL of buffered peptone water (BPW) (OXOID, Basingstoke, Hampshire, England) containing 1% glucose. The concentration of LAB was determined by making serial dilutions in buffered peptone water and plating on Lactobacilli MRS Agar (MRS) (EMD, Gibbstown, NJ). In order to metabolically activate the bacteria, the LAB was held in a 37°C incubator for 1 hour. The concentration of the LAB wash was re–evaluated post–incubation by serially diluting and plating on Lactobacilli MRS Agar. A 200 $\pm$ 10 parts per million (ppm) chlorine wash was prepared by combining 7.6 mL of sodium hypochlorite germicidal bleach (The Clorox Company, Oakland, CA) with 2.0 L of sterile tap water. The mixture was stirred and the concentration of total chlorine was determined using Hanna Instruments HI 95771 Ultra High Range meter (Hanna Instruments, Woonsocket, RI). Instructions provided by the manufacturer were

followed. If the total chlorine concentration was not acceptable, the solution was adjusted and retested until the target range was achieved. A sterile tap water wash was also prepared with a total volume of 1.0 L.

Sample Preparation

Fresh spinach was obtained from a commercial grower in California. The product was shipped overnight the same day that it was harvested, arriving at Texas Tech University approximately 24 hours later. A total of 1,500 g of the spinach were weighed into sterile plastic bags (VWR, West Chester, PA). The four–strain cocktail of *Escherichia coli* O157:H7 was diluted 1:1000 in buffered peptone water (BPW) (OXOID, Basingstoke, Hampshire, England) to obtain a final concentration of $1.0x10^6$ CFU/mL and an inoculum volume of 13 L. The pre–weighed spinach was submerged in the inoculum and allowed to soak for 20 minutes to facilitate attachment. Using sterile tongs, the inoculated spinach was spread evenly across sterile drying racks in a biological safety level II hood (Fisher Hamilton model #54L925, Two Rivers, WI) and allowed to dry for one hour. After 30 minutes of drying, the spinach was flipped, to ensure uniform air exposure, and allowed to remain for an additional 30 minutes.

Upon completion of drying, 200 g of the dry, inoculated spinach were added to a poultry rinsate bag and set aside to serve as the control. The remainder of the dry spinach was weighed into 4 sterile bags, with 200 g in each bag. Spinach in each of the 4 bags was ultimately exposed to a different treatment.

All treatments were added to the rinsate bags and agitated by hand for one minute. The rinse treatments were as follows: 500 mL of the $2.0x10^8$ LAB solution, 500 mL of 200 ppm sodium hypochlorite, 500 mL of sterile tap water and a hurdle intervention that was initially rinsed with 500 mL of 200 ppm sodium hypochlorite followed by 500 mL sterile tap water and 500 mL of the $2.0x10^8$ LAB solution. Following agitation, all rinse treatments were drained into a sterile colander and transferred to a sanitized salad spinner (Farberware, Garden City, NY). The spinach was spun 20 times, transferred to a new poultry rinsate bag and set aside. Prior to beginning each replication, the salad spinners were sanitized with 95% ethanol.

Plastic rollstock used in the packaging of fresh spinach was provider by an industry contact and utilized in this study. Prior to the beginning of each replication, the oxygen–permeable rollstock was cut and sealed to create bags with the approximate dimensions of 26.0 cm" long and 11.45" wide. The seal function of a FoodSaver (Gamesaver Deluxe Plus model) was used to create all seals on the bags.

Using a sterile tongs, 25 $\pm$ 1 g were added to each pre–made spinach bag, with a total of 7 bags created per treatment. The bags were sealed and labeled with their respective replication and treatment. At the completion of packaging, all spinach bags were randomized and placed onto one of three shelves in a retail display cooler set at 7^oC. It should be noted that samples from each treatment were randomized across all three shelves and throughout the entire length of the cooler to reduce bias.

The temperature of the retail display case was continuously recorded using a continuous temperature recorder (Temprecord Temperature Recorder MKII, Auckland, New Zealand). Before beginning the study, the temperature was set to 7^{o}C and monitored throughout storage. Each shelf contained a temperature logger that was randomly placed in the case. The temperature of each shelf was retrieved from the loggers at the end of the study.

Microbiological Analysis

From each treatment and the control, one bag was randomly selected from the retail display cooler. The bags were opened with sterile scissors and 10 g of spinach were collected on Day 0, 1, 3, 6, 9 and 12. The exact sample weight was recorded and used to determine colony forming units (CFU) on a per g basis. At each time point, the spinach was stomached (Seward Model 400, Bohemia, NY) with 90 mL of buffered peptone water at 230 rpm for 2 minutes. Homogenized samples were serially diluted and quantitatively analyzed for *Escherichia coli* O157:H7 using the Neo–Grid^{TM} Method (Neogen, Lansing, MI). Neo–Grid^{TM} filters were placed on CHROMagar (CHROMagar, Paris, France) containing tellurite, cefixime, cefsulodin and novobiocin at levels of 2.5 mg/L, 25 µg/L, 5 mg/L and 5 mg/L, respectively. Each antibiotic was added to reduce the interference from other bacteria. CHROMagar plates were incubated at 37^{o}C for 24 $\pm$ 2 hours. Mauve colonies were counted as presumptive positive for *Escherichia coli* O157:H7 and agglutinated at random for confirmation using a latex agglutination kit (Remel, Lenexa, KS). The survivability of LAB was also

determined by spread plating on Lactobacilli MRS Agar (MRS) (EMD, Gibbstown, NJ). MRS plates were incubated for 24–48 hours at 37°C. All colonies were counted and presumed to be LAB.

Experimental Design and Analysis

This study was classified as a complete randomized block design. The Statistical Analysis System (SAS) software was used to analyze the data. All data was subjected to the PROC MIXED and PROC UNIVARIATE commands. The Least Squares (LS) means obtained from the PROC MIXED procedure were used to identify statistical significance between each individual treatment in comparison to the control. Additionally, the LS means of each rinse treatment was compared to one another to identify if one treatment was significantly more effective than the other. Survivability of LAB was determined for the LAB and hurdle treatments at each sampling point by calculating the mean of all replications using Microsoft Excel 2007. The Shapiro–Wilk value provided by the PROC UNIVARIATE procedure was used to determine normality of the data. The experimental procedure was replicated a total of three times.

Results and Discussion

Table 4.1 is a comprehensive table of the average survivability of LAB throughout the shelf–life study. While the post–incubation count for both the LAB and hurdle treatments was approximately 8.5 logs, when applied to the spinach counts decreased by nearly 1 log on day 0. This was expected, as combining with the spinach results in further dilution of the LAB. Comparing day 0 to day

12, recovery of LAB declined from 7.61 to 6.88 logs and from 7.54 to 6.89 logs for the LAB and hurdle treatments, respectively. Therefore, decline in LAB survivability was less than one log for both treatments and most likely did not greatly reduce their ability to control *E. coli* O157:H7.

Evaluation of the effectiveness of all treatments employed in this study is displayed in Table 4.2. In general, in treated samples, it appears as though *E. coli* O157:H7 cells steadily grew from day 0 through day 6 and then gradually declined while there was no decline in the control samples. This steady decline is most likely representative of the cells entering into death phase and this occurred more quickly in samples treated with LAB, chlorine and with the interventions in combination.

A preliminary study was conducted to determine the most effective application method for post–harvest spinach interventions. In this study, reductions in *E. coli* O157:H7 populations achieved by both rinsing and spraying were compared throughout a 72–hour time period. The data obtained indicated that a rinse was significantly more effective (data not shown) at controlling the pathogen on the surface of fresh spinach. Therefore, all treatments investigated in Objective II were applied as a rinse. Through the same preliminary study it was determined that a dose response did not occur with varying concentrations of LAB (data not shown). Therefore, a dose of $2.0x10^8$ CFU/mL was utilized as in Objective I.

The lack of a time point/treatment interaction ($p=0.6213$) allows for the effective analysis of composite LS means for each treatment. Composite LS

means illustrated in Figure 4.1 indicates that each treatment differed from the control. The LAB treatment was similar to both water and chlorine, though chlorine was significantly different than water. A statistically significant difference exists between the hurdle and all other treatments. Additionally, the hurdle treatment resulted in the greatest decline of *E. coli* O157:H7 populations in comparison to the control, with a reduction of 1.35 logs. Therefore, these data suggest that the hurdle treatment is significantly the most effective treatment in this study.

At no sampling time point were the reductions achieved by water found to be significant in comparison to the control indicating that water alone is not effective in reducing pathogens on the surface of fresh spinach during storage. After 3 days of storage the spinach receiving the LAB treatment contained significantly less pathogen ($p=0.0311$) than that of the control. However, LAB did not produce significant reductions in comparison to the control for the remainder of the study. Chlorine significantly reduced *E. coli* O157:H7 compared to the control sample on day 0 ($p=0.0298$), day 1 ($p=0.0361$), day 3 ($p=0.0686$) and day 6 ($p=0.0146$). However, on days 9 ($p=0.1005$) and 12 ($p=0.4669$) there were no significant differences in the number of pathogens recovered from the control spinach and the chlorine treated spinach. The hurdle treatment was significantly more effective than the control at reducing *E. coli* O157:H7 levels on days 0 ($p=0.0027$), day 1 ($p=0.0129$), day 3 ($p=0.0009$) and day 9 ($p=0.0072$). While chlorine also had significant reductions on 4 of the 6 sampling days, the hurdle treatment was capable of extending those reductions out to day 9, whereas the

significant reductions attained by chlorine ended after day 6. Therefore, the combination of interventions produces a greater residual effect than does LAB or chlorine by themselves.

The greatest reductions achieved by chlorine throughout the 12–day study occurred on day 0 and 1 with a decline in *E. coli* O157:H7 populations of 1.27 and 1.22 logs in comparison to the control, respectively (Table 4.2). After day 1, the total *E. coli* O157:H7 populations increased in the spinach samples treated with the chlorine. These results indicate that the effectiveness of chlorine gradually declined as the study progressed, thus the chlorine had no residual effect. While the *E. coli* O157:H7 populations present on the hurdle treatment also increased over time, this increase occurred to a lesser extent than did with the chlorine treatment, with the exception of day 6.

On the other hand, the total *E. coli* O157:H7 in spinach treated with LAB alone was significantly different from the control on days 0 and 3 indicating that the inhibition with the LAB occurs in the beginning and lacks residual effect when implemented as the sole intervention. When combining the initial effectiveness of both chlorine and LAB in reducing the pathogen we achieved the greatest overall effect in controlling *E. coli* O157:H7 in the product as demonstrated by our data. The product treated with the combination of LAB and chlorine had significantly fewer *E. coli* O157:H7 detected through 9 days of storage compared to only 6 days of protection with the chlorine and 3 days of protection with the LAB treatments alone. Additionally, comparing the increase in pathogen levels on day 0 to day 12 was an indicator of the ability of treatments to protect against

E. coli O157:H7 over time. As indicated in Table 4.2, *E. coli* O157:H7 populations present on chlorine–treated spinach increased 1.60 logs from 4.31 to 5.91 logs on day 0 to 12, respectively. Increases in *E. coli* O157:H7 populations rose 1.29 logs from 3.79 to 5.08 logs on day 0 to 12, respectively on spinach treated with the hurdle intervention. This difference provides additional evidence that the combination of interventions may be more capable of controlling the pathogen over time and should be further investigated in order to determine variables that may optimize reductions.

Lang et al. (2004) evaluated the effects of water and a 200 ppm chlorine treatment on *E. coli* O157:H7 populations on lettuce leaves and reported that treatment with water resulted in reductions of 0.44 logs, while chlorine improved reductions to 1.10 logs compared to the controls which is similar to what was found in this study. Similarly, in a 2007 study, Niemira employed water and 300 ppm and 600 ppm chlorine washes on baby spinach inoculated with *E. coli* O157:H7. The baby spinach was inoculated by vacuum perfusion into the intercellular spaces to simulate internalization of the pathogen. Data indicated that treatment with both chlorine treatments and water resulted in *E. coli* populations on the leaves that were statistically similar to the control, with less than 0.50 log reductions for all three treatments. Unlike the present study, Niemira's investigated the reduction of the internalized *E. coli* O157:H7 which would not be directly exposed to the intervention treatment.

The use of LAB as a post–harvest intervention in fresh spinach production is not a well–researched topic. However, the use of LAB, with Bovamine® Meat

Cultures in particular, has been demonstrated in fresh meat products. Smith et al. (2005) utilized the same four LAB strains that Bovamine® Meat Cultures are comprised of as an intervention for ground beef inoculated with *E. coli* O157:H7 at a concentration of $1.0x10^5$ CFU/mL and reported significant reductions of 2.0 logs and 3.0 logs compared to controls after 3 and 5 days of storage, respectively, thus being similar to our results obtained for the hurdle intervention. The difference in temperature between the two studies may be one variable that is responsible for this variation in success.

Temperature and *E. coli* O157:H7 inoculation levels are two parameters in this study that deserve further investigation. Because Bovamine® Meat Cultures consist of LAB strains specifically chosen for their ability to produce inhibitory substances without growth at refrigerated temperatures, reducing the storage temperature of LAB treated spinach may improve the success of both the LAB and hurdle treatments. Also, a more realistic approach may need to be evaluated in terms of *E. coli* O157:H7 inoculation. An inoculation level of $1.0x10^6$ CFU/mL is most likely significantly higher than spinach would ever be exposed to at any point in the production process. However, in the event that the LAB or hurdle treatments were successful at reducing populations by multiple logs, a high level of inoculation was chosen. In the future, this shelf–life study should be expanded upon to include both low and high dose inoculation levels. Furthermore, a spray or spot inoculation method may more accurately reflect inoculation by rain or irrigation and should also be considered for use in future research.

Incubation of the LAB for one hour at 37^{o}C did not drastically impact concentration levels. Prior to incubation, the culture was determined to be at an average concentration of 3.0x10^{8} CFU/mL (8.47 logs). Following incubation, the concentration was measured to be at 3.3x10^{8} CFU/mL (8.52 logs). While the increase in concentration may be negligible, the main purpose for culture incubation is to metabolically activate the LAB cells. This increases LAB activity and production of antimicrobial metabolites, thus improving their bactericidal capabilities. For future research, lengthening the incubation period may improve the effectiveness of LAB as an intervention on fresh spinach leaves. Perhaps an incubation time of 2 to 3 hours would allow for enhanced fermentation of the 1% glucose present in the BPW to lethal organic acids, bacteriocins and other metabolites proven lethal to *E. coli* O157:H7 and other pathogens.

Fluctuations in temperature among the three shelves of the display cooler may be responsible for variability in the effectiveness of treatments between sampling periods. As Figures D1 through D3 in Appendix D illustrate, temperatures reached as high as 10 to 11^{o}C and varied throughout all three replications. Therefore, sampling of a spinach bag held at extreme temperatures may skew the data for that treatment on a particular sampling day of a replication. However, the randomization of all sample bags throughout the entire display cooler should compensate for this variable. Additionally, the natural temperature fluctuations of the retail display coolers utilized in this study are likely an accurate representation of the retail environment in which fresh spinach is stored.

Warriner et al. (2003) stated that the ability of chlorine to reduce microbial populations by a maximum of <2 logs indicates the need for a more efficacious intervention. As previously mentioned the hurdle treatment was more effective than chlorine alone and resulted in the most significant reductions of *E. coli* O157:H7 populations in comparison to control populations, with the exception of day 6. There are numerous variables that can be further evaluated that may greatly improve the success of the LAB and hurdle treatments. In addition to lowering the temperature and altering inoculation levels, lengthening the agitation time will increase the amount of interaction between the leaves and treatments, thus removing more *E. coli* O157:H7 cells.

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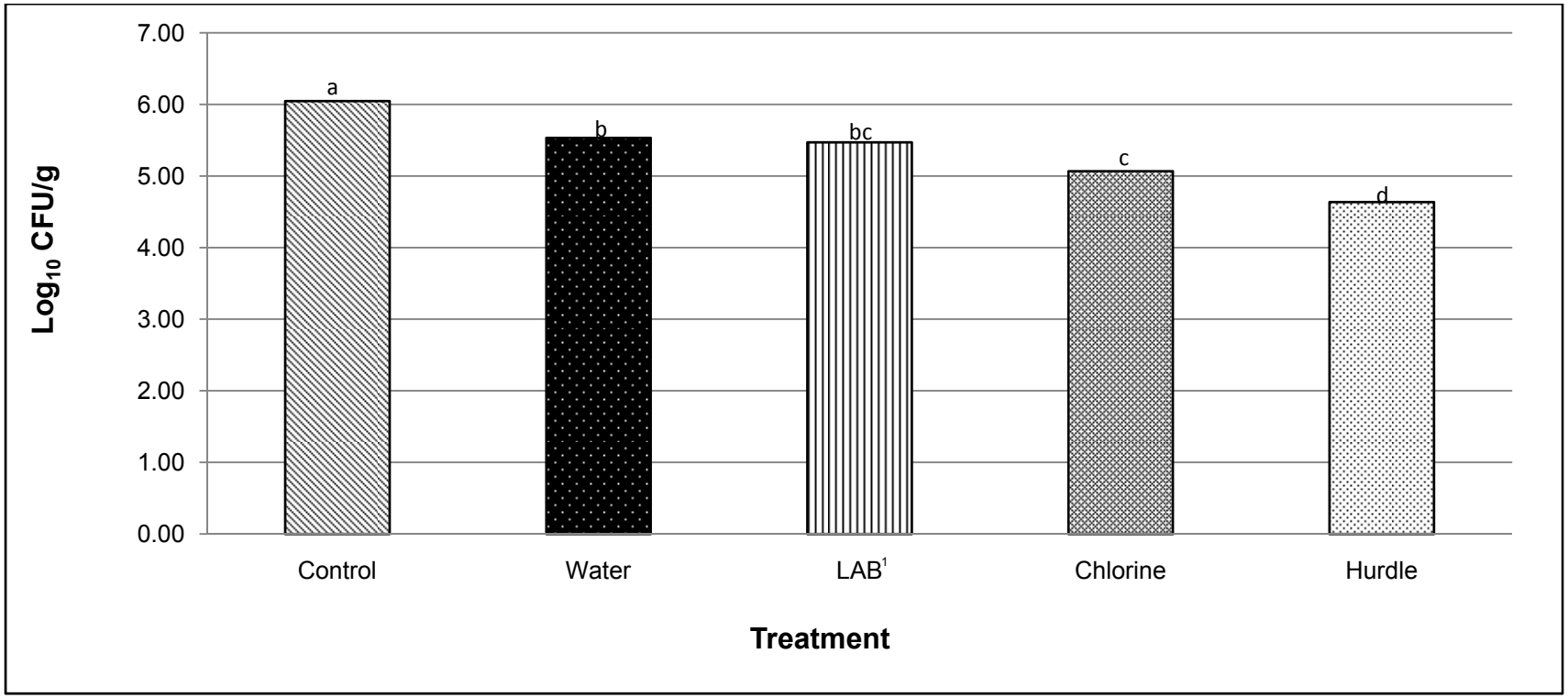

Figure 4.1. Composite Least Squares Means of *E. coli* O157:H7 levels in each spinach treatment held in a retail display cooler at a target temperature of 7°C for 12 days.

a,b,c indicates treatments that differ ($p<0.10$)

[1] LAB is representative of the Bovamine® Meat Cultures lactic acid bacteria treatment.

Table 4.1. Average survivability of lactic acid bacteria on spinach at each sampling time point for only the LAB and hurdle treatments held in a retail display cooler at a target temperature of 7°C for 12 days (Log_{10} CFU/g).

Treatment	Day 0	Day 1	Day 3	Day 6	Day 9	Day 12
LAB[1]	7.61	7.65	7.49	7.24	7.11	6.88
Hurdle	7.54	7.48	7.47	7.16	7.12	6.89

[1] LAB is representative of the Bovamine® Meat Cultures lactic acid bacteria treatment.

Table 4.2. Least Squares Means of *E. coli* O157:H7 present in each spinach treatment held in a retail display cooler at a target temperature of 7°C for 12 days (Log_{10} CFU/g).

Treatment	Day 0[y]	Day 1[y]	Day 3[y]	Day 6[z]	Day 9[y]	Day 12[y]
Control	5.58[a,BC]	5.83[a,ABC]	6.57[a,A]	6.33[a,ABC]	6.46 [a,AB]	5.50[a,C]
Water	4.78[ab,C]	5.26[ab, ABC]	6.01[ab, AB]	6.31 [a,A]	5.66 [ab, AC]	5.19 [a,BC]
LAB[1]	4.52[bc,A]	5.09[ab,ACD]	5.30[bc,ABCD]	6.25 [a,B]	5.68 [ab,BC]	5.97 [a,BD]
Chlorine	4.31[bc,A]	4.61[b,AB]	5.51[bc,BC]	4.56 [b,AB]	5.51 [ab,BC]	5.91 [a,C]
Hurdle	3.79[c,A]	4.37[b,AB]	4.56[c,AB]	5.31 [ab,B]	4.86 [b,B]	5.08 [a,B]

[a,b,c] indicates treatments that differ in each column ($p<0.10$).

[A,B,C,D] indicates days that differ in each row ($p<0.10$).

[y] indicates standard error within column is equal to 0.4256.

[z] indicates standard error within column is equal to 0.5148.

[1] LAB is representative of the Bovamine® Meat Cultures lactic acid bacteria treatment.

CHAPTER V

SUMMARY AND CONCLUSIONS

Evaluation of the efficacy of LAB as a post–harvest intervention to control *E. coli* O157:H7in fresh spinach production was multi–faceted. Due to the lack of research in this area, multiple factors were considered when designing this study. As a result, two separate objectives were investigated.

Objective I compared the efficacy of water and Bovamine® Meat Cultures to an inoculated, untreated control. Comparison of LS Means data indicated a gradual decline in *E. coli* O157:H7 populations treated by both the water and control throughout the 24–hour time period. Conversely, *E. coli* O157:H7 levels recovered from the control remained steady over time. At each sampling point, both the water- and LAB–treated spinach samples were found to be significantly different than the control. The LAB treatment produced the lowest *E. coli* O157:H7 populations, with the exception of the 8 hour sampling point, at which time the water treatment had the fewest cells. However, at no sampling point were water and LAB statistically different. Composite LS means calculated by combining all data points within a treatment identified the greatest reduction in the LAB treated spinach. Both water and LAB were statistically different from one another and the control. Therefore, the composite LS means data suggested that treatment with LAB was significantly the most effective.

Data obtained from Objective I indicates potential for the application of LAB. While reductions were not significantly different than water at each

timepoint, LAB reductions may increase under more ideal conditions. Several factors not taken into account in this study must be investigated and may optimize the efficacy of LAB. For example, determining the most appropriate application method and concentration of LAB cultures, storage temperature and LAB nutrient availability may significantly impact the success of this intervention.

To establish consumer acceptability, a triangle test was utilized to determine if the addition of LAB to fresh spinach adversely affects the sensory characteristics of fresh spinach. Forty panelists were simultaneously presented with three fresh spinach samples. Two samples were rinsed with tap water, while the remaining sample was subjected to a LAB wash. Of the 40 panelists, 16 (40%) identified the LAB spinach as being the one unique sample. With a sample size of 40 evaluated at α–levels of both 0.05 and 0.01, the LAB spinach is considered to be statistically similar to the two spinach samples washed with water. The number of discriminators, those who truly saw the difference between samples, was calculated to be a mere 4 (10%) panelists. Therefore, it is estimated that the remaining 12 participants who selected the LAB sample simply guessed.

Implementation of this triangle test has illustrated that the addition of LAB to fresh spinach is feasible from a consumer acceptance standpoint. Therefore, if effective enough from a microbiological perspective to be utilized as a post–harvest intervention, the effects on sensory should be of little concern. However, conducting a sensory test that employs a more diverse population and greater

sample size may be necessary before the intervention can become mainstream in the industry.

The knowledge gained from the previous objective was compiled and used to develop and execute Objective II. This final objective was a 12 day shelf–life study comparing the effectiveness of water, 200 ppm chlorine, $2.0x10^8$ CFU/mL LAB and a hurdle treatment. The hurdle treatment was comprised of three consecutive rinses consisting of 200 ppm chlorine, water and LAB. According to the calculated LS means values, at no sampling timepoint were chlorine and hurdle significantly different from one another. Additionally, at every timepoint, hurdle produced the greatest reductions in *E. coli* O157:H7 levels of all treatments when compared to the control populations, with the exception of day 6. However, hurdle was not considered significantly different than any other treatment at any sampling timepoint, with the exception of water on day 3. Composite LS means calculated from all data points within a treatment also indicate the hurdle treatment produced the greatest declines in populations of all the treatments. Furthermore, the combination of interventions was significantly different than every other treatment, indicating it was the most significantly effective treatment with a 1.35 log reduction from control populations.

Comparison of the LAB and chlorine LS means data, as well as the composite LS means for both treatments indicates no difference exists between the two treatments. However, when these interventions are used in combination, the reductions become significantly effective. As a result, implementation of both

LAB and chlorine in combination as a post–harvest intervention may be an efficacious alternative.

While the ideal processing and storage conditions have not yet been determined, this study provides an excellent representation of the potential LAB has as a post–harvest intervention for the fresh spinach industry. The work presented herein has laid the foundation for the implementation of LAB in spinach processing plants. The application of 200 ppm chlorine in combination with sterile tap water and LAB at a concentration of $2.0x10^8$ CFU/mL is significantly more effective than water or chlorine and LAB applied by themselves and should be considered as a potential alternative to the industry standard chlorine wash. Additionally, this study has indicated that sensory characteristics were not compromised by the application of LAB, as there was no significant difference between spinach treated with water or LAB. With future research, the use of LAB in combination with other interventions may prove to be a possible post–harvest intervention for the fresh spinach industry.

APPENDICES

APPENDIX A

RAW PLATE COUNTS

Table A1. *Escherichia coli* O157:H7 counts obtained from Objective I (CFU/g).

Replication	Treatment	Timepoint	Count	Log_{10}
1	Control	0 Minutes	150,000	5.18
1	Water	0 Minutes	21,000	4.33
1	LAB	0 Minutes	20,000	4.29
1	Control	5 Minutes	150,000	5.18
1	Water	5 Minutes	19,000	4.28
1	LAB	5 Minutes	24,000	4.38
1	Control	10 Minutes	200,000	5.31
1	Water	10 Minutes	22,000	4.33
1	LAB	10 Minutes	14,000	4.15
1	Control	1 Hour	250,000	5.39
1	Water	1 Hour	24,000	4.38
1	LAB	1 Hour	25,000	4.40
1	Control	4 Hours	170,000	5.22
1	Water	4 Hours	15,000	4.17
1	LAB	4 Hours	17,000	4.22
1	Control	8 Hours	120,000	5.08
1	Water	8 Hours	11,000	4.03
1	LAB	8 Hours	19,000	4.28
1	Control	24 Hours	140,000	5.15
1	Water	24 Hours	7,000	3.85
1	LAB	24 Hours	2,000	3.30
2	Control	0 Minutes	1,300,000	6.11
2	Water	0 Minutes	240,000	5.38
2	LAB	0 Minutes	120,000	5.07
2	Control	5 Minutes	2,300,000	6.36
2	Water	5 Minutes	260,000	5.40
2	LAB	5 Minutes	90,000	4.95
2	Control	10 Minutes	1,400,000	6.15
2	Water	10 Minutes	160,000	5.20
2	LAB	10 Minutes	100,000	5.00
2	Control	1 Hour	1,000,000	6.00
2	Water	1 Hour	120,000	5.07
2	LAB	1 Hour	40,000	4.60
2	Control	4 Hours	1,000,000	6.00
2	Water	4 Hours	150,000	5.17
2	LAB	4 Hours	40,000	4.59
2	Control	8 Hours	1,000,000	5.99
2	Water	8 Hours	100,000	5.00
2	LAB	8 Hours	80,000	4.90
2	Control	24 Hours	900,000	5.94

Table A1. Continued.

Replication	Treatment	Timepoint	Count	Log_{10}
2	Water	24 Hours	40,000	4.60
2	LAB	24 Hours	50,000	4.70
3	Control	0 Minutes	20,000	4.30
3	Water	0 Minutes	27,000	4.42
3	LAB	0 Minutes	13,000	4.11
3	Control	5 Minutes	30,000	4.46
3	Water	5 Minutes	14,000	4.15
3	LAB	5 Minutes	11,000	4.05
3	Control	10 Minutes	50,000	4.70
3	Water	10 Minutes	8,000	3.90
3	LAB	10 Minutes	7,000	3.85
3	Control	1 Hour	20,000	4.30
3	Water	1 Hour	11,000	4.03
3	LAB	1 Hour	6,000	3.77
3	Control	4 Hours	70,000	4.83
3	Water	4 Hours	5,000	3.69
3	LAB	4 Hours	5,000	3.69
3	Control	8 Hours	40,000	4.59
3	Water	8 Hours	3,000	3.47
3	LAB	8 Hours	8,000	3.90
3	Control	24 Hours	110,000	5.05
3	Water	24 Hours	6,000	3.77
3	LAB	24 Hours	3,000	3.48

Table A2. *Escherichia coli* O157:H7 counts obtained from Objective II (CFU/g).

Replication	Treatment	Timepoint	Count	Log_{10}
1	Control	Day 0	330,000	5.52
1	Water	Day 0	60,000	4.78
1	LAB	Day 0	31,000	4.49
1	Chlorine	Day 0	16,000	4.20
1	Hurdle	Day 0	9,500	3.98
1	Control	Day 1	350,000	5.54
1	Water	Day 1	110,000	5.04
1	LAB	Day 1	310,000	5.49
1	Chlorine	Day 1	45,000	4.65
1	Hurdle	Day 1	8,700	3.94
1	Control	Day 3	6,200,000	6.79
1	Water	Day 3	3,900,000	6.59
1	LAB	Day 3	72,000	4.86
1	Chlorine	Day 3	1,300,000	6.11
1	Hurdle	Day 3	5,400	3.73
1	Control	Day 6	---	---
1	Water	Day 6	---	---
1	LAB	Day 6	---	---
1	Chlorine	Day 6	---	---
1	Hurdle	Day 6	---	---
1	Control	Day 9	5,200,000	6.72
1	Water	Day 9	570,000	5.76
1	LAB	Day 9	1,500,000	6.18
1	Chlorine	Day 9	1,400,000	6.15
1	Hurdle	Day 9	370,000	5.57
1	Control	Day 12	14,000,000	7.15
1	Water	Day 12	1,400,000	6.15
1	LAB	Day 12	12,000,000	7.08
1	Chlorine	Day 12	840,000	5.92
1	Hurdle	Day 12	590,000	5.77
2	Control	Day 0	430,000	5.63
2	Water	Day 0	58,000	4.76
2	LAB	Day 0	35,000	4.54
2	Chlorine	Day 0	28,000	4.45
2	Hurdle	Day 0	4,100	3.61
2	Control	Day 1	830,000	5.92
2	Water	Day 1	500,000	5.70
2	LAB	Day 1	41,000	4.61
2	Chlorine	Day 1	67,000	4.83
2	Hurdle	Day 1	11,000	4.04

Table A2. Continued.

Replication	Treatment	Timepoint	Count	Log_{10}
2	Control	Day 3	2,800,000	6.45
2	Water	Day 3	15,000,000	7.18
2	LAB	Day 3	340,000	5.53
2	Chlorine	Day 3	230,000	5.36
2	Hurdle	Day 3	94,000	4.97
2	Control	Day 6	9,800,000	6.99
2	Water	Day 6	4,000,000	6.60
2	LAB	Day 6	1,900,000	6.28
2	Chlorine	Day 6	120,000	5.08
2	Hurdle	Day 6	4,400,000	6.64
2	Control	Day 9	2,600,000	6.41
2	Water	Day 9	360,000	5.56
2	LAB	Day 9	300,000	5.48
2	Chlorine	Day 9	140,000	5.15
2	Hurdle	Day 9	20,000	4.30
2	Control	Day 12	110,000	5.04
2	Water	Day 12	90,000	4.95
2	LAB	Day 12	2,400,000	6.38
2	Chlorine	Day 12	220,000	5.34
2	Hurdle	Day 12	10,000	4.00
3	Control	Day 0	400,000	5.60
3	Water	Day 0	62,000	4.79
3	LAB	Day 0	34,000	4.53
3	Chlorine	Day 0	19,000	4.28
3	Hurdle	Day 0	6,000	3.78
3	Control	Day 1	1,100,000	6.04
3	Water	Day 1	110,000	5.04
3	LAB	Day 1	150,000	5.18
3	Chlorine	Day 1	22,000	4.34
3	Hurdle	Day 1	130,000	5.11
3	Control	Day 3	2,900,000	6.46
3	Water	Day 3	18,000	4.26
3	LAB	Day 3	330,000	5.52
3	Chlorine	Day 3	110,000	5.04
3	Hurdle	Day 3	97,000	4.99
3	Control	Day 6	300,000	5.48
3	Water	Day 6	670,000	5.83
3	LAB	Day 6	1,100,000	6.04
3	Chlorine	Day 6	7,200	3.86
3	Hurdle	Day 6	3,100	3.49

Table A2. Continued.

Replication	Treatment	Timepoint	Count	Log_{10}
3	Control	Day 9	1,800,000	6.26
3	Water	Day 9	460,000	5.66
3	LAB	Day 9	250,000	5.40
3	Chlorine	Day 9	170,000	5.23
3	Hurdle	Day 9	53,000	4.72
3	Control	Day 12	20,000	4.30
3	Water	Day 12	29,000	4.46
3	LAB	Day 12	28,000	4.45
3	Chlorine	Day 12	3,000,000	6.48
3	Hurdle	Day 12	290,000	5.46

APPENDIX B

TRIANGLE TEST HUMAN SUBJECTS PROPOSAL

Cover Sheet for Human Subjects Proposal 50/090

Texas Tech University
Protection of Human Subjects Committee (IRB)

2007 OCT 16 PM 3:44
RESEARCH SERVICES

Title: Effect of Lactic Acid Producing Bacteria on the Sensory Characteristics of Fresh Spinach

Date: 10/15/07

- Human subjects proposals can be approved only for TTU faculty
- Students are to be listed as Co-PIs and must have a faculty PI
- Use additional sheets if necessary

Faculty PI: Mindy Brashears
Co PI: Sara Gragg
Co PI:

Department: Animal and Food Sciences
Phone: 742-2805
Mail Stop: 42141
E-mail: mindy.brashears@ttu.edu

Checklist (Include all the following items)

- [x] Cover Sheet (this form)
- [x] Claim of Exemption **OR** / Expedited Form **OR** / Full Board Summary
- [x] Proposal (items I through V)
- [x] Proofread Consent/Assent Form or / Consent Waiver Form
- [x] Recruiting Materials
- [x] Questionnaire(s)/Surveys
- [ ] Sponsored projects information*
- [ ] Other (misc. items that need tot be included)

Signatures:

[redacted]
Faculty PI

[redacted]
Co-PI (use additional sheets if necessary)

[redacted]
Department Chair or Dean

Requested Review:

- [x] Exemption Claim
- [ ] Expedited Review
- [ ] Full Board Review

Proposal involves:

- [ ] Minors
- [ ] Prisoners
- [ ] Pregnant Women/Fetuses
- [ ] Institutionalized Individuals
- [ ] Mentally Handicapped
- [x] Other

Submission:
Please submit to:
Donna Peters (742-3884)
Institutional Review Board for the Protection of Human Subjects
Texas Tech University, Office of Research Services
203 Holden Hall * MS 1035

Sponsored Project:

- [ ] ***If this HS protocol is to be used with a sponsored project:**

Include a copy of the technical part of the sponsored project proposal (if multi-task proposal, only relevant sections are needed)

Please allow 10 working days for approval of this request.

Reviewer Approval:

CLAIM FOR EXEMPTION
FOR REVIEW BY THE
HUMAN SUBJECTS PROTECTION COMMITTEE

Notice

Advertising, recruitment of subjects, mailing or distribution of surveys, and the collection of data may begin only after this claim has received approval (allow 10 days for processing). The Committee may, upon review of this claim, deny the request for an exemption and route the proposal for review.

Faculty PI's Last Name: Brashears Abbreviated Title: Effect of lactic acid

First 4 words of proposal title

BASIS OF CLAIM FOR EXEMPTION. Federal regulations and/or University policy require that in order for research to be exempt from review at least one of the following blocks (1-4) must be checked.

Note: Limitations for exemptions for children: Exemptions cannot be granted for: (a) projects with children as subjects that involve interview or survey procedures or (b) research where public behavior is observed and the investigator participates or interacts with the children. These projects require expedited or full review.

_____ 1. The research will be conducted only in established or commonly accepted educational settings (like classrooms) **AND** it involves normal educational practices such as research on regular and special educational instructional strategies, or research on the effectiveness of, or the comparison among, instructional techniques, curricula or classroom management methods.

_____ 2. The research involves the use of only the following techniques Check the applicable technique(s):

a. _____educational tests (cognitive, diagnostic, aptitude, achievement), or

b. _____survey or interview procedures, or

c. _____observing the public behavior of subjects,

AND (one of the following must be checked):

aa. _____ the information obtained will be recorded in such a manner that subjects *cannot* be identified *directly or through identifiers linked to the subjects*, or

bb. _____ if any disclosure of the subjects' responses outside the research could not reasonably place the subject at risk of criminal or civil liability, or be damaging to the subjects' financial standing, employability, or reputation (e.g., information regarding illegal or immoral conduct, drug or alcohol use, sexual behavior, mental illness, or other possibly personally embarrassing subjects), or

cc. _____ the subjects are elected officials or candidates for public office.

_____ 3. The research is limited to the collection or study of existing data, documents, records, pathological or diagnostic specimens under one of the following conditions: (one of the following must be checked):

a. _____ they are available to the public or

b. _____ they recorded by the investigator in such a manner that subjects cannot be identified, directly or indirectly, through identifiers linked with the subjects.

__X__ 4. Another provision of 45 CFR 46.101 (2). Please identify the subsection and describe in detail how the category applies to the proposed research.

STATEMENT OF RISK:

The undersigned certify that they believe that the conduct of the above described research creates no risk of physical or emotional harm or social or legal embarrassment to any subject. Any modifications that (a) change the research in a substantial way, (b) might change the basis for exemption, or (c) might introduce any additional risk to subjects should be reported to the IRB, before they are implemented, in the form of a new claim for exemption or a proposal for expedited or full board review.

[redacted] 10/14/07

Signature of TTU Faculty Principal Investigator Date

[redacted] 10/15/07

Signature of Co-Investigator Including Students Date

[redacted]

Signature of Co-Investigator Including Students Date

Signature of Co-Investigator Including Students Date

Claim for Exemption
Addendum

1. Another Provision of 45 CFR 46.101 (2).

According to 45 CFR 46.101 (b) "**Unless otherwise required by department or agency heads, research activities in which the only involvement of human subjects will be in one or more of the following categories are exempt from this policy:**

> **(6) Taste and food quality evaluation and consumer acceptance studies, (i) if wholesome foods without additives are consumed or (ii) if a food is consumed that contains a food ingredient at or below the level and for a use found to be safe, or agricultural chemical or environmental contaminant at or below the level found to be safe, by the Food and Drug Administration or approved by the Environmental Protection Agency or the Food Safety and Inspection Service of the U.S. Department of Agriculture.**

This research will utilize taste and food quality evaluation. Wholesome foods will be consumed (control) or treatments will be added at or below the level found to be safe by the FDA, EPA or USDA-FSIS. Moreover, the conventionally grown bagged spinach will be purchased from a commercial grocery store that follows ServSafe® guidelines.

Request for the Use of Human Subjects in Research
Texas Tech University

Project Investigators: M.M. Brashears, and S.E. Gragg
Department of Animal and Food Sciences

Project Title: Effect of Lactic Acid Producing Bacteria on the Sensory Characteristics of Fresh Spinach

I. Rationale

The Problem and Present Knowledge.

Escherichia coli O157:H7 has increasingly become a problem in fresh spinach products. The current industry sanitation standards are only successful in reducing the pathogen load by 1-2 logs. Current research ongoing in our lab has showed improved reductions in spinach treated with lactic acid producing bacteria. Therefore, the next logical step is to determine if the lactic acid producing bacteria have an adverse affect on the sensory characteristics of conventionally grown, bagged fresh spinach.

The data from this project will be beneficial in numerous ways: 1) Identification of safety interventions superior to those currently utilized in the spinach industry; 2) processors will have evidence to illustrate that this treatment is effective in reducing *E coli* O157:H7 without changing quality and consumer acceptability.

Aim of the Study.

To determine the effect of lactic acid bacteria (Bovamine® Meat Cultures) on the sensory characteristics of fresh spinach.

Importance of the Knowledge Obtained.

The information obtained will be disseminated to the scientific community, industry personnel, and government officials through publications in scientific journals, abstracts and presentations at scientific meetings. The information will be used to identify future research needs and provide officials with needed information to evaluate current spinach safety interventions.

II. Subjects

(a) Populations:

i. Consumer Taste Panels: Panelists (n=20 to 40) will consist of volunteers from the Animal and Food Sciences Department, as well as other individuals affiliated with Texas Tech University. Participants must be 18 years of age or older.

(b) Recruitment:

i. Consumer Taste Panels: Consumers will be solicited verbally and and through e-mails sent to faculty, staff and graduate students of

the Animal and Food Sciences Department at Texas Tech University.

(c) Benefits:

i. Consumer Taste Panels: There will be no benefit to taste panelists by participating in this taste panel.

III. Procedures

1. Conventionally grown bagged fresh spinach will be purchased from a commercial grocery store that operates under ServSafe® guidelines and transported to Texas Tech University and refrigerated in the sensory laboratory kitchen. The bags of fresh spinach will be combined (in order to reduce variability) and divided into thirds, with one third receiving the lactic acid bacteria (Bovamine® Meat Cultures) treatment, while the other two thirds will remain untreated.
2. (a) Consumer taste panelists will be recruited from the Animal and Food sciences Department for the study. The spinach will be treated with the lactic acid bacteria (Bovamine® Meat Cultures), allowed to dry, refrigerated and served cold to panelists. The untreated spinach will also be served cold to panelists housed in individual booths under red lighting. Panelists will be provided an expectorant cup and palate cleanser. Sensory characteristics will be quantified by their ability to determine the sample that is different, using a triangle test in which two samples (untreated spinach) are the same and one is different (lactic acid bacteria).
   (b) The research project involves visual and consumption of spinach samples only. The activity (triangle test) is judged by the authors to have minimal risks (or risks that do not exceed those associated with observing and consuming fresh product in every-day life). Panelists will be randomly assigned a number to maintain the confidentiality of their responses.
   (c) Panelists will be provided with candy at the completion of each panel. Consumption of the candy is optional. Panelists will volunteer their time and will receive no benefits for participation.

IV. Adverse Events and Liability

This research will utilize taste and food quality evaluation. Wholesome foods will be consumed (control) or treatments will be added at or below the level found to be safe by the FDA, EPA or USDA-FSIS. Moreover, the spinach will be purchased from a commercial retail grocery store. Therefore we feel the risks are not more than "minimally beyond the ordinary risks of daily life." Additionally, we feel the use of random number identification assures the confidentiality of participant responses.

V. Consent Form – Attached
VI. Attachments – Sensory Ballots

ORIGINAL APPROVAL

INSTRUMENT TO OBTAIN INFORMED CONSENT

The following document contains important information concerning participation of human subjects in research at Texas Tech University. Dr. Mindy Brashears is responsible for this research project and can be reached at 806-742-2805 if you have any questions. Please sign this consent form only after reading all information carefully.

- I understand that my role in this study is one of a sensory panelist and that I will observe palatability traits for flavor, off-flavor, mouth-feel and texture in spinach leaves in order to distinguish any differences among samples as part of a scientific research project.
- I understand that the purpose and objectives of this study are to determine the effect lactic acid producing bacteria have on the sensory characteristics of pre-packaged, conventionally grown fresh spinach leaves.
- I understand that this study will take place between October of 2007 and November, 2007.
- I understand that the time I will spend participating in this study (observing samples and filling out evaluation forms) will be approximately 30 minutes and on a one-time basis. I will be informed if additional participation time will be necessary.
- I understand that the benefit of participating in this study is the advancement of research in Food Science and that no risks are involved with participation in this study above the inherent risks associated with observing fresh spinach.
- I understand that my participation in this study is confidential and that my name will be entered as a code in data analysis to ensure confidentiality.
- I understand that participation in this study is completely voluntary and that I may decide to discontinue participation at any time.
- I understand that significant new findings during research that may relate to my health or willingness to participate in this study will be provided immediately upon discovery so that I may decide whether or not to continue participation in the study.
- I understand that the Food and Drug Administration (FDA) may inspect any FDA sponsored research.

Dr. Mindy Brashears will answer any questions you have about the study. For questions about your rights as a subject or about injuries caused by this research, contact the Texas Tech University Institutional Review Board for the Protection of Human Subjects, Office of Research Services, Texas Tech University, Lubbock, Texas 79409. Or you can call (806) 742-3884.

This consent form is not valid after October 31st, 2008.

I have read and understand the explanation provided to me. I have had all my questions answered to my satisfaction, and I voluntarily agree to participate in this study. I have been given a copy of this consent form.

_____ _____

Signature of participant Date

[redacted]

_____ _____

Mindy Brashears, PhD Date

FINAL APPROVAL

INSTRUMENT TO OBTAIN INFORMED CONSENT

The following document contains important information concerning participation of human subjects in research at Texas Tech University. Dr. Mindy Brashears is responsible for this research project and can be reached at 806-742-2805 if you have any questions. Please sign this consent form only after reading all information carefully.

- I understand that my role in this study is one of a sensory panelist and that I will observe palatability traits for flavor, off-flavor, mouth-feel and texture in spinach leaves in order to distinguish any differences among samples as part of a scientific research project.
- I understand that the purpose and objectives of this study are to determine the effect lactic acid producing bacteria have on the sensory characteristics of pre-packaged, conventionally grown fresh spinach leaves.
- I understand that I will consume fresh spinach leaves that contain lactic acid bacteria at the level of 10^8, which is less than the FDA – approved level of 10^9.
- I understand that this study will take place between October of 2007 and December, 2007.
- I understand that the time I will spend participating in this study (observing samples and filling out evaluation forms) will be approximately 30 minutes and on a one-time basis. I will be informed if additional participation time will be necessary.
- I understand that the benefit of participating in this study is the advancement of research in Food Science and that no risks are involved with participation in this study above the inherent risks associated with observing fresh spinach.
- I understand that my participation in this study is confidential and that my name will be entered as a code in data analysis to ensure confidentiality.
- I understand that participation in this study is completely voluntary and that I may decide to discontinue participation at any time.
- I understand that significant new findings during research that may relate to my health or willingness to participate in this study will be provided immediately upon discovery so that I may decide whether or not to continue participation in the study.
- I understand that the Food and Drug Administration (FDA) may inspect any FDA sponsored research.

Dr. Mindy Brashears will answer any questions you have about the study. For questions about your rights as a subject or about injuries caused by this research, contact the Texas Tech University Institutional Review Board for the Protection of Human Subjects, Office of Research Services, Texas Tech University, Lubbock, Texas 79409. Or you can call (806) 742-3884.

This consent form is not valid after October 31st, 2008.

I have read and understand the explanation provided to me. I have had all my questions answered to my satisfaction, and I voluntarily agree to participate in this study. I have been given a copy of this consent form.

_____ _____

Signature of participant Date

_____ _____

Mindy Brashears, PhD Date

Recruiting Material

To be displayed in the Animal and Food Sciences Building and e-mailed to all faculty, staff and graduate students in the department.

Sensory Taste Panel

Please participate in a spinach taste panel today (insert date) at (insert time) in the sensory laboratory of the Animal and Food Sciences Department. A treat will be provided for all panelists. Thank you for participating!

Triangle Test Answer Sheet

Name_____

Date_____

Panelist Number_____

Instructions:

1. Taste samples from left to right. Two samples are identical and one is different.
2. Select the different sample and indicate by placing an X next to the code of the different/odd sample.
3. If you are unable to determine a difference you must guess.

Samples on Tray	Odd Sample	Comments
_____	_____	_____
_____	_____	_____
_____	_____	_____

APPENDIX C

CALCULATIONS UTILIZED FOR TRIANGLE TEST

CALCULATIONS TO DETERMINE NUMBER OF CORRECT RESPONSES REQUIRED FOR STATISTICAL SIGNIFICANCE AT THE α–LEVEL OF 0.05 & 0.01[A]

$$z = (k\text{-}1(1/3)n) / \sqrt{(2/9)n}$$

z = standard normal distribution value

n = number of assessments = 40

k = minimum number of correct responses required for significance

---

α=0.05

z=1.645

$$1.645 = (k\text{-}1(1/3)40) / \sqrt{(2/9)40}$$

$$1.645 = (k\text{-}1(13.333) / \sqrt{8.88}$$

$$1.645 = k\text{–}13.333 / 2.9814$$

$$\boldsymbol{k = 18}$$

α=0.01

z = 2.326

$$2.326 = (k\text{-}1(1/3)40) / \sqrt{(2/9)40}$$

$$2.326 = (k\text{-}1(13.333) / \sqrt{8.88}$$

$$2.326 = k\text{–}13.333 / 2.9814$$

$$\boldsymbol{k = 20}$$

[A] Meilgaard, M., Civille, G.V., Carr, B.T. 1999. Statistical Tables. P. 353–375. *In* Meilgaard, M., Civille, G.V. (ed). Sensory Evaluation Techniques. 3rd ed. CRC Press LLC. Boca Raton, FL.

CALCULATIONS TO DETERMINE THE NUMBER OF DISCRIMINATORS[A]

Discriminator: Panelist who sees the true difference and selects the correct "odd" sample

Non–Discriminator: Panelist who sees no difference and guesses

$$C = D + {}^{1}/_{3}(N–D)$$

C = Correct Responses D = Discriminators N = Sample Size

$$16 = D + {}^{1}/_{3}(40–D) = {}^{2}/_{3}D + 13.3$$

$$D = 4.05 = \textbf{4 Panelists–10\%}$$

[A] Lawless, H.T., Heymann, H. 1999. Discrimination theories and advanced topics. P. 140–172. *In* Lawless, H.T., Heymann, H. (ed) Sensory Evaluation of Food. Kluwer Academic/Plenum Publishers, Norwell, MA.

APPENDIX D

TEMPERATURE DATA OF RETAIL DISPLAY COOLER

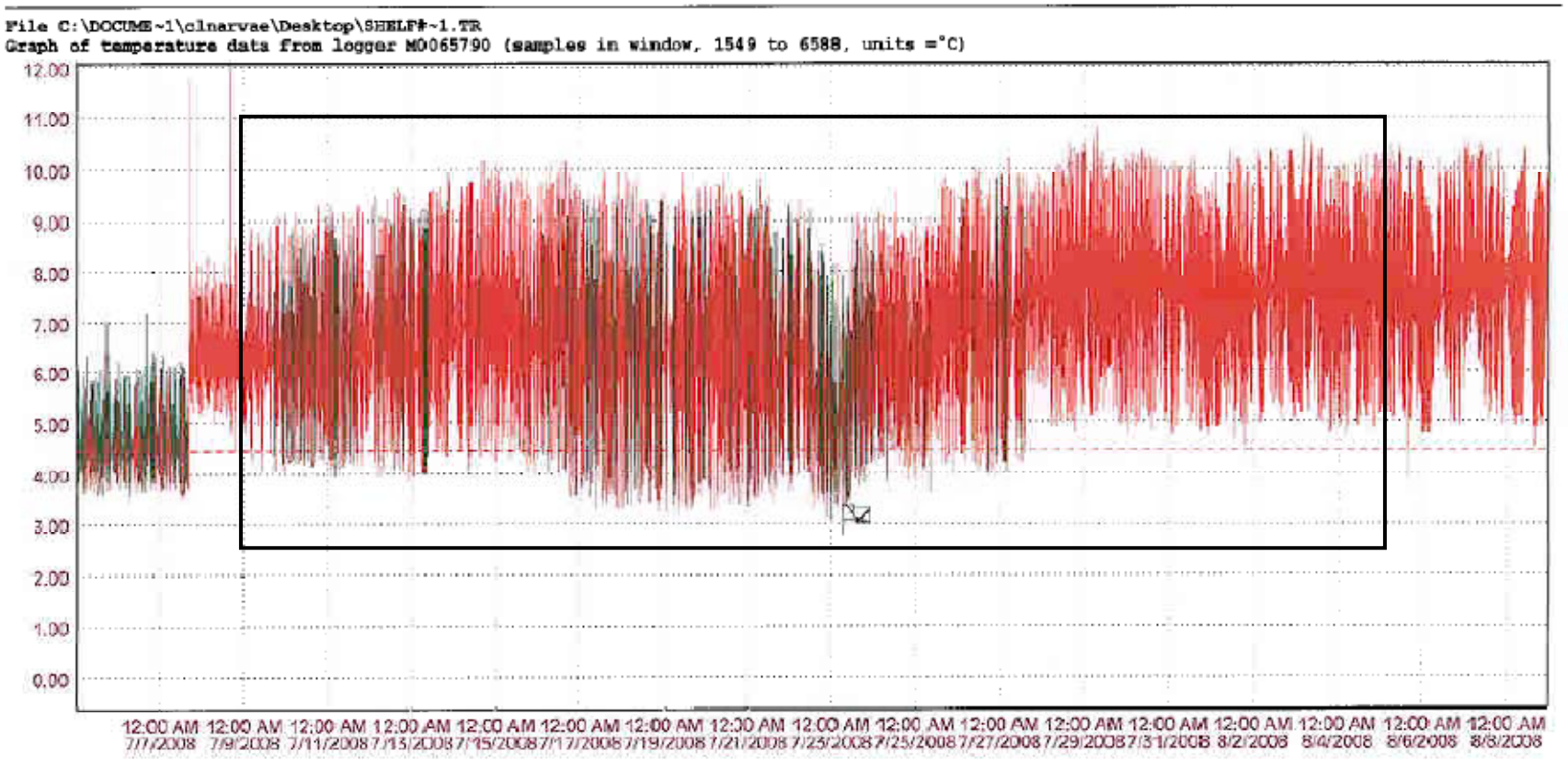

Figure D1. Temperature records obtained from shelf #1 of the display cooler employed for all replications of Objective II from July 9th through August 4th, 2008.

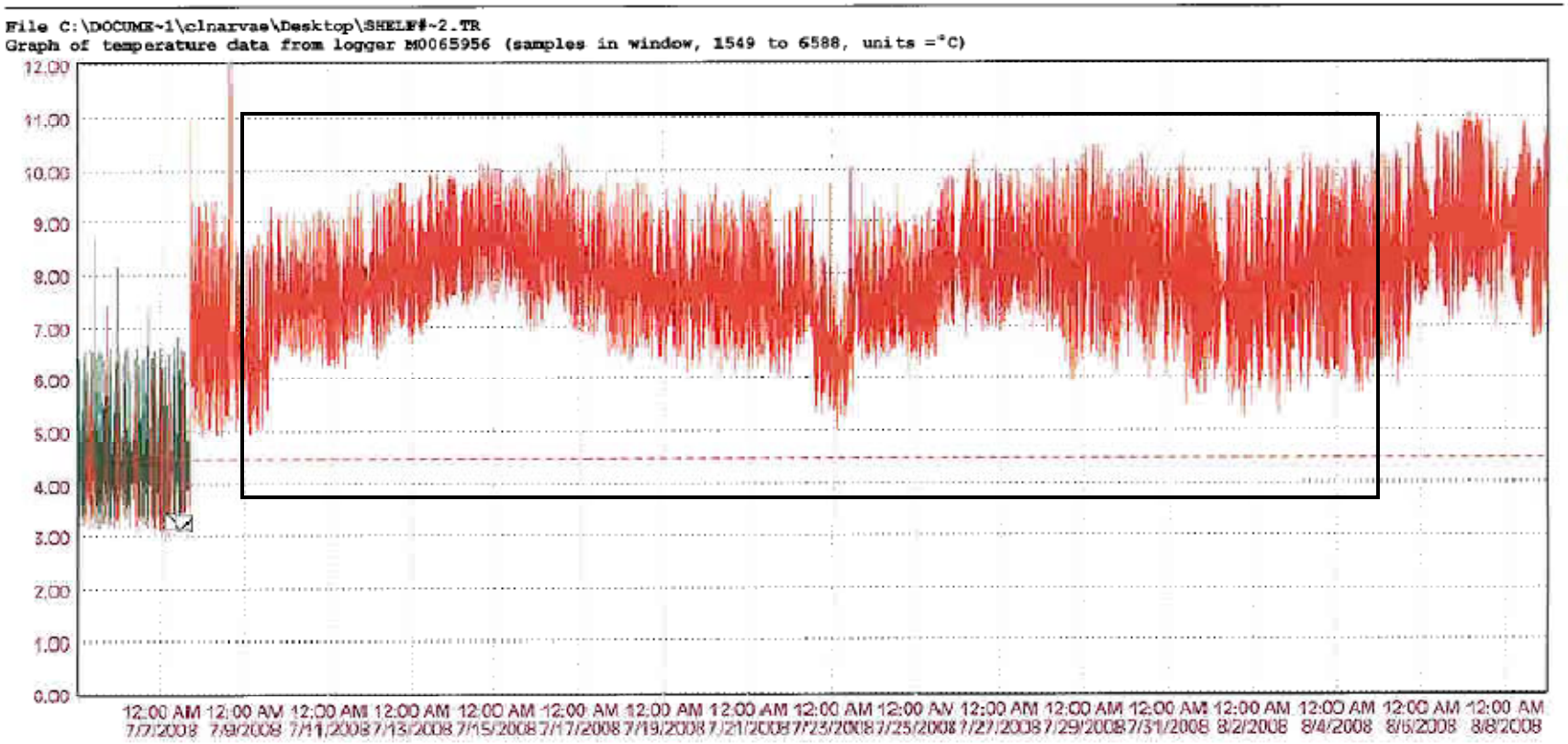

Figure D2. Temperature records obtained from shelf #2 of the display cooler employed for all replications of Objective II from July 9th through August 4th, 2008.

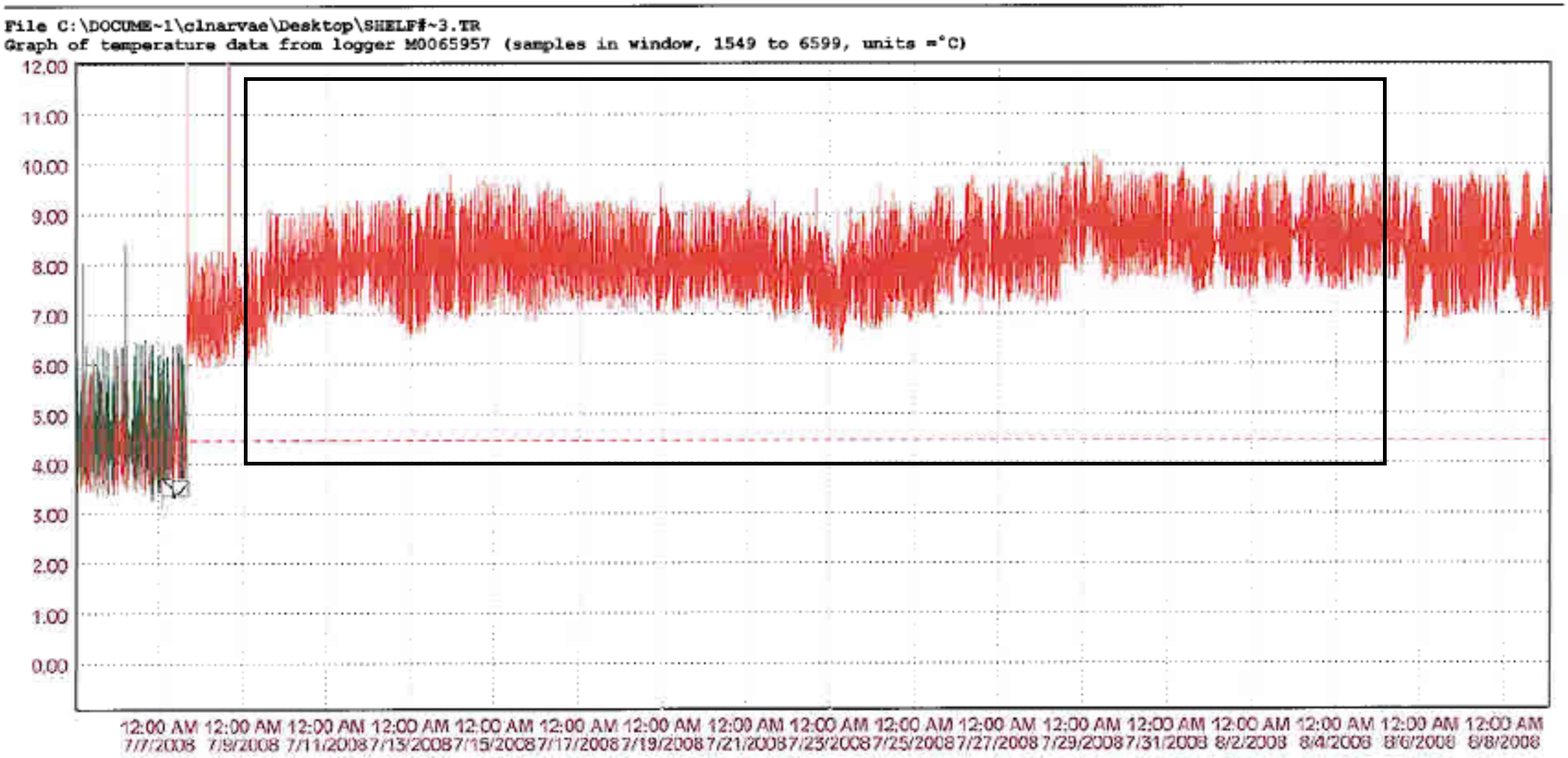

Figure D3. Temperature records obtained from shelf #3 of the display cooler employed for all replications of Objective II from July 9th through August 4th, 2008.

APPENDIX E

ADDITIONAL FIGURES

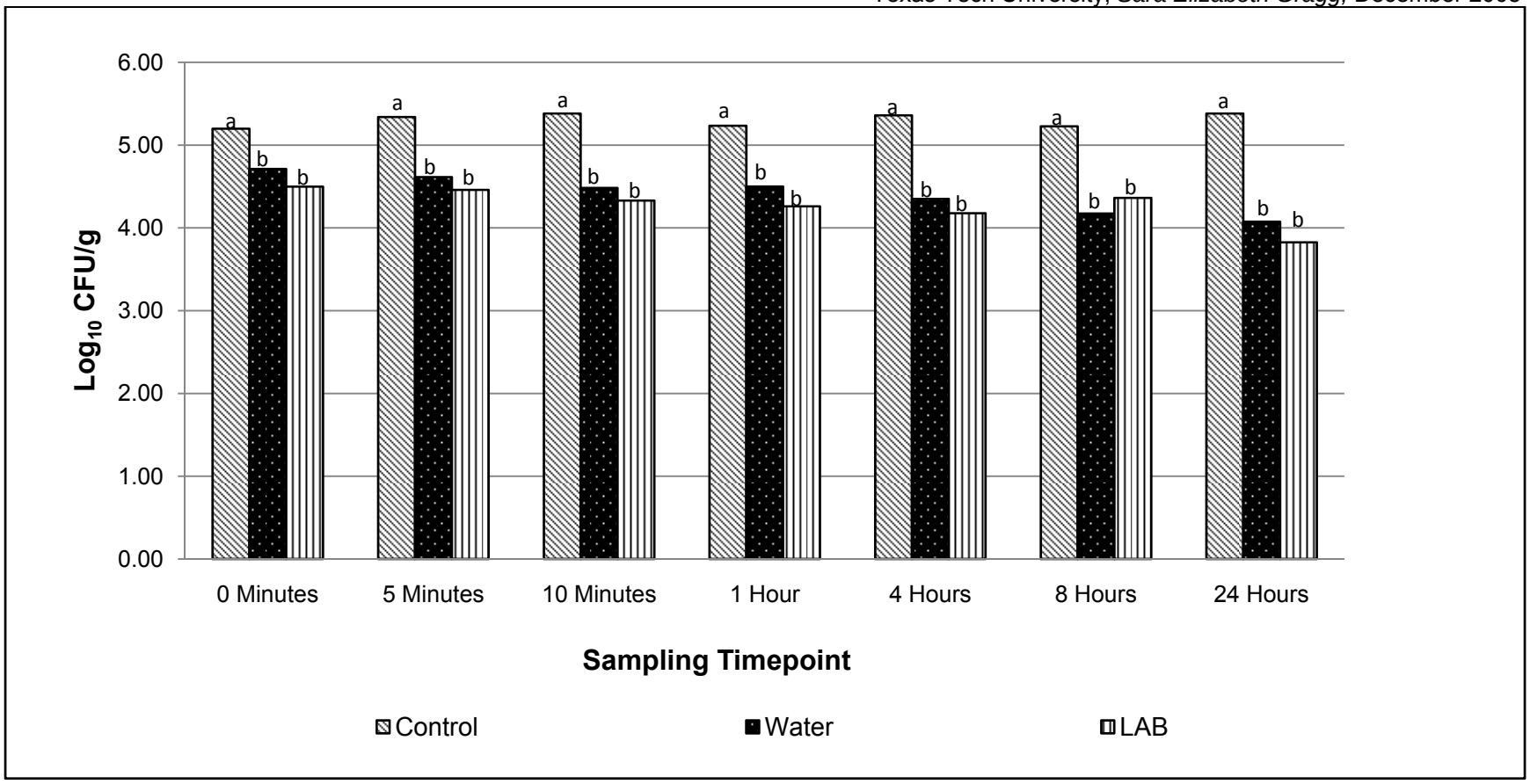

Figure E1. Least Squares Means of *E. coli* O157:H7 levels in each spinach treatment held at a target temperature of 7°C for 24 hours as obtained during Objective I.

^{a,b} indicates treatments that differ within each day ($p<0.05$).

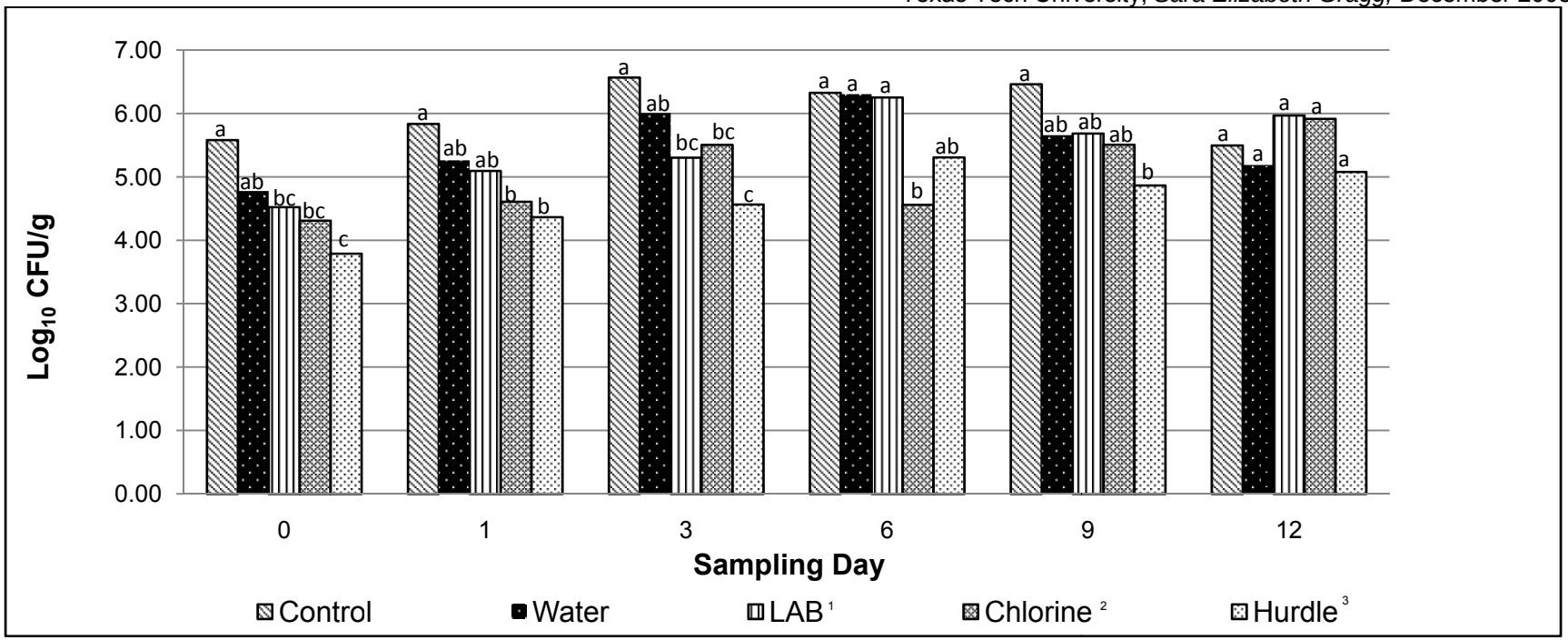

Figure E2. Least Squares Means of *E. coli* O157:H7 levels present in each spinach treatment held in a retail display cooler at a target temperature of 7°C for 12 days as obtained during Objective II.

a,b,c indicates treatments that differ within each day ($p<0.10$).

[1] LAB is representative of the Bovamine® Meat Cultures lactic acid bacteria treatment.

[2] Chlorine applied at a concentration of 200 parts per million (ppm).

[3] Combination treatment consisting of 200 ppm chlorine and Bovamine® Meat Cultures.

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