Investigating pre-harvest and postharvest interventions to control foodborne pathogens and surrogates on lettuce

by

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Abstract

Leafy greens have been recognized as vehicles for transmission of foodborne pathogens and an effective pre-harvest intervention to control them is currently lacking. After harvest, lettuce is often subjected to chlorinated water to reduce the microbial load in the water and on the lettuce tissue. While moderately effective, there is also a need for improved postharvest interventions.

The purpose of Objective I was to 1) determine potassium bisulfate efficacy at reducing populations of *Escherichia coli* (*E. coli*) and *Listeria innocua* (*L. innocua*) when applied pre-harvest to lettuce, and 2) assess the impact on product quality at harvest. Potassium bisulfate reduced *E. coli* populations on inoculated lettuce by 1.32 $\log_{10}$ CFU/g (P=0.0002) and *L. innocua* by 1.18 $\log_{10}$ CFU/g (P=0.0017). No detectable differences were observed in color (P>0.05); however, brown spots were observed on various leaves sprayed with potassium bisulfate.

The purpose of Objective II was to employ a blend of benzalkonium chloride, acetic acid, and methyl paraben (BAM) as a postharvest wash on romaine and iceberg lettuce and to 1) determine efficacy at reducing populations of *Listeria monocytogenes* (*L. monocytogenes*), *E. coli* O157:H7 and *Salmonella*, 2) measure changes in aerobic bacteria throughout the shelf life, and 3) quantify benzalkonium chloride and methyl paraben residues post-washing.

To quantify efficacy of BAM reducing pathogenic bacterial populations, fresh-cut romaine and iceberg lettuce were inoculated with *L. monocytogenes*, *E. coli* O157:H7, or *Salmonella* and washed in BAM at concentrations of 0%, 1%, 2% or 3% for one or five minutes. When plated on recovery media, contact time and wash concentration was not significant (P>0.05) for *Salmonella* on either product. Concentration was significant (P=0.0189) for *L. monocytogenes* on romaine; however, the greatest reduction observed was <1.0 $\log_{10}$ CFU/g. The 3% wash significantly reduced *E. coli* O157:H7 on romaine by 1.75 $\log_{10}$ CFU/g, which is 0.66

$\log_{10}$ CFU/g better than the 0% wash. Following washing, wash water was analyzed and data demonstrate that all wash concentrations significantly ($P \leq 0.05$) reduced each foodborne pathogen by >2.0 $\log_{10}$ CFU/g in the wash water.

To quantify benzalkonium chloride and methyl paraben residues, as well as changes in aerobic bacteria and product quality, fresh-cut romaine and iceberg lettuce were subjected to a 1 minute wash in BAM at concentrations of 0%, 1%, 2%, or 3% and immediately sampled to determine aerobic populations and product quality. Concentrations 0% and 2% were also packaged into retail storage bags and sampled on days 0, 3, 5, and 7. Residues were quantified on these days as well. On day 0, aerobic populations did not vary according to wash concentration ($P>0.05$). With regards to shelf-life data, the 2% wash significantly reduced ($P=0.0203$) aerobic bacteria on romaine lettuce; however, no significant difference was observed on iceberg lettuce ($P=0.0819$). With regards to overall visual appearance of romaine or iceberg lettuce, no significant difference was detected between 0% and 2% BAM washes for each day throughout the shelf-life study ($P>0.05$). Methyl paraben and benzalkonium chloride residues were <5.0 and <10.0 ppm, respectively, on both products on each sampling day.

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Chapter 1 - Introduction

The United States government defines produce as any fruit, vegetable, or nut (*19*). Produce, and their related safety, is regularly in the news, which warrants investigation into effective food safety interventions for these products (*18*). Of the over 9.6 million cases of foodborne illness with a known etiologic agent, 45.9% were produce-related; the highest of any other food commodity group (*19*, *21*). The Centers for Disease Control and Prevention (CDC) estimates that overall foodborne illness is much higher, at around 48 million cases annually, due to underreporting and underdiagnoses (*19*, *21*). When extrapolating the 45.9% of produce-related outbreaks to this CDC estimation, it can be estimated that over 22 million people become sick from contaminated produce annually. Complicating matters, produce can be compromised anywhere along the farm-to-fork continuum with a foodborne pathogen. The farm-to-fork continuum can be described as the cycle of production, distribution, and consumption of fruits, vegetables, and nuts. Pre-harvest, postharvest, postharvest processing, and consumer practices are the key components of the farm-to-fork continuum.

In a 2010 Nutritional Reviews® article, University of Massachusetts professor David Nyachuba hypothesizes why foodborne illness, including cases with produce as the etiologic agent, is still so high given intense research and preventative efforts in the last thirty years (*15*). In Nyachuba's argument, the United States' push for more fruits and vegetables in the American diet is likely a main culprit for the rise in related foodborne outbreaks. This is supported by a 2008 United States Department of Agriculture (USDA) report indicating a 19% total increase in fruit and vegetable consumption from the years 1970-2005 (*23*). That same USDA report indicates that the per capita fruit and vegetable consumption in 2005 is still nearly 45% below their recommended mark. As a result, the United States government is continuing to push for

more consumption through nutrition assistance programs (e.g. food stamps), food guidance systems (e.g. *MyPyramid*), and education outreach initiatives which may perpetuate produce related foodborne illness (*3*). There is clearly a need for improving preventative measures as well as research into viable antimicrobial interventions throughout the farm-to-fork continuum.

Recent reports recognize that the underlying cause of foodborne illness is dynamic and multilateral. It is not solely because more produce is available to consumers; moreover, there is a growing susceptible population. In present day, the elderly population, as well as those with weakened or compromised immune systems, continues to grow. Recent studies suggest as many as 30% of the U.S. population fits into one of those categories (*9*, *14*). Others with heightened risk for foodborne illness, such as those with below average immunity, include children and pregnant mothers (*9*). As populations with increased susceptibility to foodborne pathogens rise, so does overall foodborne illness. This may partially explain why an estimate of 22 million foodborne illnesses associated with produce every year is a feasible approximation.

The food system continues to evolve so that fewer farms are experiencing the burden of feeding more people (*23*). As a result, existing farmers must grow greater amounts of food. It has been suggested that this may be contributing to foodborne illness outbreaks, as a large amount of product will be affected if contaminants (e.g. pathogenic microorganisms) enter the produce production chain. This large amount of product can then affect a large population. Dr. Robert Gravani of Cornell University has stated that, "in our complex food supply chain, with the multiple handling of produce during harvesting, sorting, washing, transportation, and storage, there is a greater chance of production contamination and temperature abuse to occur" (*9*). Produce is not exempt from this statement, as these products often go through washing, distribution, shipping (perhaps multiple times), and display before reaching the consumer.

Not only is foodborne illness a serious public health issue, but it also comes with serious economic ramifications. A 2011 cost-of-illness model from The Ohio State University, which has been accepted by the USDA, estimates the cost of foodborne illness to be approximately $51 billion annually (*22*). This model includes physician care, hospital services, laboratory testing, productivity loss, and a variable of uncertainty, which attempts to account for the wide underreporting and underdiagnoses of foodborne illness (*22*). What this model does not include, however, is the cost to the food industry, affected consumer confidence, and litigation disputes; meaning the true cost of foodborne illness extends far beyond $51 billion annually (*7*).

To further examine the severe implications of even a single foodborne outbreak, the 2006 *E. coli* O157:H7 outbreak of spinach will be used as an example. On September 14, 2006, the Food and Drug Administration (FDA) made an announcement to all U.S. retailers to stop selling all fresh, bagged spinach (*5*). This notification arose from the CDC advice after fifty illnesses and eight cases of kidney failure were reported across the country, all of which had fresh spinach as the suspected vehicle of transmission (*10*). Over the next two weeks, the FDA slowly began isolating the source of contaminated spinach by targeting California suppliers, with emphasis placed on spinach grown in Monterey, Santa Clara, or San Benito, California (*5*). As might be expected, spinach sales immediately plummeted, taking nearly fifteen months to recover to pre-outbreak sales levels (*5*). While spinach sales from all growers (California or otherwise) plummeted, growers of other leafy greens also experienced a decrease in sales over the next three months (*4*). Canada, which imports the largest amount of U.S. leafy greens, briefly blocked trade of leafy greens, and the market remained low until June 2007 (*4*). With so much at stake and a tarnished reputation, California leafy green growers joined together in 2007 to form the California Leafy Green Products Handler Marketing Agreement (LGMA), a collaborative effort

with universities, food safety specialists, farmers, and government officials to create a validated food safety program for the growing of leafy greens (*2*). This effort aims to target sources of microbial contamination, survey farms, establish safe worker practices and establish government audits for participating farms (*2*). The mission of the LGMA recognizes that prevention and control of foodborne pathogens on produce needs to be holistic and at every step of the growing/production process.

Farms with the most progressive and rigorous food safety standards are not immune to microbial contamination due to the inherent fact that the majority of produce is grown outside, where pathogens are naturally occurring, and lacks a major lethality step during processing and preparation before consumption (*6*). This creates a challenging, dynamic dilemma, which establishes a market for antimicrobial interventions throughout the farm-to-fork continuum. Currently, the availability of pre-harvest antimicrobials for fruit, vegetable, and nut growers is limited. Processers of fruits and vegetables commonly add chlorine to wash water; however, it is intended to kill pathogens in the water, rather than on the surface of plant tissue (*12*). Other research has been done on postharvest washes, but none have been successful enough to change the market standard of chlorine. A likely reason is that chlorine remains cheap, yet moderately effective at controlling pathogens. However, introducing a safe, more effective postharvest antimicrobial to washing may significantly reduce foodborne illness related to produce.

A gap exists with regards to suitable antimicrobials designed for pre-harvest and postharvest applications. A single outbreak can not only destroy the livelihood of the implicated farm, but also can make consumers ill and in severe cases, cause death. Even growers of similar products can be negatively impacted economically. Past outbreaks have strengthened our food

system, but there is still opportunity for improvement based on the frequency in which outbreaks occur.

The overall objective of this study was to evaluate a pre-harvest and a postharvest antimicrobial intervention with the aim of reducing foodborne pathogen populations, such as *E. coli*, *L. monocytogenes*, and *Salmonella*, on lettuce. The first objective was to determine if a 0.25% (w/v) solution of potassium bisulfate could be applied at either one week, two days, or one week and two days before harvest, and effectively reduce populations of the foodborne pathogen surrogates *E. coli* and *Listeria innocua* (*L. innocua*) on lettuce without negatively impacting product quality at harvest. The second objective evaluated a blend of benzalkonium chloride, acetic acid, and methyl paraben as a postharvest antimicrobial wash to reduce foodborne pathogens and aerobic bacteria on romaine and iceberg lettuce without negatively impacting product quality or resulting in chemical residues on the product.

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Chapter 2 - A Review of the Literature

2.1 Background and Significance

Foodborne illness is not a new issue. In fact, foodborne pathogens may have infected notable people of the past. Some theorize that Alexander the Great, the 335 BC Macedonian king and conqueror of the Persian Empire, contracted the foodborne pathogen *Salmonella enterica* subspecies Typhi that ultimately caused his premature death (*5*). Other notable people have also fallen victim of foodborne illness including U.S. president Zachary Taylor, novelist Rudyard Kipling, and aircraft innovator Wilbur Wright (*4*). In fact, the delirium and hysteria associated with the infamous Salem Witchcraft Trials is thought to have been rooted in a toxic fungus growing in consumed grain products (*4*). These historical examples came from a time before knowledge of disease causes and prevention, foodborne and alike. It was not until the middle nineteenth century when research by John Snow, working on a London outbreak of cholera, and Robert Koch, who further researched bacteria and is credited with Germ Theory, were able to link microscopic creatures to disease (*25*). Germ theory not only paved the way for enhanced public health awareness regarding how diseases spread but also ignited research into impactful interventions including vaccines, antibiotics, and antimicrobials. This understanding of disease is an important reason, among many, why the life expectancy of developed countries has increased in the last 150 years (*76*).

While overall disease incidence is down over the last 150 years, it begs the question why foodborne illness continues to plague public health today? To partially answer that question, an understanding of both the past and present food system is needed. The industrialization of the United States (and the world) in the late nineteenth century led to a massive efflux of farmers into factories as laborers (*41*). The growing U.S. population demanded remaining farmers to

"specialize, mechanize, simplify, and routinize" (*41*). This meant famers had to be more efficient and produce more food while lowering cost. This industrialized system enabled the United States to develop one of the better economies of the world and provide food and clothing in abundant amounts to all its citizens, which, by itself, isn't inherently bad (*41*). This system eventually spread around the world and created the global food market we know today. While the luxuries and abundance of food from this globalized market are unparalleled in human history, they have also brought unintended consequences to food safety. A foodborne outbreak originating from a farm now often means a large amount of product is contaminated, thus reaching a greater number of consumers (*15, 60*). This often leads to outbreaks involving many people in very different geographic locations, as contaminated products are also often distributed all over the world, only exacerbating the problem (*3, 15*). Researchers also hypothesize that pathogens are evolving into new serovars, partly due to large-scale farming and the broad ecologies seen around the world (*15*). Foodborne pathogens are becoming more virulent and more resilient to environmental stress and antimicrobials. These reasons at least partially explain why foodborne illness remains a challenge for public health. With increased fruit and vegetable consumption over the last forty years, produce is becoming increasingly implicated in outbreaks.

2.2 Sources of Foodborne Pathogen Contamination

Examining the production chain for produce, all agrarian steps in the production of raw produce is termed "pre-harvest" (*79*). This includes planting, irrigating, harvesting, and other processes. "Postharvest" is the term used to describe the steps after the raw commodity has been harvested, which include (but are not limited to) storage, transportation, washing, and other processing necessary to get to market (*79*). While contamination can also happen in retail establishments or consumer kitchens, it has been recognized and agreed upon that pre-harvest

and postharvest environments are the most common areas for foodborne pathogen contamination based on recent quantitative microbial risk assessments (QMRA) (*61*). Therefore, producers and researchers alike focus on pre- and postharvest as presenting the most logical opportunities for prevention and the application of antimicrobials. Currently, postharvest processing is the most common point for application of antimicrobials (particularly chlorine washes); however, the pre-harvest setting is probably of biggest concern for foodborne pathogen introduction (*56*).

Pre-harvest risks for microbial contamination most commonly originate from animals in the form of feces or contaminated water (*63*). It is well understood that animals, particularly ruminants, can harbor pathogenic *E. coli* and *Salmonella* spp. in their natural flora but can also contain *L. monocytogenes* (*63*). Animals are therefore highly discouraged from being proximal to growing produce fields. It is atypical for farmers to allow roaming livestock in production fields; however, wildlife can be difficult to deter, even with fencing (*11*). Nets and covers can be used to control birds, which are important, as their feces have been shown to harbor both *Salmonella* spp. and *E. coli* O157:H7 (*63*). Experimental research also reports certain invertebrates (e.g., house flies, aphids, thrips, *et cetera*) as possible vectors of pathogen transfer, although the true role and the practical risk are currently unknown (*11*, *63*).

Animal waste has one major route of contamination that has been recognized as a vector for disease: irrigation water. In fact, irrigation water may be the key contributor to pathogen contamination of produce (*47*). Epidemiological research confirms that the quality (and safety) of irrigation water is directly related to the safety of growing produce (*11*). Agriculture is inherently heavily dependent on water, and many opportunities are available for it to become microbiologically compromised. The source of contamination largely depends on the source of water. Surface water (e.g., from lakes, rivers, and streams) can become contaminated with

pathogens from wildlife, agricultural run-off, human waste, and industrial effluent (*61*). It is, therefore, crucial for farmers to test their irrigation water regularly for pathogens, as no practical water disinfection method has been developed to accommodate the large volumes needed for a large farm setting (*11*). Ground water (e.g. from wells) is typically thought of as posing less risk for pathogens, but contamination can still occur in the same manner as surface water contamination, meaning testing is equally important (*11*). Research suggests that the method of irrigation may also be important in controlling risk of foodborne pathogens. Surface drip and overhead irrigation pose increased risk for microbial contamination, compared with furrow (corrugated) and surface irrigation, presumably because water is coming into direct contact with produce (*11*, *63*). However, method of irrigation and foodborne pathogen risk is controversial and currently the subject of additional research efforts (*11*). Contaminated on-farm water systems can find their way onto produce beyond irrigation as well. Fertilizers, herbicides, and pesticides are prepared with water that directly makes plant contact.

The soil in which produce is grown is another consideration when evaluating foodborne pathogen risk associated with produce (*63*). This particularly applies to produce grown in close proximity to the ground, such as leafy greens, melons, and root crops—further explaining their elevated risk (*49*). The soil ecosystem can harbor many foodborne pathogens including: *L. monocytogenes*, *Bacillus cereus*, *Clostridium* spp., and others (*61*). Farmers that use animal waste as fertilizer can have a broadened profile of pathogens, including pathogenic *E. coli* and *Salmonella* spp. if waste is not composted correctly (*61*). Farmers often use manure as it has shown to increase water-holding capacity, increase aeration, decrease soil erosion, and increase nutrient density (*42*). Once contaminated, enteric pathogens can survive upwards of 260 days in soil, depending on soil temperature, moisture, and source of contamination (*61*). Current research

suggests lower temperatures and higher moisture are more conducive to enteric pathogen survival (*42*).

It is clear that when fruits, vegetables, or nuts are growing on a farm, the potential for the introduction of foodborne pathogens is present. Improperly composted manure, farm water, and soil are the major inoculum sources for the introduction of pathogens, but many others do exist to a lesser extent. Many items are still hand harvested by humans, which presents a risk that pathogens carried by a human may be transmitted onto produce (*11*). Even produce that is harvested by machines is at risk for pathogen introduction, as harvesting machines constantly come into contact with the soil and water (*61*). Other less frequent, but possible, sources of on-farm contamination include: the bins in which harvested produce is stored, dust, and air pollution (*11*, *61*). Foodborne pathogens pose a dilemma to producers, as they cannot ensure everything they grow is pathogen free. To minimize risk, growers of fruits, vegetables, and nuts need to be aware of potential sources of pathogen introduction and develop a plan to prevent introduction to growing commodities. Fortunately, the U.S. government is making it easier for growers by issuing advised on-farm practices as well as the introduction of good agricultural practices (GAPs).

After harvest, produce is transported, processed, and distributed to market. Transporting offers risks with regards to the containers holding the produce, but also with anything that comes into contact with the product (e.g., water drippings, dust, *et cetera*). Depending on the commodity, processing presents additional risks for product contamination. More specifically, contamination may occur by means of the water for washing, the machines for slicing, and any packing equipment bundling produce together (*56*).

2.3 The Food Safety Modernization Act and Produce Safety

On January 4, 2011, President Barack Obama signed into law the Food Safety Modernization Act (FSMA), reforming over 70 years of food safety law when Franklin D. Roosevelt signed the Food, Drug, and Cosmetic Act in 1938. The FSMA includes many sections aimed at shifting our food safety system from reactionary legislation to prevention. Among the sections, the FSMA includes a Produce Safety rule that sets standards for the growing, harvesting, packing, and storage of produce destined for fresh markets. Importantly, all standards are backed by scientific literature (*10*).

As water is associated with transmission of human foodborne pathogens to produce, the Produce Safety rule includes water testing specifications for growers of fresh produce. Testing parameters and allowable limits of generic *E. coli* for untreated surface and ground water have been set forth by the Produce Safety rule. It should be noted that generic *E. coli* cannot be detected in any of these samples if the water directly contacts produce, is to be used on food contact surfaces, is used for the washing of hands, or is used for the irrigation of sprouts. This ruling does not apply to growers who use municipal water but to those who use untreated surface or ground water (*10*).

Additional regulations in the Produce Safety rule address other common routes of human pathogen transmission on a farm setting. Growers who use raw manure on-farm are now mandated to wait at minimum 120 days between the application and the harvest of crops that come in direct contact with soil. Crops that do not contact soil have a 90 day interval. While the FDA is currently conducting a risk assessment to determine the appropriate time to eliminate pathogens from raw manure, these parameters are based on standards outlined in the USDA's National Organic Program. Additional sections in the Produce Safety rule specify animal (both

wild and domestic) exclusion standards, the sanitation of all equipment that may come into contact with produce (including bins and tools), and an entire section dedicated to the growing of sprouts (*10*).

The Produce Safety rule of the FSMA also addresses the health and hygiene of farm workers who come into direct contact with produce. Farms are to prevent sick employees from working, give adequate restroom and hand-washing facilities, and prevent visitors from possibly introducing contaminants on the farm (by means of exclusion, hand-washing, *etc*) (*10*)

The FSMA final rule for Preventive Controls for Human Food requires FDA food processing facilities to "establish and implement a food safety system that includes an analysis of hazards and risk-based preventive controls" (*10).* For example, all facilities covered by this rule are to generate and follow a HACCP-based food safety plan addressing all potential hazards of processing. Operations that fall under the FSMA definition of a 'farm' will not be subject to this rule. The FDA does offer assistance to industry to improve comprehension and implementation of FSMA requirements. There are many exemptions to these FSMA rules, particularly for very small farms, particularly those whose produce is destined for processing (e.g. canning), and very small processors (*10*).

2.4 Pathogens Implicated with Leafy Greens

The major foodborne pathogens associated with leafy greens are indicative of the major sources of contamination. As feces play a predominant role in contamination, many of the foodborne pathogens seen in outbreaks are endogenous to excrement and are part of the natural flora of animals (*59*). The most predominant pathogens in animal feces are *Salmonella* spp. and *E. coli*, which at least partially explains their frequent implication in produce outbreaks. *Listeria monocytogenes*, a ubiquitous environmental pathogen that can be a major problem with

processors, is also commonly implicated in produce outbreaks (*1*). Norovirus, which is associated with an estimated 20% of produce outbreaks, is also a problem in the produce sector; however, introduction typically occurs in consumer's hands and during preparation (*23*). Other foodborne pathogens have been implicated in produce outbreaks including: *Campylobacter jejuni*, Hepatitis A, *Cyclospora cayetanensis* (a protozoan parasite), *Giardia lamblia*, and *Staphylococcus aureus* (*10, 59*). It has been established that *Salmonella* spp., *E. coli*, and *L. monocytogenes* are the primary outbreak culprits and deserve the most attention with regards to prevention and intervention efforts (*59*).

A CDC epidemiological study by Herman et al. (2015) researched leafy greens outbreaks from the years 1973-2012. Data were collected from volunteering local, state, and territorial health departments across the United States. This study found that from 1973-2012 a total of 606 (an average of four per year) foodborne outbreaks were associated with contaminated leafy greens. The results of this study revealed that 42.9% of leafy greens outbreaks were caused by Norovirus, 8.1% by shiga toxin-producing *Escherichia coli* (STEC), 5.3% by various *Salmonella* serovars, and 2.3% by *Shigella*. A large number (34.2%) of leafy greens outbreaks never had a confirmed pathogen. Improving surveillance to better determine implicated pathogens will focus preventative efforts in the future (*37*).

2.5 Mechanisms of Pathogenicity

As *Salmonella* spp., *E. coli*, and *L. monocytogenes* are the most common bacterial causes of produce outbreaks; they will be the focus for pathogenicity. Each microbe will be followed from ingestion to illness.

Ingested *Salmonella* pass through the stomach where many cells succumb. However, some *Salmonella* cells are capable of surviving the acidic environment via an acid tolerance

response (ATR) (*64*). This ATR includes lysine carboxylase converting available lysine into cadaverine and ammonia, ultimately raising the pH of the area immediately surrounding the cell, which helps *Salmonella* cells to tolerate the low pH of stomach acid (*64*). Another mechanism *Salmonella* uses is the up-regulation of acid-shock protein (ASP) genes, which function to remove excess hydrogen ions from the cytoplasm and assist in preventing destruction of an acidified cytoplasm (*64*). Cells able to survive the stomach acid travel to the lower gastrointestinal (GI) tract where they use their flagella to attach to an epithelial cell (*29*). *Salmonella* cells must also compete with endogenous microflora, but capable cells attach to epithelial cells and use a needle like apparatus, called a type-three secretion system (T3SS), to inject proteins into the cytosol of the intestinal epithelial cell (*29, 57*). Injected proteins ultimately lead to a reshuffling of the host's outer membrane (*29*), and the *Salmonella* cell invades. More specifically, once inside the GI tract, *Salmonella* invades at the M cells and through the Peyers patches, where it is engulfed by the phagosome vacuole (*46*). *Salmonella* then releases more proteins, namely SifA, to protect itself from host defenses and allow replication (*29, 46*). Cells are ultimately released to the bloodstream where they are isolated to the spleen, lymph nodes, liver, gall bladder, or bone marrow (*46*). This leads to symptoms of gastroenteritis including diarrhea and cramps and possible complications such as meningitis, endocarditis, and osteomyelitis (*7*). Since *Salmonella* can enter the gall bladder, one strain (*Salmonella* Typhi, i.e. Typhoid fever) can remain in the body undetected in the infected individual, who remains asymptomatic and sheds cells in their feces (*34*). Such was the case for the famous early twentieth century cook Mary Mallon, otherwise known as Typhoid Mary (*48*). Mary Mallon was perhaps the most notable asymptomatic *Salmonella* Typhi carrier and she is estimated to be

responsible for as many as 50 fatalities (*48*). Her refusal to stop cooking, as well as her changing aliases, makes exact figures difficult to ascertain.

Pathogenic *E. coli* is responsible for one of the largest foodborne outbreaks to date: the 2006 outbreak involving spinach. *Escherichia coli* can be categorized into a number of different groups: enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enteroaggregative (EAEC), diffusely adhering (DAEC), and enterohemorrhagic (EHEC); each grouped based on virulence, pathogenicity, symptoms, and serotypes. Serotypes are differentiated based on antigens present on the surface of the capsule (K), flagella (H), and cell wall, otherwise known as the somatic antigen (O). Most *E. coli* strains are differentiated based on their O and H antigens. Enterohemorrhagic *E. coli* (EHEC) causes the most cases of foodborne illness (*59*).

Pathogenic EHEC have an array of virulence factors that enable the infection of susceptible hosts. Similar to *Salmonella*, EHECs have a certain degree of acid tolerance and the capability of surviving stomach acid (pH ~ 2), which enables a small number of cells (10-100) to infect a host (*40*). Three acid-resistance systems are known for *E. coli*. The least known mechanism is controlled by a sigma factor, RpoS, and is a general gene product that is induced by any stressor (*12*). It is currently thought that RpoS induces a gene that protects proteins from acid damage, but more research is needed (*13*). The other two acid-resistance systems involve the decarboxylation of either of the amino acids glutamate or arginine, thus consuming cytoplasmic protons and creating antiporter molecules that further rid the cytoplasm of hydrogen ions (*13*).

Upon reaching the lower GI tract, an *E. coli* cell docks itself on top of an intestinal microvillus and creates a pedestal-like lesion (*59*). Intimin, an outer membrane binding protein,

is made and presented on the *E. coli* surface (*57*). *Escherichia coli* then injects a Tir protein into the microvillus, via a T3SS. The Tir protein acts as the intimin receptor, which allows for firm adhesion to the microvillus (*59*). All genes required for microvillus attachment are collectively known as the locus of enterocyte effacement (LEE) island and are imperative for *E. coli* infections (*59*). What separates pathogenic from non-pathogenic *E. coli* is not only the ability to attach to a microvillus but the production of one or two shiga toxins, otherwise known as Stx. Two variants of the toxin exist, Stx1 and Stx2, and are related to the toxin produced by *Shigella dysenteriae* (*54*). The shiga toxins are located on the *E. coli* chromosome; however, they are associated with a lambdoid prophage that inserted itself into the *E. coli* genome some time ago from *Shigella* (*54*). This lysogenic phage only decides to go lytic (i.e. remove itself from the chromosome and turn into an active virus) when DNA damage has occurred in the *E. coli* cell (*54*). The phage not only excises itself out of the *E. coli* chromosome, but also causes substantial upregulation of the Shiga toxin (*54*). While the effected *E. coli* cell does lyse, the Shiga toxin destroys the attacking host leukocytes and neutrophils (*54*). It has been suggested that this fratricide is for the greater good of the entire population (*54*). Throughout this process, infected individuals may experience severe cramps, and diarrhea, which is often bloody—a hallmark symptom of a Shiga-toxin producing *E. coli* infection (STEC). Severe cases lead to kidney failure in the form of hemolytic uremic syndrome (HUS) or clotting of the blood vessels by means of thrombotic thrombocytopenic purpura (TTP) (*61*).

Another foodborne pathogen commonly implicated in produce outbreaks, *L. monocytogenes*, has the highest untreated mortality rate at between 15-25% (*27, 61*). This ubiquitous organism can grow between -0.4°C to 50°C and is known to be a potent biofilm former (*38*). It is an organism that food growers and manufactures monitor, and it is very

difficult to remove from environments once present (*61*). *Listeria monocytogenes* has unique virulence factors that aid in evading the immune system. Further, *L. monocytogenes* can invade highly sensitive areas such as the spinal cord, brain, and the placenta of pregnant mothers (*61*).

Listeria monocytogenes regulates initial virulence factors by the use of a DNA binding protein, PrfA, and only allows dissociation from the DNA with either an acidic or temperature (37°C) stressor—both of which occur when ingested in the human body (*45*). When PrfA dissociates, the operon of virulence genes is upregulated and the gene products are produced. Gene products include key invasive proteins, namely, internalin (*45*). Upon reaching the lower gastrointestinal tract, *L. monocytogenes* uses the internalin protein to bind to E-Cadherin of host epithelial cells, and causes host cell membrane reshuffling to invade (*45*). After passing through the intestinal epithelial cells, *L. monocytogenes* cells are engulfed by macrophages where they are sequestered within the vacuole for death (*61*). *Listeria monocytogenes* cells, however, are able to avoid death, and replicate inside a macrophage vacuole, leading to an escape mediated by listeriolysin-O and phosphatidylinositol phospholipase C (*61*). Cells unable to escape are avirulent, making this step crucial for survival and dissemination within the host (*35*). Upon escaping the macrophage vacuole, *L. monocytogenes* cells upregulate the protein ActA and recruit host actin to bind to a scaffolding on one end of the *Listeria* cell (*35*). Enough actin creates a "tail," which causes *L. monocytogenes* to spin, ultimately creating movement inside the macrophage cytosol (*35*). This movement propels cells out of the current macrophage into adjacent cells where it can start replicating again (*35*). In this way, *L. monocytogenes* cells can propel themselves into the host bloodstream, meninges, and, in a pregnant woman, the placenta (*61*). Those experiencing listeriosis should only expect to feel mild flu-like symptoms throughout this process, followed by intense illness depending on where the bacteria invades (*61*).

2.6 Microbial Attachment to Plants

The surface of the average plant tissue is composed of a multilayered hydrophobic cuticle that provides protection from infection, insect damage, and water loss (*31*). Stomata, pore openings on the surface of the tissue, allow gas exchange and reduce water loss (*31*). Trichomes, which are hair-like appendages that project from the plant cuticle, provide plant tissue defense from insects in the way of chemical and physical repellant (*31*). Bacteria tend to cluster around stomata and trichomes (*23*). Research shows that bacteria on the surface of a plant, also known as epiphytes, will aggregate around wounds on the plant due to nutrient leakage (*23*). The hydrophobic cuticle is believed to make it more difficult for bacterial cells to attach; however, cells able to attach may have increased resistance to antimicrobials (*31*). This may explain why it is generally recognized that postharvest produce washes are meant for disinfecting the water rather than the plant tissue (*17*). Microbes that attach to plants must also survive stresses including: the aerobic environment, temperature changes, humidity changes, poor nutrient availability, varying water availability, and ultraviolet light (*18*).

Understanding how foodborne pathogens attach to produce is important for human health and food safety. A complete understanding is important in order to effectively approach pre- and postharvest habits and microbial removal. Microbes utilize a combination of van der Waals forces, hydrophobic interactions, and hydrogen bonding to achieve firm attachment (*68*). Bacterial polysaccharides and proteinaceous pili act as the bacterial attachment points to the hydrophobic plant surface (*23*). Some microorganisms produce a complex, jelly-like matrix called an extracellular polymeric substance (EPS) that may also aid in plant attachment (*66*). Evidence suggests that the level of attachment on a plant tissue is dependent on the serovar of the pathogen (*44*). Jeter and Matthysse (2007) found that strains of pathogenic *E. coli* capable of

causing diarrhea in humans were also able to attach to plant tissue more readily compared to non-diarrheic strains (*44*). This suggests that human epithelial attachment genes may play a role in attaching to plant tissue (*18*). Research also suggests that the side of the leaf may be important for attachment as the abaxial (underside) showed greater attachment compared to the adaxial (top); perhaps showing the abaxial side is prone to fewer stressors (*21*). Others hypothesize bacteria prefer colonizing the abaxial side of the leaf due to more trichomes and stomata (*14*).

Biofilm formation can also occur on the surface of plant tissues (*66*). A biofilm is a highly resistant, complex network of cells that enables nutrient flow and waste removal (*66*). The increased resistance is due to the reduced ability for antimicrobials to penetrate the biofilms. Encompassed cells also have a slower growth rate, thus reducing uptake of any antimicrobial introduced to a biofilm (*26*). This enables cells to be tolerant of conditions that are sub-optimal or otherwise lethal (*23*). Biofilms are formed when a large number of associated bacteria "communicate" with each other via signal molecules. This is known as quorum sensing. Bacteria constantly secrete these signal molecules into the environment, and when the concentration of them is high enough, cells recognize this and an up-regulation of genes takes places, which manifests into a biofilm (*23*). Biofilms are very difficult to rid from a surface, including the plant tissue surface.

2.7 Past Research on Postharvest Antimicrobials

Researching viable alternatives to chlorine sanitizers as a postharvest processing aid have been numerous and ongoing. Chlorine sanitizers are effective at removing pathogens from water, but are not overly efficacious at removing pathogens attached to lettuce tissue. Chlorine sanitizers are also prone to organic loading (the reduction in free chlorine by binding to organic matter—reducing overall efficacy), and are sensitive to temperature and pH. Therefore,

alternatives have been researched in the past including: ozone, peroxyacetic acid (PAA), hydrogen peroxide, and quaternary ammonium compounds (QAC) (*62, 75*).

Ozone has been shown to be effective at reducing *Salmonella*, *L. monocytogenes*, and *E. coli* by greater than 3.0 $\log_{10}$ CFU/g in free wash water. Research using ozone as a produce wash has reported positive results, although its overall ability to reduce foodborne pathogens has not been significantly better than chlorine. Research has also demonstrated that ozone can be corrosive to machinery and maintaining concentration in a water system can be difficult. It is also dangerous to humans so a ventilated processing facility would be required (*62*).

Peroxyacetic acid (PAA) has been evaluated as a postharvest produce wash: however, research is conflicted regarding its ability to remove foodborne pathogens and data are often confidential by companies. One study found a 2.0 $\log_{10}$ CFU/g reduction of *L. monocytogenes* on cut-salad mixtures when exposed to 90 ppm PAA for 15 seconds, however, no significant difference was found compared to a 100ppm chlorine wash. Peroxyacetic acid has been shown to inhibit bacterial growth better than chlorine, which may increase the shelf life of products exposed to it (*62*).

Hydrogen peroxide has been evaluated at 1% and 2% concentrations on many different produce items, including fresh-cut lettuce. Its efficacy has been shown to not be any better than chlorine, which has discouraged use. It also has been shown to negatively impact the sensory quality of lettuce, further discouraging use (*62*).

Quaternary ammonium compounds (QAC) have been evaluated as a produce postharvest wash. Research into QAC shows effectiveness to be best against gram positive microorganisms with optimal activity between a pH of 6-10. Efficacy is significantly worsened in an acidic environment (pH <6.0) or in the presence of surfactants. A proprietary (company has not

disclosed formulation) QAC solution of 200ppm demonstrated moderate effectiveness as a postharvest wash by reducing total aerobic bacteria by 95% (1.30 $\log_{10}$ CFU/g) on the surface of lettuce, compared to 60% (0.40 $\log_{10}$ CFU/g) of the water control. The FDA does not currently allow QACs to be used commercially as they need to determine if produce treated with them are safe for consumption (*62*).

2.8 Surrogate Microorganisms

In food microbiology and food safety, a surrogate can be defined as a nonvirulent, model microorganism that mimics survival and growth of a pathogenic microorganism of interest. Their use stems from the need to not introduce pathogens to a specific environment, but to study how pathogens behave in that environment (*72*). For example, lactic acid may prove to be an effective intervention to control pathogenic *E. coli* in a laboratory setting, but in-plant validation is needed to ensure efficacy translates to the setting and environment of its intended use. Due to the safety risk, pathogens would never be allowed in a facility where food production takes place, but surrogates may be allowed for research purposes. The primary benefit of using surrogates is that they are safer to work with compared to pathogens (*19*) and, thus, are not a risk to human health.

Choosing an appropriate surrogate for a pathogen can be a difficult task. Often, there is not a perfect nonvirulent model organism for the intended research pathogen. Busta et al. (2003) argue the best surrogate is a nonvirulent strain of the pathogen. However, when access to such a microorganism is not possible, characteristics of a good surrogate include: 1) growth kinetics that can be used to predict pathogen, 2) differentiated between microflora, 3) susceptibility of injury similar to pathogen, 4) easily grown to high numbers (*19*). The use of surrogates is important to research in order to evaluate how pathogens would behave in a particular environment or matrix without using the pathogens themselves.

2.9 Risk of Fresh-Cut Lettuce

Due to the chopping process, fresh-cut lettuce is injured and, thus, releases intercellular fluids into the environment (*36*). These intercellular fluids have been shown to be nutritive to bacteria and may allow growth of foodborne pathogens, particularly when exposed to temperature abuse. *Listeria monocytogenes* and *E. coli* have both been shown to grow on fresh-cut lettuce (*36, 50*). Temperature control becomes paramount at controlling growth on any fresh-cut produce item, but particularly for lettuce. As stated by Harris et al., "survival of foodborne pathogens on produce is significantly enhanced once the protective epidermal barrier has been broken." (*36*). Additionally, the shredding process may introduce foodborne pathogens as it may process hundreds of pounds of lettuce between washes. A contaminated head of lettuce may transfer pathogens to the shredding machinery, which may subsequently transfer pathogens to all lettuce shredded until the machinery is adequately washed (*36*).

Fresh-cut lettuce may also be further prone to water infiltration, particularly when a large difference exists between the temperature of the water and lettuce itself (*36*). A common procedure is to not allow the temperature difference to increase beyond 10°F, as infiltration greatly increases above this temperature (*62*). Internal gas pressure, as well as the hydrophobic lettuce surface, typically does not allow this unless a large temperature difference exists between the product and water. However, internal gas pressure and the hydrophobic surface are greatly reduced on a cut product leading to greater water infiltration susceptibility (*36*). This poses a challenge, as water harboring foodborne pathogens may infiltrate cut lettuce tissue where nutrients are present for bacterial growth. Additionally, internalized bacteria are less susceptible to antimicrobial interventions, as the lettuce is a barrier to exposure.

The addition of a sanitizer to a fresh-cut lettuce (or other leafy greens) operation is a necessary step to prevent the transfer of foodborne pathogens. Studies generally suggest the commonly used sanitizer chlorine does not significantly reduce common foodborne pathogens on lettuce tissue compared to a water control (*62*). As stated by Parrish et al., "there are no known mitigation strategies to completely remove pathogens once contamination has occurred while maintaining produce freshness" (*62*). Chlorine, and many other sanitizers, do effectively destroy pathogens in water, so their addition is still important to processing environments, at they reduce the transfer of pathogens between products (*62*).

2.10 Potassium Bisulfate Mode of Action and Past Research

Potassium bisulfate (IUPAC name potassium hydrogen sulfate) is a colorless crystal that emits a mild sulfuric odor. This odor occurs because it is the potassium salt of sulfuric acid. According to its safety data sheet (SDS), its purest form (≥95% by weight) may cause severe skin burns, eye irritation, inhalation dangers, and severe complications if consumed (*8*). Potassium bisulfate is, by definition, a weak acid; however, with a uniquely low pKa of 1.9, it is much stronger than other weak acids in its ability to donate hydrogen atoms in solution (*8*).

The commercial use of potassium bisulfate has been relatively limited; moreover, most research has been done with its cation counterpart sodium bisulfate. Some fertilizer companies have taken advantage of potassium bisulfate's ability to deliver potassium to plant systems; however, minimal work has been done to determine antimicrobial capacity. Antimicrobial activity is by and large the same as other acidic antimicrobials. It is generally recognized that small acidic compounds can pass through the lipid cell wall of bacteria and dissociate in the cytoplasm—already dissociated compounds are thought to be unable to cross the cell wall (*63*). As bacterial cells require very specific pH ranges in their cytoplasm, the dissociation of an acid,

and the subsequent acidification of the cytoplasm, causes the cell to halt unnecessary energy-dependent reactions and upregulate transmembrane proteins that rid the cytoplasm of the excess protons. These excess protons change the charge of amino acids and drastically change protein structure (known as denaturing), which likely leads to cell death. Furthermore, cells use transmembrane proteins to remove excess protons, but this can lead to metabolic exhaustion if cells remain in an acidic environment too long. There is some debate that the accumulation of the anion is also harmful to cells, but conclusive evidence has not been established (*67*).

During 1997, in an effort to reduce *Salmonella* Typhimurium, sodium bisulfate was used as a carcass intervention on freshly slaughtered chickens (*80*). The research team tested two concentrations, 5% and 10%, three levels of spray pressure, and spray times of 30 seconds and 90 seconds. Pressure was of little significance in reducing *Salmonella* Typhimurium populations, but the 90 second spray time (of a 10% solution of sodium bisulfate) achieved approximately a 1.0 $\log_{10}$ CFU/g greater reduction in populations compared to the 30 second spray time (*80*). Under the most rigorous spraying conditions of 827 kPa for 90 seconds, a 2.58 $\log_{10}$ CFU/g reduction in *Salmonella* Typhimurium was achieved (*78*). The study concluded that contact time and the concentration affected the results the most. This study showed potential for potassium bisulfate in other applications to reduce *Salmonella* and other enteric microorganisms.

2.11 Benzalkonium Chloride Mode of Action and Past Research

Benzalkonium chloride is better known as part of QAC group of compounds. Quaternary ammonium compounds contain a quaternary nitrogen (thus creating a positive net charge of the compound) and usually contain at least one major hydrophobic constituent (*33*). Specifically, benzalkonium chloride is a mixture of *n*-alkyldimethylbenzyl ammonium chloride where the *n*-alkyl group can vary in length. The most common homologues are typically blended, and have

either a twelve, fourteen, or sixteen length carbon chain alkyl group. These three lengths have been found to be most effective against gram positive and gram negative bacteria (*33*). Benzalkonium chloride is also effective at killing most viruses, protozoan, and fungi. Homologues with an alkyl chain of less than four, or greater than eighteen, have very little antimicrobial effect, if any (*33*). It is this combination of large hydrophobic molecules, and the net positive charge, that are hypothesized to be responsible for benzalkonium chloride antimicrobial activity (*28*). The net positive charge of benzalkonium chloride is attracted to the negative charge of teichoic acids and the lipopolysaccharide for gram positive and gram negative bacteria, respectively (*28*). The large hydrophobic group allows firm attachment to the lipophilic cell wall. It is this reason that benzalkonium chloride is commonly used as an antimicrobial in food processing plants, clinical settings, and increasingly in domestic households (*28*). Benzalkonium chloride also has a natural propensity to penetrate porous surfaces better than other common antimicrobials (*28*). An example benzalkonium chloride molecule can be found in Appendix A.

In 1989, Japanese scientists found benzalkonium chloride to attach to the outer cell membrane of bacterial cells causing the cell membrane to be completely removed from the cell (*70*). This causes a very weak bacterial cell that ultimately succumbs to apoptosis or cytoplasmic leakage. In 2005, United Kingdom scientists found the positive charge on the quaternary nitrogen to attract and attach to the phospholipids of a cell membrane (*33*). Following attachment, the hydrophobic tail of benzalkonium chloride penetrates into the cell membrane core, creating a firm hold. At low concentrations, the consequences are two-fold. This firm benzalkonium chloride attachment increases the surface pressure against the cell and also decreases permeability of the cell membrane, thus decreasing flow of molecules in and out (*33*).

At higher concentrations, benzalkonium chloride can dissolve the cell membrane and enter the cellular cytoplasm, causing coagulation, and ultimately leading to cell death (*28*).

As benzalkonium chloride is capable of entering a bacterial cytoplasm, cells with competent efflux pumps are hypothesized to be not as sensitive to benzalkonium chloride. The role of an efflux pump is to rid the bacterial cytoplasm of molecules the cell does not want in the intracellular space. *Listeria monocytogenes,* a notably resilient microorganism, demonstrates increased resiliency to benzalkonium chloride, compared to other bacteria, likely due to such efflux pumps (*28*). Iranian scientists were able to disable the efflux pumps associated with *L. monocytogenes*, resulting in a lower minimum inhibitory concentration of benzalkonium chloride—meaning *L. monocytogenes* became more susceptible to benzalkonium chloride without a competent efflux pump (*28*). Biofilm formation is also hypothesized to reduce benzalkonium chloride efficacy against *L. monocytogenes*, although this holds true for nearly every other biocide.

2.12 Methyl Paraben Mode of Action and Past Research

Methyl paraben, a methyl ester of *p*-hydroxybenzoic acid, is a preservative that has been in the United States for the last sixty years and can be found in nearly 22,000 cosmetic products (*4, 73*). It is a relatively safe preservative that is commonly used due to its antimicrobial activity (particularly against yeasts and molds), stability, and solubility (*73*). The FDA has given methyl paraben, among other parabens, generally recognized as safe (GRAS) status with a limit in food of 0.1% (*74*). Methyl paraben is also found in pharmaceuticals where it is used as a preservative. Methyl paraben is non-carcinogenic, non-genotoxic, non-irritating, and generally, non-toxic (*74*). Parabens also offer no perceptible odor or taste, exhibit no change in pH or color, and are active across a large spectrum of temperatures and pH (*77*). However, parabens have been accused of

causing dermatitis and inflammation at the site of application, which has caused many online bloggers to discourage use (*73*). The FDA has ongoing investigations into parabens, but is routinely concluding that they are safe to use. Contact irritation may occur for "those with the most sensitive skin" (*74*). An example methyl paraben molecule can be found in Appendix A.

Methyl paraben can enter through the cell membrane of bacteria and cause a general disruption leading to lipid membrane failure (*74*). The cell can't survive without a properly working cell membrane. For prokaryotic cells, reports suggest methyl paraben binds to cellular oxidative enzymes causing respiratory failure (*73*). Further, research with *E. coli* and *Bacillus subtilis* have indicated both DNA and RNA binding properties leading to reduced cellular translation (*74*). In summary, methyl paraben needs to cross the cell membrane to affect a cell and cause death (*73*). This is why cells with efficient efflux pumps can tolerate a higher concentration of methyl paraben compared to cells lacking efflux pumps (*73*).

Studies on multiple animals, including humans, have shown methyl paraben to be non-toxic, hence its GRAS status. There has been no evidence of accumulation in the body and research suggests nearly 90% of ingested methyl paraben is excreted in urine within a twenty-four hour period (*74*). Only at the highest doses (10% w/v), administered topically, has experimental research shown it to cause mild skin irritation in laboratory rats (*74*). As a result, methyl paraben was accepted in 1974 FAO/WHO Expert Committee on Food Additives as an acceptable preservative in foods, cosmetics, and pharmaceuticals (*74*).

2.13 Chlorine Sanitizer Mode of Action and Past Research

In the food industry, chlorine is the most commonly used antimicrobial in wash water systems. It is cheap, effective, and does the necessary task of reducing microbial populations in the water. The United States Environmental Protection Agency (EPA) and California

Department of Pesticide Registration (DPR) have approved three forms of chlorine disinfectant that postharvest processors can use on produce items. Calcium hypochlorite is the most commonly used disinfectant in the produce industry mainly due to price and storage capability. Sodium hypochlorite is the most commonly used form for small processors and is the same active ingredient in household bleach. It is typically more expensive than the other two sources, as it is liquid-based and requires larger shipping containers. Chlorine gas is the least expensive option and is best suited for large processors. It needs to be monitored closely with constant addition for optimal use (*75*).

Chlorine mechanism of action towards microorganism has not been fully elucidated to date (*9*). Chlorine molecules are electronegative in aqueous solution and many hypothesize that these molecules oxidize the peptide bond between certain amino acids (*52*). Some propose that chlorine breaks the bond between nucleotides, effectively cutting DNA or RNA (*9*). Others hypothesize that chlorine bonds to amino acids, which chlorinates large proteins and renders them ineffective (*9*). The CDC further defines the mode of action to include: decreases uptake of nutrients, oxidation of respiratory components, decrease in adenosine triphosphate (ATP) production, and reduces DNA synthesis (*9*). The multiple modes of action make chlorine very difficult for microorganisms to develop resistance.

Without the presence of organic matter, it has been shown that less than five parts per million (ppm) of free chlorine is enough to kill vegetative bacteria (*9*). Spores of *Bacillus. atrophaeus* are killed at just 100 ppm chlorine with a five minute contact time, while twenty-five different viruses were killed at a 200 ppm available chlorine concentration after ten minutes (*9*). *Salmonella*, *Staphylococcus aureus*, and *P. aeruginosa* are killed in a 100 ppm solution of free chlorine and a ten minute contact time. These parameters indicate that chlorine solutions are

active at relatively low concentrations against spores, viruses, and most common pathogens. However, the contact time shown effective in studies can be difficult to achieve in produce processing facilities.

A 2009 Spanish survey reported that many postharvest processors do not use chlorine-based sanitation optimally, leading to excessive residue or ineffective microorganism kill (*32*). This is because water quality, temperature, organic matter, product, and concentration greatly affect the activity of chlorine-based sanitizers. The number one suggestion to processors is to use clean, potable water during processing. Subsequent water quality testing is also necessary. Organic loading is also important when working with a chlorine-based sanitation system, as free chlorine molecules react with all organic material (e.g. dirt, bacteria, and plant tissue) that enters the water. This reduces free chlorine concentrations, which reduces the overall concentration in wash water, thus, reducing efficacy over time. Additionally, higher temperatures can lead to further volatilization of chlorine and reduced antimicrobial activity. For these reasons, it is important to monitor chlorine concentration, replace wash water as needed, or filter out organic material. The monitoring of chlorine concentration is guided by standard operating procedures (SOPs), but typically is done more often as product goes through a washing system (*75*).

The use of chlorine in wash water has been an important advancement for the produce industry in the last forty years. It is thought that the fresh cut market for salads and other pre-washed vegetables would be nonexistent if not for chlorine-based postharvest washes (*32*). There is little evidence suggesting that a chlorinated water wash (when used appropriately) affects quality or shelf life; an important consideration for any postharvest wash (*47*). Some hypothesize there to be an increase in shelf life, as the chlorination may reduce total bacteria on a plant

surface (*47*). Chlorine compounds are also cheap, do not leave residue, and are effective at killing bacteria in aqueous solution.

There is ongoing research into discovering alternatives for chlorine-based sanitation of fresh fruits and vegetables. Among the top reasons is its lack of ability to target organisms on a plant tissue surface. It effectively destroys bacteria in wash water but does a poor job targeting bacteria attached to plant tissue. Chlorine-based washes also need to be monitored constantly to ensure adequate concentration. Due to organic loading, washes are prone to losing effectiveness without maintenance. There is also speculation that high chlorine concentrations, combined with high organic matter (through heavy use), may form volatile compounds such as trihalomethane or other carcinogenic by-products (*9*).

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Chapter 3 – An Investigation into Potassium Bisulfate as a Pre-Harvest Lettuce Antimicrobial Intervention targeting *Escherichia coli* and *Listeria innocua*

3.1 Introduction

Both *Listeria monocytogenes* (*L. monocytogenes*) and *Escherichia coli* O157:H7 are highly virulent pathogens that have been implicated in produce outbreaks. A 2015 study by the Centers for Disease Control and Protection (CDC) indicates that, from 2003-2012, *Escherichia coli* O157:H7 (*E. coli*) was implicated in 39 outbreaks linked to produce, 29 of which originated from leafy greens (*14*). From 2009-2011, the CDC documented twelve outbreaks of *L. monocytogenes*, two of which were produce-related (*25*). However, 2011 saw the deadliest *L. monocytogenes* outbreak in over 90 years when a cantaloupe outbreak sickened 125 people and killed 33 (*19*). Consumers rely on growers effectively implementing on-farm safety practices to minimize foodborne pathogen contamination. This is particularly important for produce consumed raw, as foodborne pathogens are not visible to the naked eye, and washing does very little to remove them (*4*). Cooking is an option to destroy potential pathogens, but this is not always desirable depending on the produce item.

Preventing foodborne pathogen contamination on produce is challenging because fruits and vegetables are primarily grown outdoors and subject to possible contamination throughout production, distribution, and consumption. In addition to being consumed raw, some products also have minimal to no packaging to protect against contaminants (*22*). Additionally, it has been generally accepted that contamination is most likely to occur pre-harvest, or during initial processing steps, making antimicrobial interventions most logical and impactful at these points

(*22*). Research now suggests that foodborne pathogens, such as *E. coli* O157:H7, may adhere more strongly to plant tissue than other produce tissues (5). Currently, the only way growers can reduce the possibility of contaminating their produce with foodborne pathogens is to utilize preventative measures on-farm.

Potassium bisulfate has been effective at controlling various enteric organisms; thus, it may be a suitable pre-harvest intervention to control enteric microorganisms such as *E. coli* O157:H7 (*16*, *29*). Potassium bisulfate is the potassium salt of sulfuric acid, and has a mode of action similar to other acids. Undissociated acids can translocate into the cytoplasm of susceptible cells and dissociate, thus causing acidification (*17*). The stressed cell is forced to expel energy to remove excess cytoplasmic protons, which may ultimately lead to cell death due to metabolic exhaustion (*17*).

According to current literature, potassium bisulfate has not been investigated as an intervention for food products. However, previous research explored the use of sodium bisulfate as an intervention for meat products. In this study, meat slurries were inoculated with either *Salmonella* or *E. coli* O157:H7, sprayed with sodium bisulfate to reach a final pH of 2.81, and stored at ambient temperature over a period of twenty-eight days. Within thirty minutes post spraying, *E. coli* O157:H7 populations were reduced by 1.29 $\log_{10}$ CFU/g, with similar reductions observed for *Salmonella* spp. populations in the meat slurries (*17*). At seven days and beyond, both *Salmonella* spp. and *E. coli* populations were reduced to <5 CFU/g (*17*). This suggests that applying sodium bisulfate may significantly reduce gram negative, enteric microorganisms in a meat matrix given adequate exposure time.

In the present study, potassium bisulfate was chosen in lieu of sodium bisulfate, as potassium bisulfate may be able to supply potassium, a key biochemical nutrient, to plants (*10*).

This would make potassium bisulfate a fertilizer, which may be seen favorably to those considering use. Potassium-based fertilizers are regularly applied to plants to promote early growth, disease resistance, and efficient water usage (*25*).

The overall objective of this study was to determine if a 0.25% potassium bisulfate solution applied pre-harvest to lettuce is efficacious at reducing *E. coli* or *L. innocua* without negatively impacting lettuce quality at harvest.

3.2 Materials and Methods

Preliminary Data Collection

Potassium bisulfate concentrations of 0.25% and 0.50% (w/v) were sprayed once on mature lettuce and observed over the course of four days. Lettuce sprayed with 0.50% potassium bisulfate had brown speckling indicating phytotoxicity. By day four, the 0.25% potassium bisulfate treatment had no noticeable effect on the leaves. As a result, 0.25% potassium bisulfate was evaluated in this study. This preliminary study was performed in late fall, outdoors at the Kansas State University Olathe Horticulture Research and Extension Center (Olathe, Kansas).

Listeria innocua (ATCC® 33090™) and *E. coli* (ATCC® 1427™) surrogate strains were evaluated to compare potassium bisulfate susceptibility to pathogen strains. A single USDA *L. monocytogenes* isolate and a single *E. coli* O157:H7 strain were used. This preliminary study was performed using microplate susceptibility testing to expose a standardized cell density to specific potassium bisulfate concentrations. Results from this preliminary study revealed that the surrogate strains had the same susceptibility to potassium bisulfate as the pathogenic strains (data not shown). Thus, *L. innocua* (ATCC® 33090™) and *E. coli* (ATCC® 1427) were used as models for the foodborne pathogens *L. monocytogenes* and *E. coli* O157:H7, respectively.

Study Design

Mature, inoculated lettuce plants were randomly assigned to one of seven treatments. Six lettuce plants were assigned to each treatment group. The treatments were as follows: 1) untreated, inoculated control, 2) 0.25% w/v potassium bisulfate applied one week before harvest, 3) sterilized water applied one week before harvest, 4) 0.25% w/v potassium bisulfate applied two days before harvest, 5) sterilized water applied two days before harvest, 6) 0.25% w/v potassium bisulfate applied one week and two days before harvest, 7) sterilized water applied one week and two days before harvest. The spray applications were applied to the drip point. The 0.25% w/v potassium bisulfate was prepared with sterilized water; however, the concentrated potassium bisulfate was not a sterilized product. A total of six replications were completed in this study. Figures 3-1 to 3-3 are plot maps summarizing this experimental design.

Growth of Lettuce

Pelleted Tropicana lettuce seeds (Johnny's Selected Seeds, Winslow, Maine) were planted on January 20, 2015 and grown in a greenhouse at the Kansas State University Olathe Horticulture Research and Extension Center (Olathe, Kansas). On February 12, Lettuce seedlings were transplanted to individual plastic pots. Lettuce was allowed to mature until March 9 (foliage ~15cm across), at which point they were inoculated. Lettuce was watered daily via overhead irrigation until inoculation, and afterwards, watered from below by filling trays holding lettuce pots with water. Briefly, the plastic containers containing the growing lettuce plants were placed in another solid container that would hold water and allow dry soil to soak. This was done instead of overhead irrigation to eliminate the possibility of irrigation removing attached cells from the lettuce plants.

Inoculation of Lettuce Plants

Stock cultures of *L. innocua* (ATCC® 33090™) and *E. coli* (ATCC® 1427™) stored at -80°C in the Kansas State University-Olathe food safety laboratory were removed from frozen storage and thawed using ice water. One mL aliquots of *L. innocua* (ATCC® 33090™) and *E. coli* (ATCC® 1427™) were aseptically transferred to individual sterile bottles, each containing 750 mL sterile Buffered Peptone Water (BPW) (Remel, Lenexa, Kansas), and incubated for 18-24 hours at 37°C. Overnight cultures were then transferred to a hand spray bottle, which was used to inoculate growing lettuce until the drip point. Briefly, the inoculum was applied until the leaves were fully saturated and additional applied liquid would drip off the plant; thus, ensuring complete and uniform coverage. Each lettuce plant was inoculated with either *L. innocua* (ATCC® 33090™) or *E. coli* (ATCC® 1427™) nine days prior to harvest. Approximately six hours after inoculation, random leaf samples were collected and transported to the food safety laboratory at Kansas State University-Olathe for microbiological sampling. After acceptable titer determination, the application of treatments proceeded.

Application of Treatments

Seven days prior to harvest (March 10, 2015), water and potassium bisulfate treatments were applied to the growing lettuce. Briefly, a 0.25% w/v potassium bisulfate was prepared in sterile tap water and poured into a hand-held spray bottle. Sterile tap water was also poured into a hand-held spray bottle. Treatments were sprayed onto the lettuce until the drip point to ensure uniform coverage of the leaf. On March 15, 2015, tap water and 0.25% w/v potassium bisulfate treatments were prepared and applied to all lettuce plants requiring a second application of 0.25% w/v potassium bisulfate or water two days prior to harvest. This second treatment application was also applied to the drip point. Fresh sterile tap water and 0.25% w/v potassium bisulfate solutions were prepared each day and for reach replication.

Harvest & Microbiological Analysis

On the day of harvest (March 17, 2015), all six lettuce plants in a treatment group were torn off at the base of the plant using latex gloves (Aurelia, Aurora, IL) and placed in zip top bags. They were immediately transported to the food safety laboratory at Kansas State University-Olathe on ice. Twenty-five gram samples of lettuce were randomly taken from each treatment bag and stomached (Stomacher® 400 Circulator, Seward, Davie, Florida) with 225mL of buffered peptone water (BPW) (BD BBL™, New Jersey) at 230 RPM for sixty seconds. Homogenized samples were serially diluted (1:10) using BPW as the diluent and the appropriate dilutions were spread plated. *Listeria innocua* (ATCC® 33090™) inoculated samples were plated on oxford medium base (BD BBL™, New Jersey) with added *Listeria* selective supplement (OXOID, Basingstoke, Hampshire, England). Colonies presenting with a black clearing were counted as *Listeria innocua* following incubation at 37°C for 18-24 hours. *Escherichia coli* (ATCC® 1427™) inoculated lettuce samples were plated on MacConkey agar (Remel, Lenexa, Kansas) and colonies exhibiting a mauve color were counted following incubation at 37°C for 18-24 hours.

Quality Analysis

Non-inoculated lettuce was grown and treated in the same manner as in the microbiological study in order to investigate the impact of 0.25% w/v potassium bisulfate on postharvest lettuce quality. The quality analysis portion of this study was performed in a greenhouse during late spring and into early summer. Lettuce quality was determined by visual inspection and quantifying color using a Minolta Chroma Meter CR-400 (Minolta, Ramsey, NJ). The treatments of potassium bisulfate applied one week and two days before harvest, and corresponding water control, were removed from this experiment because lettuce samples

observed during the inoculation study demonstrated that even a single application of 0.25% potassium bisulfate was burning the leaves. This is contrary to the 4-day preliminary investigation, possibly due to the increased contact time on the plants (7 days). To maintain quality, harvested lettuce samples (in zip top bags) were immediately stored at 4°C for approximately one hour until analysis. Overall quality was assessed by visual inspection and color.

Visual Inspection: Lettuce quality was evaluated subjectively using ratings scales (*6*). Briefly, the scale was from 1-9. More specifically, 1 = severe wilting and discoloration; 3 = more yellow than green, decay noticeable; 5 = noticeable loss of green and water loss; 7= slight loss of green color, non-objectionable water loss; to 9 = fresh appearance, crisp and no decay. Twenty leaves were randomly selected from each sample for evaluation. Samples were randomly evaluated by marking bags with a designated color that corresponded with their plot in the greenhouse in order to reduce bias and to evaluate lettuce leaves without knowledge of treatment. A copy of the lettuce quality rating scale can be found in Appendix B.

Color. Ten random leaves from each sample were chosen and evaluated for color on the adaxial side of the leaf, on both sides of the midrib. The color indices L*, a*, and b* (the Hunter Chroma Scale), were determined with a Minolta Chroma Meter CR-400 (Minolta, Ramsey, NJ). The L-axis, between 0-100, represents the lightness of the lettuce (100 = white, 0 = black). The a-axis represents the green and red aspects of the leaf with a positive value signifying red, and a negative value signifying green. The b-axis represents the yellow and blue aspects of color with a positive number being yellow and a negative number representing blue.

Statistical Analysis

This study was categorized as a randomized block design and microbiology data were subjected to the MIXED procedure with LSMEANS of Statistical Analysis Software (SAS, Version 9.4, Cary, NC). The pdiff option was used to determine differences in LSMEANS and evaluate statistical significance at the $P=0.05$ threshold. All experimental procedures were replicated six times; however, attachment populations for both *E. coli* and *L. innocua* were not adequate for two replications, which were subsequently removed from all analyses. Quality data were analyzed using a One-Way ANOVA with Tukey's Multiple Comparison test (Statistical Analysis Software Version 9.4, Cary, NC) using LSMEANS to compare treatments and determine statistical significance of the $P\leq0.05$ threshold.

3.3 Results and Discussion

Microbial Reduction Study

The application of water at any point before harvest did not significantly ($P>0.05$) impact *E. coli* populations at harvest. As shown in Figure 3.4, potassium bisulfate was moderately effective at reducing *E. coli* populations on lettuce, with 1.32 $\log_{10}$ CFU/g less *E. coli* than the control when applied one week prior to harvest. Because *E. coli* populations were significantly reduced ($P=0.0002$) when potassium bisulfate was applied one week prior harvest in comparison to the control, it stands to reason that these reductions should maintain or increase when potassium bisulfate is applied at one week and two days before harvest. However, as Figure 3.4 illustrates, the additional treatment two days before harvest did not demonstrate efficacy. While it is possible that variations in attachment might be responsible for the population differences between these two treatments, it's also important to consider environmental exposures and the metabolic state of the cell. A 1995 study by Arnold and Kaspar subjected *E. coli* O157:H7 cells to acidic environments (media adjusted to pH of two) at various points (e.g. lag phase) during

growth (*3*). The authors concluded that *E. coli* O157:H7 cells that were starved during stationary phase demonstrated greater acid tolerance than cells actively growing. When potassium bisulfate was applied one week before harvest, *E. coli* cells were two days removed from inoculation, and that inoculum was applied with the growth medium BPW. That growth medium may have provided enough nutrients for the *E. coli* cells to maintain some degree of metabolic activity. Thus, perhaps the *E. coli* cells remained susceptible to the initial potassium bisulfate treatment. However, the additional potassium bisulfate treatment, which was applied two days prior to harvest, was sprayed on what were likely starved *E. coli* cells in stationary phase that had been stressed with the prior exposure to potassium bisulfate. Therefore, it is possible that the second potassium bisulfate treatment rehydrated any residual nutrients; thus, allowing the stressed, and potentially more acid-tolerant, *E. coli* cells to recover for two days before harvest.

It should also be noted that lettuce tissue damage (i.e. burning) was observed following application of the 0.25% w/v potassium bisulfate treatment. Thus, it could be hypothesized that this damage resulted in nutrient leakage, and/or *E. coli* gained access to the internal portion of the plant through the damaged areas. It is possible that tissue damage caused by the second application of 0.25% w/v potassium bisulfate was excessive enough for this to occur.

As shown in Figure 3.5, potassium bisulfate potassium bisulfate demonstrated moderate efficacy as a pre-harvest intervention to reduce *L. innocua* populations on lettuce. When applied twice (one week and two days before harvest), 0.25% w/v potassium bisulfate significantly (P=0.0017) reduced *L. innocua* populations on inoculated lettuce by 1.18 $\log_{10}$ CFU/g in comparison to the control. Although not statistically significant (P=0.1333), it is noteworthy that applying water twice (one week and two days before harvest) reduced *L. innocua* populations by 0.50 $\log_{10}$ CFU/g in comparison to the control. Two applications of 0.25% w/v potassium

bisulfate were significantly (P=0.0482) more effective than two applications of water; however, a marginal population difference of 0.68 $\log_{10}$ CFU/g was observed between the two treatments. These data suggest that multiple applications of 0.25% w/v potassium bisulfate are needed to significantly reduce populations of *L. innocua*; however, the improved benefit over two applications of water must be considered.

This study is novel in that very little research has been done on the application of an antimicrobial on growing lettuce. The limited research completed to date focused more on applying an antimicrobial pre-harvest to control decay causing microorganisms rather than foodborne pathogens. One study aiming to control yeasts, molds, and total aerobic bacteria used aloe vera gel as an antimicrobial on lettuce (30). The authors reported ~1.0 $\log_{10}$ CFU/g reduction in aerobic bacteria when applied to lettuce one week before harvest. In comparison, the 0.25% potassium bisulfate achieved in excess of a 1.0 $\log_{10}$ CFU/g reduction in *E. coli* and *L. innocua* when applied one week before harvest or one week and two days before harvest, respectively. Castillo et al. (2004) suggest that surface antimicrobials may not reduce bacteria more than two $\log_{10}$ CFU/g on plant tissue due to the complex nature of tissue and the possibility of pathogen internalization (*7*). By the standards of Castillo et al., 0.25% potassium bisulfate was moderately effective at reducing populations of *E. coli* and *L. innocua*.

It is also important to mention that the microorganisms used for inoculation had a longer time to attach to the lettuce tissue and were likely in a different metabolic state when potassium bisulfate was applied just two days before harvest. This is an important factor to consider when determining efficacy and feasibility, as it is impossible to determine when contamination occurs in a field.

One consideration for future research would be to combine potassium bisulfate with another antimicrobial compound. Studies show that using multiple antimicrobials is generally more effective than when the antimicrobials are used individually (*9*, *18*). Additionally, the concentrations of each antimicrobial can often be reduced (compared to lethality observed at a particular concentration when used individually) when compounds act synergistically, meaning overuse, and possibly resistance, may become less common (*5*). Research studies also find synergistic compounds offer greater efficacy if each compound has a different mode of action to a target microbe (e.g. a combination of two acidic compounds won't be as effective synergistically as other possible combinations) (*9*).

Due to the hydrophobic nature of plant surfaces, lettuce leaves can only hold so much liquid before excess drips off. Increasing the liquid carrying capacity of the lettuce leaf may increase efficacy, as a greater amount of antimicrobial would be retained on the leaf surface. Introducing a surfactant to improve leaf coverage and potassium bisulfate adherence may improve antimicrobial efficacy and minimize treatment coalescence. This may also positively impact by product quality by minimizing burning on the leaf where the 0.25% w/v potassium bisulfate presumably coalesced. Tween 80 and Tergitol are common food grade surfactants but the FDA also lists calcium lignin sulfonate, methyl glucoside, poloxalene, and sodium lauryl sulfate (*1*).

Visual Inspection

A distinct brown speckling on various samples sprayed with of 0.25% w/v potassium bisulfate was observed, regardless if spraying occurred one week or two days before harvest. Other studies using various fertilizers reported the same result when the concentration was too high (*3*). In an effort to bypass this problem, Azeem et al. (1996) suggests using a lower

concentration multiple times, or to utilize a controlled release approach (*3*). Due to the hydrophobic nature on the surface of plant tissues, the application of water or 0.25% w/v potassium bisulfate results in coalescence on the surface of the leaf. Presumably, the acidic nature of potassium bisulfate caused "burning" where the treatment coalesced on the lettuce surface (Figure 3.6) (*15*). As expected, lettuce sprayed with water prior to harvest exhibited little to no quality damage during postharvest quality analyses (Figure 3.7). As Figure 3.8 illustrates, no significant difference in visual quality was observed among treatments. This was likely due to the fact that not every leaf exhibited potassium bisulfate damage.

Color

The results from the color analyses are highlighted in Figures 3-9 – 3-11. No significant differences ($P>0.05$) in color were observed among the treatments. Although burning was observed for lettuce treated with 0.25% w/v potassium bisulfate, this did not appear to impact color quality of the lettuce. As expected, no color difference was observed for untreated and water sprayed lettuce. Potassium is not known to contribute to lettuce color (unless there is a deficiency); thus, improvements in greening (lower a* values) were unlikely given the short time between application and harvest (*10*).

3.4 Conclusions

This was a preliminary study evaluating the efficacy of applying 0.25% w/v potassium bisulfate as pre-harvest intervention on lettuce to reduce populations of the foodborne pathogen surrogate microorganisms *L. innocua* and *E. coli*. Results from this study demonstrate that potassium bisulfate was moderately efficacious as a pre-harvest intervention to control *L. innocua* and *E. coli* on lettuce. Further research is necessary to determine if efficacy can be optimized such that tissue burning is minimized while microbial populations are further reduced.

Because of leaf burning, more resilient produce may be better suited for future research. This study demonstrated potential in using potassium bisulfate as a pre-harvest produce intervention; however, further research is necessary before 0.25% w/v potassium bisulfate can be recommended as a pre-harvest intervention on lettuce.

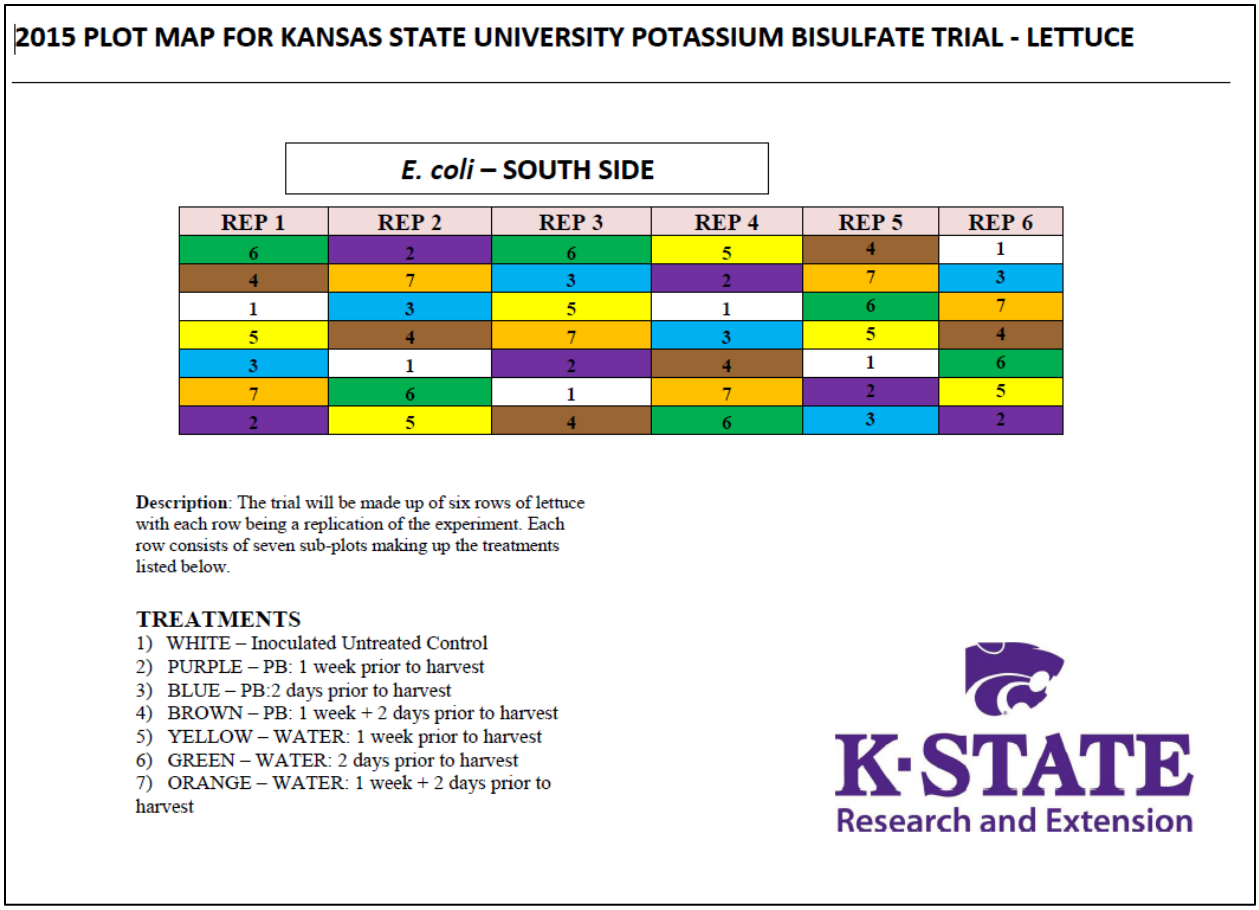

Figure 3-1. Plot map used for *Escherichia coli* inoculated lettuce.

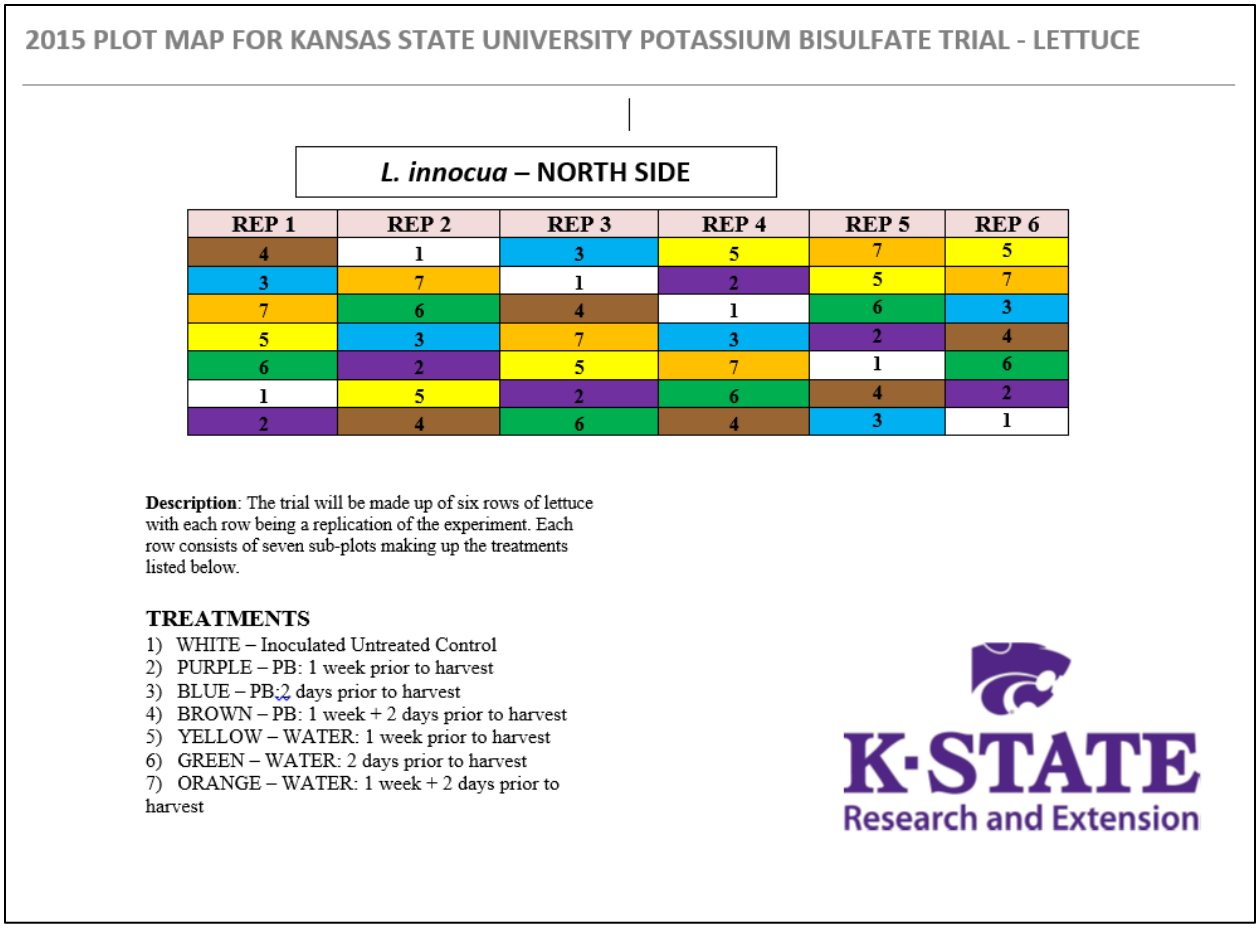

Figure 3-2. Plot map used for *Listeria innocua* inoculated lettuce.

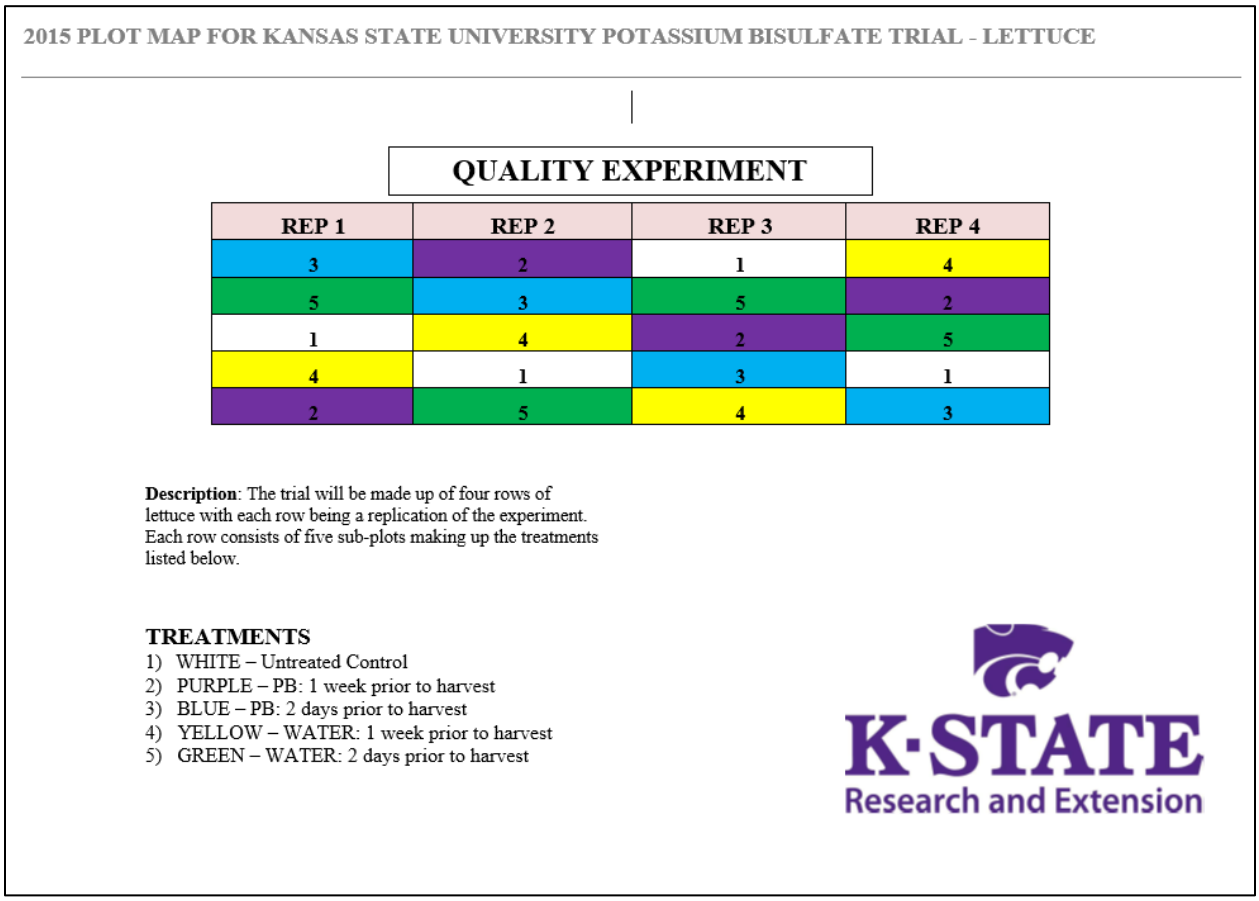

Figure 3-3. Plot map used to assess quality of uninoculated lettuce after treatment.

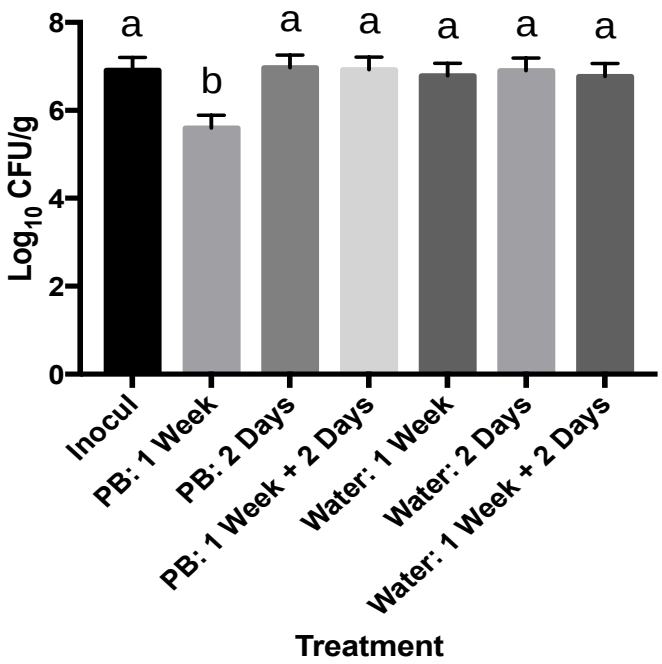

Figure 3-4. *Escherichia coli* populations at harvest on lettuce treated with water and potassium bisulfate (PB).

Error bars represent standard error of the mean.

[a,b] Indicates treatments that differ statistically ($P \leq 0.05$).

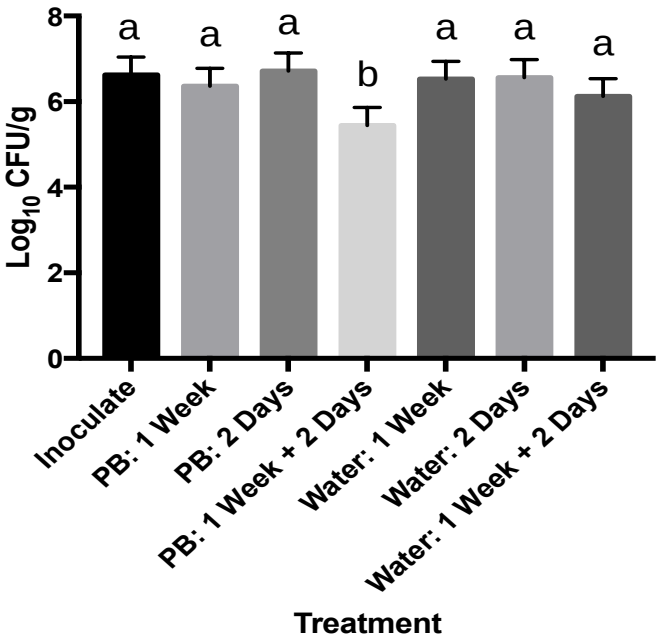

Figure 3-5. *Listeria innocua* populations at harvest on lettuce treated with water and potassium bisulfate (PB).

Error bars represent standard error of the mean.

[a,b] Indicates treatments that differ statistically ($P \leq 0.05$).

Figure 3-6. Lettuce samples sprayed with 0.25% potassium bisulfate seven days before harvest.

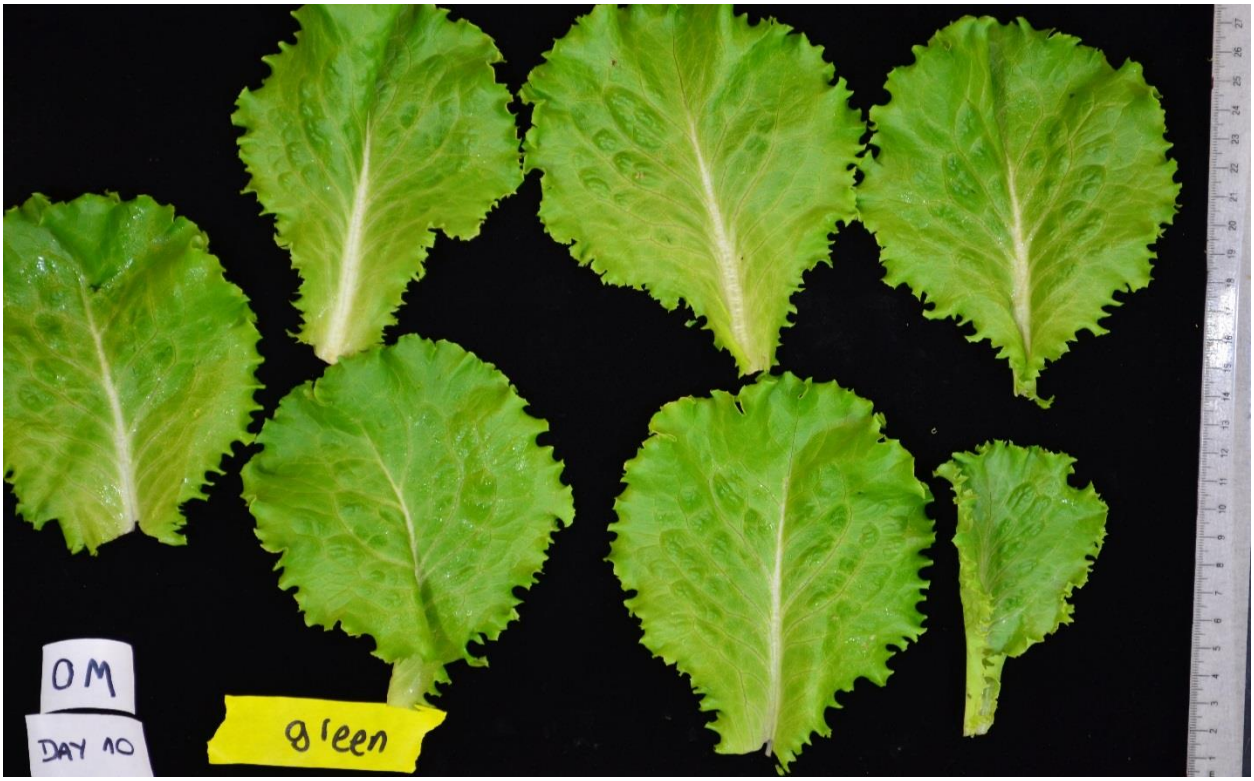

Figure 3-7. Lettuce samples sprayed with water two days before harvest.

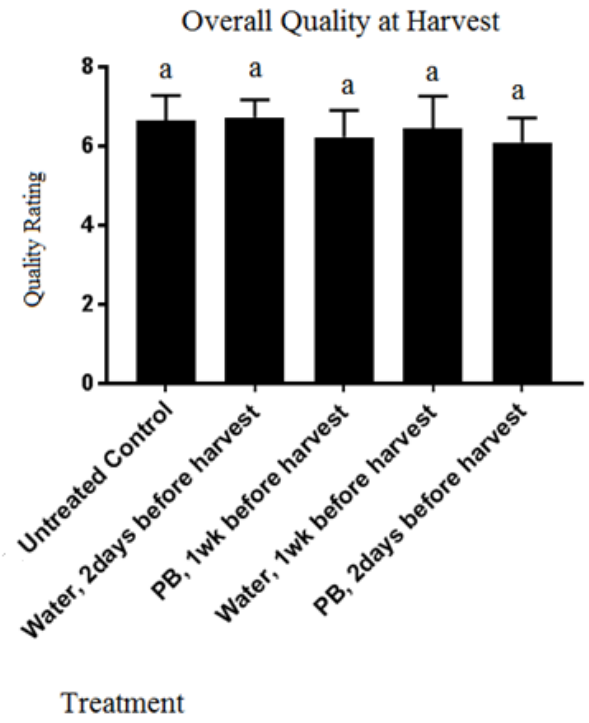

Figure 3-8. Overall quality of lettuce subjected to 0.25% w/v potassium bisulfate and water either one week or two days before harvest.

Error bars represent one standard deviation.

[a] Indicates treatments that differ statistically ($P \leq 0.05$).

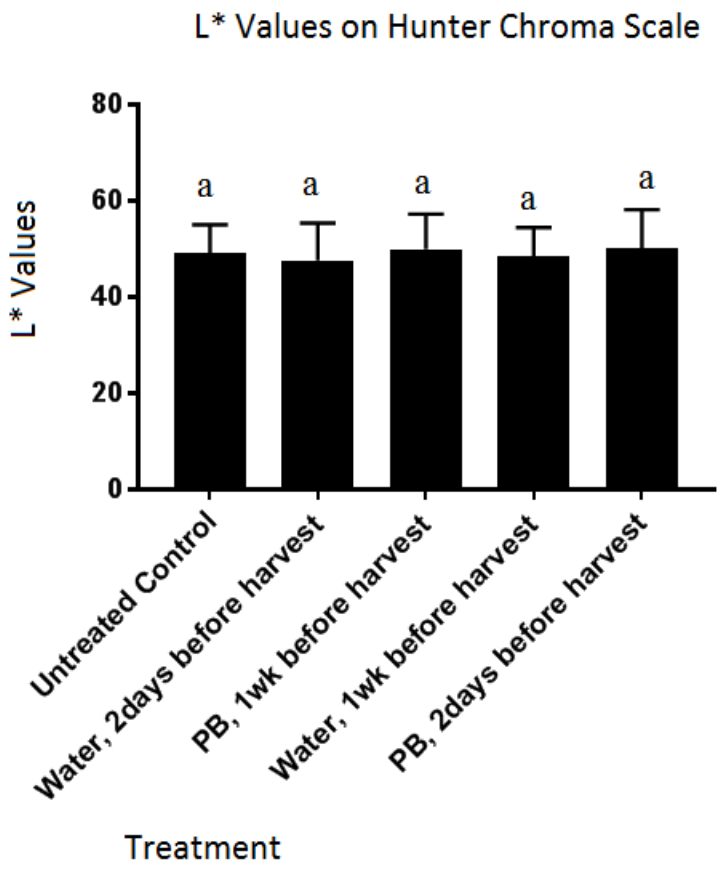

Figure 3.9. L* values at harvest for lettuce subjected to 0.25% w/v potassium bisulfate and water either one week or two days before harvest.

Error bars represent one standard deviation.

[a] Indicates treatments that differ statistically ($P \leq 0.05$).

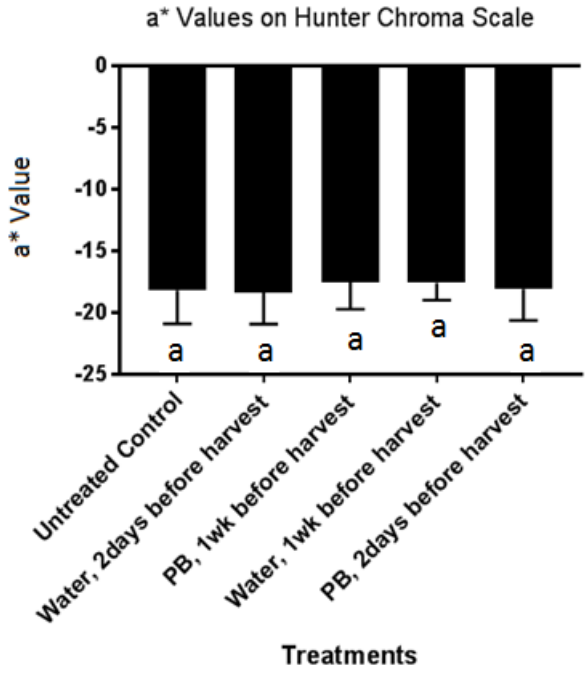

Figure 3-10. a* values at harvest for lettuce subjected to 0.25% w/v potassium bisulfate and water either one week or two days before harvest.

Error bars represent one standard deviation.

[a] Indicates treatments that differ statistically ($P \leq 0.05$).

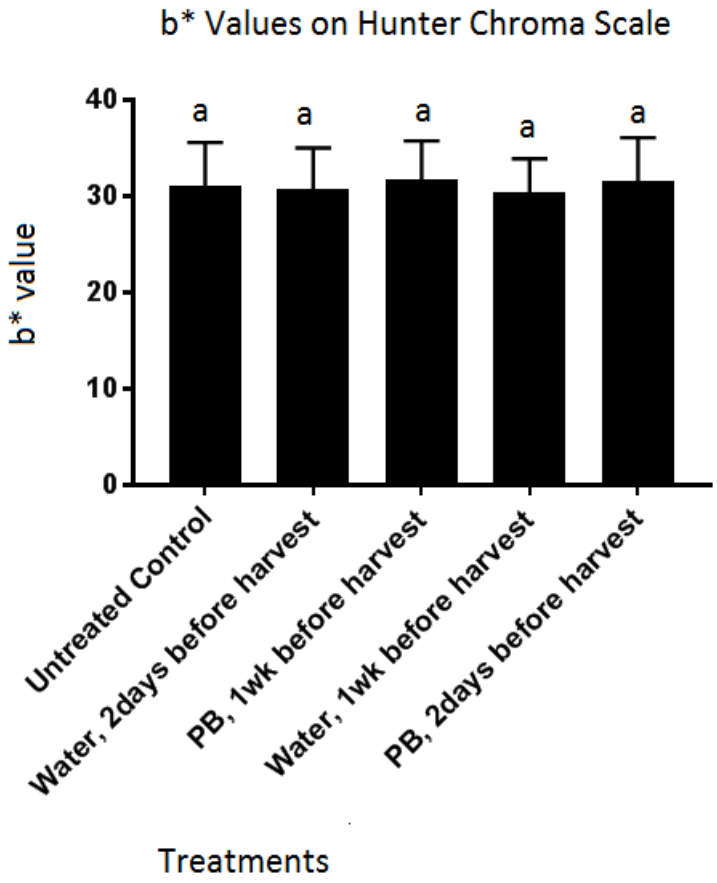

Figure 3-11. b* values at harvest for lettuce subjected to 0.25% w/v potassium bisulfate and water either one week or two days before harvest.

Error bars represent one standard deviation.

[a] Indicates treatments that differ statistically ($P \leq 0.05$).

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Chapter 4 – An Investigation into the Efficacy of Benzalkonium Chloride, Acetic Acid, and Methyl Paraben in Combination as a Postharvest Intervention to control *Escherichia coli, Salmonella* spp., and *Listeria monocytogenes* on Lettuce

4.1 Introduction

A 2010 epidemiological study by Berger et al. reported that past research efforts on foodborne pathogen transmission were focused on foods of animal origin (e.g., poultry, beef, pork); however, according to Painter et al. (2013), foodborne illness attributed to produce exceeds all foods of animal origin combined (including cases caused by egg and dairy products) (*8*, *24*). This is primarily due to the increased fruit and vegetable consumption observed in the past fifty years, but is also because most produce items (particularly leafy greens) are consumed in a raw state (*8*). Currently, the only common measure to reduce foodborne pathogens on produce is the use of a post-harvest chlorinated water wash. However, the efficacy of chlorine is not substantial; thus, alternatives should be evaluated (*9*).

Postharvest processing of fruits and vegetables is recognized as a potential contamination point for foodborne pathogens, and water is the most likely vehicle of transfer, particularly when sanitizer is absent, or when the sanitizer is inactivated (*2, 22*). In a postharvest processing operation, water can be used in the way of flumes, tanks, and spray washes, and can be directly contaminated via the source, or indirectly contaminated by the introduction of contaminated product. The industry has addressed this by adding a chlorine-based antimicrobial to reduce the potential for cross-contamination from water to produce (*2*). Ultimately, the goal of using

chlorinated wash water is to prevent cross contamination of organisms from produce wash water to the product, but also to prevent contaminated produce from contaminating the wash water (*30*). In general, chlorinated washes are not intended to reduce organisms on produce itself. That being said, current research has demonstrated a 90-99% reduction (1-2 $\log_{10}$) in total aerobic bacteria can be expected on produce when a properly prepared chlorinated wash (fluctuation depends on product, concentration, contact time, etc.) is used (*9*). A "properly prepared" solution typically infers a free chlorine concentration between 75-200 ppm, with a three to five minute contact time, and at a temperature that facilitates product cooling (~50°F) (*30*). The product and wash water are to not be more than 10°F apart (i.e. wash water cannot be more than 10^{o}F cooler than the product), as this may lead to an influx of water into the product, potentially causing foodborne pathogens to enter the product. Eliminating 90-99% (1-2 $\log_{10}$) of aerobic bacteria is a reduction that may seem effective; however, it must be considered that some foodborne pathogens have infectious doses as low as a few cells (*22*). Therefore, even if 1% of bacteria are not killed by a postharvest wash, a legitimate risk for foodborne illness may exist. This establishes a need for further research evaluating the effectiveness of alternative postharvest washes in order to achieve microbial reductions in excess of two logs.

Benzalkonium chloride is a cell membrane disrupter that has activity against gram negative and gram positive microorganisms. According to Fazlara et al. (2012), benzalkonium chloride is particularly effective against *Listeria monocytogenes* (*L. monocytogenes*) and *Escherichia coli* (*E. coli*), but *Salmonella* Typhimurium also exhibited a high degree of susceptibility (*15*). Benzalkonium chloride has been studied as a postharvest wash on uninoculated radishes, cilantro, parsley, and basil (*15*). When applied at 92 ppm for fifteen minutes, total aerobic bacteria on the plant tissue were reduced by approximately 1.2 $\log_{10}$

CFU/g; however, they did not report a significant difference compared to the water control. Washing parsley, cilantro, and basil with 92 ppm benzalkonium chloride, followed by a secondary wash of either peracetic acid or hydrogen peroxide improved efficacy compared to just a single postharvest treatment. When used in tandem, two separate antimicrobial washes were able to achieve up to a 2.8 $\log_{10}$ CFU/g reduction in total aerobic bacteria on the plant tissue, further demonstrating the added benefit of employing hurdle technology (*15*).

In 2004, The American College of Toxicology evaluated eighteen studies attempting to determine the safety of benzalkonium chloride by studying concentrations ranging from 0.00045% to 0.01% subjected to humans daily (*20*). Two of the eighteen studies found human subjects to be negatively impacted by benzalkonium chloride exposure when used as a nasal spray. However, the overall conclusion of the study was that benzalkonium chloride "appears to be safe and well-tolerated for both long- and short-term clinical use" (*20*). At the present time, benzalkonium chloride is not generally recognized as safe (GRAS), but it has been evaluated (*5*).

Methyl paraben has the benefit of exhibiting both a mild fungicidal and bactericidal effect (*29*). While antimicrobial efficacy is important, an evaluation of residue remaining on the plant tissue post washing is also critical. According to the Code of Federal Regulations (CFR) Chapter 21 §184.1490, the limit in or on a food stuff must not exceed 0.1% (*4*).

Acetic acid has also been studied as a postharvest antimicrobial. A 0.5% acetic acid dip for two minutes was able to reduce *E. coli* O157:H7 and *L. monocytogenes* on fresh-cut iceberg lettuce by 1.2 $\log_{10}$ CFU/g and 0.8 $\log_{10}$ CFU/g, respectively (*31*). No significant difference was reported between a two and five minute contact time. Acetic acid residue was not evaluated in this study as, according to CFR Chapter 21 §184.1005, it is GRAS (up to 0.15% on lettuce) and is readily water soluble, minimizing concern of residue on romaine or iceberg lettuce.

The overall objective of this study was to investigate the use of a Marvel Technologies™ USA, LLC formulated blend of benzalkonium chloride, acetic acid, and methyl paraben called Free N Clear™ (FNC) as a novel postharvest intervention on fresh-cut romaine and iceberg lettuce. The specific research objective was to determine the antimicrobial efficacy of FNC against *L. monocytogenes, E. coli,* and *Salmonella* on the lettuce product as well as in wash water. A secondary objective was to quantify benzalkonium chloride and methyl paraben residues on lettuce after washing with FNC and rinsing with potable water. Visual quality was also evaluated to determine if FNC negatively impacted lettuce quality.

4.2 Materials and Methods

Preliminary Data Collection

Preliminary data was collected to determine the concentrations of both methyl paraben and benzalkonium chloride in 1%, 2%, and 3% FNC wash solutions, as prepared in the following Washing of Cut Lettuce section. When 100% FNC was prepared according to manufacturer guidelines (Marvel Technologies™,Franklin, TN), 0.04 lb benzalkonium chloride solution (containing 50% benzalkonium chloride), 0.04 lb methyl paraben, and 0.08 lb of acetic acid were added to a container and tap water was added to a final volume of one gallon. When prepared accordingly, the concentration of benzalkonium chloride in 1%, 2% and 3% FNC wash solutions was nearly double of the theoretical concentrations (theoretical concentrations for 1%, 2%, and 3% FNC solutions are 23.5 ppm, 47 ppm, and 73.5 ppm, respectively). In an effort to explain this, 100% FNC was prepared in a 250 mL volumetric flask and 1%, 2%, and 3% FNC was compared to the concentration made according to manufacturer guidelines. Further, 1%, 2%, and 3% FNC wash solutions were made from each solution into 100 mL, 250 mL, 1,000 mL, or 2,000 mL volumetric flasks and the resultant concentrations were compared. Results are shown

in Appendix C. Based on these preliminary data, the theoretical concentrations of benzalkonium chloride were much closer to the prepared concentrations when 100% FNC was mixed in a 250 mL volumetric flask, compared to when FNC was prepared according to manufacturer guidelines, which specifies preparation in a one-gallon volume. These results suggest, that even with high agitation and heat, benzalkonium chloride does not seem to evenly disperse in large volumes of water. This non-uniformity of benzalkonium chloride mixing should be further evaluated for large-scale uses with high volumes of water.

Study Design

Three individual studies were accomplished in this objective: a shelf life, residue analysis, and pathogen reduction study. Iceberg and romaine lettuce was procured in 25 lb cardboard boxes from a local produce supplier in Kansas City, Missouri. The produce was not washed or processed in any way before use in these studies. Romaine and iceberg lettuce was stored at 4°C overnight. The pathogen reduction study utilized its own box each of romaine and iceberg lettuce while separately purchased boxes were both used for the shelf life and residue studies (i.e., the same boxes of romaine and iceberg lettuce were used for the shelf life and residue analysis studies). One box each of romaine and iceberg lettuce was used for all three replications for the pathogen reduction study. The boxes of romaine and iceberg lettuce for the shelf life and residue analysis were used for all three replications for each study. It is important to note that several heads of lettuce were stored in each box and individual heads of each lettuce type were randomly assigned to a single replication.

4.2.1 Shelf Life

Washing of Cut Lettuce

Concentrated FNC solution was prepared by combining 0.08 lb acetic acid, 0.04 lb methyl paraben, and 0.04 lb of USP-BAC (50% benzalkonium chloride) into one gallon of water. This one gallon of concentrated FNC solution was used to prepare 0%, 1%, 2%, and 3% wash concentrations by mixing with five gallons of tap water to make individual treatment solutions. Fresh five-gallon treatment solutions were prepared for each of the three replications. Romaine and iceberg lettuce were cored and chopped into approximately 1" x 1" squares. Two hundred grams of romaine and iceberg lettuce samples, separated into individual slotted containers, were completely submerged for one minute in the five gallon FNC wash solutions. The same wash solution was used to wash iceberg and romaine lettuce. Following FNC treatment, each 200 g batch of lettuce was completely submerged in tap water for one minute as a secondary wash prior to being spun dry in a salad spinner of 10.25 inches in diameter (Prepworks®, Kent, Washington). Pulling the salad spinner cord fully ten times standardized this procedure. A diagram illustrating how the washing of lettuce was performed can be found in Appendix D.

Preparation of Packaged Lettuce

The 0% and 2% concentrations of FNC were evaluated throughout the shelf life because 2% FNC is the washing concentration that Marvel Technologies™ is targeting for commercial use, and a 0% FNC wash to serve as the control. After dewatering in the salad spinner, five duplicate bags each of 0% and 2% FNC washed lettuce were weighed to approximately 55 g in a standard lettuce bag film (The American Packaging Corporation, Rochester, NY). The film structure was the following: 75Ga OPP/Ink/Adhesive/1.65mil LLDPE with a target oxygen transmission rate of 85 cc/100in^2/day. Bags were then sealed using a FoodSaver® (Sunbeam Products, Neosho, MO) vacuum sealer; however, the vacuum function was not used in this study. Sealed bags, for each wash, replication, and lettuce type, were immediately transferred to a 4°C

walk-in cooler, randomly assorted in a clear plastic container, and stored throughout the seven day shelf life study. The walk-in cooler had a single 2' x 2' window exposed to fluorescent lighting for 24 hours a day. Fluorescent lighting from the walk-in cooler itself was on no more than 30 minutes per day throughout the study. Figure 4-1 illustrates the temperature and relative humidity inside the walk-in cooler throughout the seven-day period. Starting on day zero (the day of washing), samples were visually observed once a day for seven days using previously developed quality methodology found in Appendix B (11, *26*).

Microbiological Analysis

Immediately after the dewatering step, fresh-cut romaine and iceberg lettuce washed in 0%, 1%, 2% and 3% FNC were sampled for total aerobic bacteria, coliforms, and generic *E. coli*. Microbiological analysis was also completed for the 0% and 2% FNC samples on days 3, 5, and 7 by randomly selecting a sealed bag from the walk-in cooler. This bag was terminal and not used for the remainder of the study. At each sampling point, approximately 25-30 g of washed lettuce was stomached (Stomacher® 400 Circulator, Seward, Davie, Florida) with 225 mL of Dey Engley Neutralizing Broth (BD BBL™, New Jersey) at 230 RPM for sixty seconds. Homogenized samples were serially diluted (1:10) using peptone water as the diluent and the appropriate dilutions were plated on Aerobic Plate Count Petrifilm™ (3M™, Maplewood, Minnesota) in duplicate. Dilutions were also plated on *E. coli*/coliform (ECC) Petrifilm™ (3M™, Maplewood, Minnesota) in duplicate. All Petrifilm™ were incubated at 35^{o}C for 24-48 hours.

Appearance Evaluation

Fresh-cut iceberg and romaine appearance was scored immediately after washing on day 0, and from sealed bags washed in 0% and 2% FNC on days 2-7. Grading was performed by visually inspecting all sample bags and closely evaluating defects under fluorescent lighting.

Special attention was given to the cut edges of lettuce, as deterioration was most pronounced around these areas. Two trained personnel individually graded each bag, and an overall conclusion was recorded for each bag. Appearance ratings were averaged for all bags within a treatment (i.e. replications and duplicates were averaged) to generate an individual overall appearance score for the day of grading.

Romaine lettuce quality was evaluated subjectively using previously developed rating scales (*11*). Briefly, the scale was from 1-9, where 1 = severe wilting and discoloration; 3 = more yellow than green, decay noticeable; 5 = noticeable loss of green and water loss; 7= slight loss of green color, non-objectionable water loss; to 9 = fresh appearance, crisp and no decay. The romaine lettuce quality scale can be found in Appendix B.

Iceberg lettuce quality was evaluated subjectively using previously developed rating scales (*26*). This method used the Karlruher Schema nine point scale where 9 = excellent and 1 = very bad (*26*). The quality scale used can be found in Appendix B.

Statistical Analysis

All experimental procedures were repeated a total of three times. All day zero total aerobic plate count log reductions were calculated by comparing the populations recovered from each experimental wash compared to the unwashed control. Log reductions for total aerobic plate count populations on romaine and iceberg lettuce treated with 0% and 2% FNC were calculated by comparing the treated lettuce populations throughout the shelf life to populations from an unwashed control sample on day zero. All log reductions collected from the shelf life study were then used for statistical analyses using GraphPad Prism Version 6.0 (La Jolla, CA). More specifically, total aerobic bacteria populations after washing (0%, 1%, 2%, or 3% FNC) on day zero were evaluated using a One-Way ANOVA with Tukey's multiple comparison test.

Significance was evaluated at the P=0.05 threshold. Total aerobic bacteria populations throughout the shelf life study were evaluated using a Two-Way ANOVA with Tukey's multiple comparison test. The main effects of concentration and day, as well as the concentration x day interaction, were also evaluated. Significance was determined at the P=0.05 threshold. Methyl paraben and benzalkonium chloride wash water concentrations, both before and after washing, were determined by calculating the average and standard deviation for all three replications using Microsoft Excel (Microsoft, Redmond, WA). Coliform and generic *E. coli* populations fell below the limit of detection (~0.5 $\log_{10}$ CFU/g); therefore, these data have a standard error of zero and statistical analyses could not be performed.

4.2.2 Residue Analysis

Samples

Fresh-cut romaine and iceberg lettuce was evaluated for total benzalkonium chloride and methyl paraben residue immediately after washing in 0%, 1%, 2%, or 3% FNC on day zero, and for 0% and 2% FNC washed lettuce on days three and seven. Samples for residue analysis were taken from the shelf life study. On days three and seven, lettuce samples for residue analysis were taken from the same destructive bag as was used for the microbial analysis for the shelf life study. Five gram samples of randomly selected lettuce were placed in a 50 mL conical tube and immediately frozen until the time of analysis.

Benzalkonium Chloride Chromatographic System

The procedure for determining benzalkonium chloride in wash waters and fresh-cut romaine and iceberg lettuce leaves was based on a published paper by Diez et al. (*13*). A Waters™ (Milford, MA) Acquity UPLC with an Atlantis® T3 3.0-µm 2.1 x 100 mm analytical column was used to separate benzalkonium chloride. The flow rate was set at 0.400 mL/minute

and the column temperature was set at 40°C. The mobile phases were (A) water and (B) methanol, each containing 0.1% of formic acid to improve ionization. A gradient program was used and started at 35% A and gradually went to 0% A (100% B) over three minutes. This was held for two minutes to remove potential matrix interference from the column. Therefore, the total run time for each sample was five minutes. A 1µL loop was used for injection. All samples were detected using an Acquity QDa detector (Waters, Milford, MA) using mass spectrometry.

Mass Spectrometer Settings

The mass of 304 daltons was analyzed and corresponded with the benzalkonium chloride molecule that has a twelve carbon alkyl chain. Ionization was performed in positive mode with a cone voltage of 15 V. The capillary voltage was set at 1.5 kV.

Extraction

To accurately quantitate benzalkonium chloride in romaine and iceberg tissue, an extraction was required. The extraction protocol was in accordance with QuChERS, per the previously published method (*13*). The 5 g lettuce sample was placed into a 20 mL Nalgene (Rochester, NY) centrifuge tube followed by the addition of 15 mL of acetonitrile with 1% acetic acid. The lettuce and acetonitrile with acetic acid solution was hand-mixed vigorously for one minute. The DisQue™ (Waters, Milford, MA) salt mixture (6 g anhydrous magnesium sulfate and 1.5 g sodium acetate) was added to the solution and then vigorously hand-mixed for another thirty seconds. This mixture was centrifuged at 1,630 x g at 4 °C for 5 minutes to obtain a well-defined solid-liquid phase separation. A 1 mL aliquot was then transferred to a clean-up tube consisting of 150 mg of magnesium sulfate and 50 mg of a primary secondary amine (PSA) bonded silica. Further centrifuging at 5,590 x g for 1 minute followed in order to obtain another solid-liquid phase separation. The supernatant was removed with a 1.0 mL plastic syringe

(Thermo Fisher Scientific, Waltham, MA) and filtered through a 0.20 micron PTFE filter (VWR International, Radnor, PA) before being placed in a 2 mL glass vial (Waters, Milford, MA) where UPLC analysis would occur. Extracted samples in vials were stored at 4°C until analysis.

Analysis of Lettuce Samples for Benzalkonium Chloride Residue

The residual amount of benzalkonium chloride in fresh-cut lettuce was determined after washing with various concentrations of FNC, rinsing with potable water, and dewatering by creating a standard curve to evaluate the peak area of known concentrations. As lettuce contains many compounds that may interfere with detection by the UPLC machine, the standards must be as representative of the unknown samples as possible. Therefore, the extraction protocol was followed using unwashed, fresh lettuce and the supernatant from the finished extraction was used as the diluent for preparing standards. Not only did this create the most representative standard, but it is also supported in the literature as a method published by Diez et al. (*13*). Briefly, a stock FNC solution (100% concentration) was diluted to 1,000 ppm of benzalkonium chloride and used to make standards for analysis. From the 1,000 ppm solution, known concentrations were created with the lettuce extraction matrix, and these solutions were then injected into the UPLC as standards. A standard curve was generated from these standards, which was used to determine the concentration of unknown samples. More specifically, the peak area generated from the unknown sample could then be fit into the standard curve equation to quantify the concentration of benzalkonium chloride in the lettuce tissue samples. All solutions were created using volumetric flasks to ensure accuracy.

FNC Wash Water Benzalkonium Chloride Analysis

Wash water samples were taken before and after romaine and iceberg lettuce were washed during the shelf life and pathogen reduction studies to confirm benzalkonium chloride

concentrations. Using a serological pipette, water was taken from a random area of the washing solution and placed into a 15 mL conical tube. Wash water samples were immediately frozen to -20°C and saved until the time of analysis. The UPLC chromatographic system settings are described above in section Benzalkonium Chloride Chromatographic System.

Using Standards to Determine Unknown Concentrations in Wash Waters

To determine free benzalkonium chloride in wash water solutions, a stock FNC solution (100% concentration) was diluted in distilled water to prepare standards of a known benzalkonium chloride concentration. Figure 4-2 shows an example benzalkonium chloride chromatogram. Figure 4-3 illustrates the standard curve and equation used to determine free benzalkonium chloride in unknown FNC wash solutions. Methyl paraben standards were prepared in the same manner as benzalkonium chloride. Figure 4-4 shows an example methyl paraben chromatogram in FNC wash water. Figure 4-5 shows the standard curve and equation used to determine free methyl paraben in unknown solutions. As Figures 4-2 and 4-4 illustrate, both benzalkonium chloride and methyl paraben generate individual, clear peaks that can be integrated and assembled into a standard curve. The integrated peak area corresponds to the known concentration; moreover, when an unknown concentration is injected, the peak area generated is then inserted into the standard curve equation to generate a value for the unknown.

Methyl Paraben Chromatographic System

The chromatographic system parameters for analyzing methyl paraben in wash waters and washed romaine and iceberg lettuce was provided by Marvel Technologies™. A Waters™ BEH C18 1.7µm 2.1 x 50 mm was employed to facilitate separation of methyl paraben. The mobile phases contained (A) water and (B) methanol, each containing 0.1% formic acid to improve ionization, and were run isocratically with a 50% mixture of each solvent. The flow rate

was 0.400 mL/minute for three minutes. A 1µL loop was used for injection. The chromatographic system was equipped with a binary solvent manager, and a photodiode array detector (PDA), set at 254 nm, which was used to detect methyl paraben. Empower 3 Software developed by Waters™ was used to identify and quantitate samples.

Analysis of Lettuce Samples for Methyl Paraben Residue

Methyl paraben residue in both fresh-cut romaine and iceberg lettuce was conducted in the same manner as benzalkonium chloride. Because a published method did not exist for this analysis, a preliminary study was conducted in order to validate methyl paraben extraction from lettuce. Briefly, known concentrations of methyl paraben were introduced onto the lettuce surface and the extraction was performed. After extraction, methyl paraben samples (ranging from 0-15 ppm were injected and peak areas were compared to methyl paraben standards prepared from the extraction supernatant of unwashed lettuce. A percent recovery was determined to be 99-101% for methyl paraben concentrations (data not shown). These preliminary data demonstrate that the extraction procedure would not impact the true methyl paraben concentration in romaine and iceberg lettuce, which validated efficacy of the methods. Additionally, unknown concentrations on lettuce could be effectively elucidated using the methods described above in section Extraction.

Statistical Analysis

Methyl paraben and benzalkonium chloride wash water concentrations, both before and after washing, were determined by calculating the average and standard deviation for all three replications using Microsoft Excel (V. 2013, Redmond, WA). Methyl paraben and benzalkonium chloride residues on iceberg and romaine samples fell below the limit of detection (<5.0 ppm); thus these data have a standard error of zero and statistical analyses could not be performed.

4.2.3. Pathogen Reduction Study

Inoculum Preparation

Two strains of *L. monocytogenes* (B-33054; Cucumber isolate; USDA ARS; and B-33245; Environmental isolate; USDA ARS), *Salmonella* Typhimurium (ATCC 14028), *Salmonella* Newport (ATCC 6962), and two strains of rifampicin resistant *E. coli* O157:H7 (ATCC 43890 and 43895) were removed from frozen storage (-80°C) and activated by transferring one cryobead into 10 mL Tryptic Soy Broth (TSB; BD BBL™, New Jersey) and incubated at 37°C for 24 h. A 100 µL aliquot of each *Listeria* culture (total of 200µLl) was combined with 200 ml of TSB and incubated at 37°C for 24 h. The same transfer was done for both *Salmonella* and both *E. coli* O157:H7 cultures, separately. Following incubation, cultures were centrifuged (5,520 x *g*; -4°C; 15 min) and the resulting pellets were rehydrated with 100 ml 0.1% peptone water. One milliliter from each of the rehydrated pellets (total of 3 ml) was combined with 150 ml 0.1% peptone water to create a combined master inoculum. Five ml of the combined master inoculum was then sprayed onto 1000 g of lettuce. Inoculum concentrations were enumerated for each bacterium separately and for the mixed master inoculum. This was accomplished using serial dilutions of 0.1% peptone water and plated onto oxford medium base (BD BBL™, New Jersey) with *Listeria* selective supplement added at ½ strength (OXOID, Basingstoke, Hampshire, England), xylose lysine deoxycholate (XLD; Remel, Lenexa, Kansas), and sorbitol MacConkey ($SMAC_{rif}$; 100 ppm rifampicin added; Remel, Lenexa, Kansas) agars. The plates were then incubated at 37°C for 24 h.

Inoculation of Lettuce

Whole romaine and iceberg lettuce was purchased from a local produce supplier in Kansas City, Missouri. Lettuce was cored and chopped in the ~1 in x 1 in squares. One-thousand

gram batches of each lettuce type were inoculated with a mixed cocktail containing two strains of *Listeria monocytogenes*, *Salmonella* Typhimurium, *Salmonella* Newport, and two strains of *E. coli* O157:H7. Lettuce batches were inoculated using a light spray mist procedure to target 7 $\log_{10}$ CFU/g of product. Lettuce was held at room temperature for 30 min, to allow bacterial attachment, before FNC wash treatments were applied.

Washing of Cut Lettuce

Concentrated FNC solution was prepared by combining 0.08 lb acetic acid, 0.04 lb methyl paraben, and 0.04 lb of USP-BAC (50% benzalkonium chloride) with 1 gallon of water. The concentrated FNC solution was mixed with tap water to prepare four individual five gallon treatment solutions (0%, 1%, 2%, and 3%). New five gallon treatment solutions were prepared for each of the three replications. Two hundred grams of inoculated romaine or iceberg lettuce were placed in slotted containers and completely submerged for either 1 min or 5 min in the different treatment solutions. Following FNC treatment, each 200 g batch of lettuce was completely submerged in tap water for 1 min to simulate a secondary wash prior to being spun dry in a salad spinner of 10.25 inches in diameter (Prepworks®, Kent, Washington). Pulling the salad spinner cord fully ten times standardized this procedure.

Microbiological Sampling of Lettuce

Following washing, ~25 g samples of lettuce were randomly stomached (Stomacher® 400 Circulator, Seward, Davie, Florida) with 75 mL Dey Engley Neutralizing Broth (DNB; BD BBL™, New Jersey) at 230 RPM for sixty seconds. Homogenized samples were serially diluted (1:10) using 0.1% peptone water as the diluent and the appropriate dilutions were spread plated. Samples were plated on Tryptic Soy Agar, (TSA; Remel, Lenexa, Kansas) that was overlaid with selective media after six hours of incubation. The TSA+overlay media allowed for the recovery

of injured cells, which provides a more conservative estimate of pathogen reductions. More specifically, *Listeria monocytogenes* samples were plated on TSA with an overlay of oxford medium base (BD BBL™, New Jersey) with *Listeria* selective supplement added at ½ strength (OXOID, Basingstoke, Hampshire, England). Colonies that turned black with a zone of clearing were counted as *L. monocytogenes*. *E. coli* O157:H7 samples were plated on TSA with an overlay of Sorbitol MacConkey agar with 100 ppm rifampicin (SMAC; Remel, Lenexa, Kansas) and colorless colonies were counted. *Salmonella* samples were plated on TSA with an overlay of Xylose Lysine Deoxycholate (XLD) agar (Remel, Lenexa, Kansas), and colonies exhibiting a black color were counted. All plates were incubated at 37°C for 18-24 hours.

Microbiological Sampling of Wash Water

The wash water solutions for each concentration were sampled after 5 minutes, while inoculated produce was submerged, to establish microbial populations in the FNC wash water. At each time point, 20 ml of FNC wash water was removed and combined with 20 ml of double strength DNB. Samples were serially diluted with 0.1% peptone water and plated onto both selective and recovery media as previously described.

Benzalkonium Chloride and Methyl Paraben Concentrations in Wash Waters

A 10 mL sample of each wash water solution was collected both before and after product washing in order to determine benzalkonium chloride and methyl paraben concentrations in each wash solution. Wash water samples were immediately frozen to -20°C and saved for future analysis. Benzalkonium chloride and methyl paraben concentrations in the wash waters were analyzed as previously described.

Statistical Analysis

All experimental procedures were replicated a total of three times. Log reductions were calculated by comparing the populations recovered from each experimental wash to the inoculated, untreated control. These log reductions were then used for the statistical analyses. Data collected from all three replications were analyzed using the MIXED procedure of Statistical Analysis Software (SAS 9.4; Cary, NC) with the Satterthwaite approximation. The RANDOM statement was used to account for variability between replications. For each pathogen, the main effects (media, concentration, and contact time) and the interactions were evaluated for statistical significance at the P=0.05 threshold. The best model was constructed by means of backwards elimination. Due to a large degree of variation in pathogen recovery between the two media types, media type was removed from the model and data were subsequently analyzed for each individual media type (concentration, contact time, and the concentration x contact time interaction).

4.3 Results/Discussion

4.3.1 Shelf life Study

Concentrations of benzalkonium chloride and methyl paraben in all FNC wash waters are located in Tables 4-1 and 4-2.

Appearance

Overall product appearance throughout the seven day shelf life study can be found in Figures 4-6 for iceberg lettuce and 4-7 for romaine lettuce. Based on Figures 4-6 and 4-7, there was no difference (P>0.05) in appearance detected for romaine and iceberg lettuce treated with either 0% or 2% FNC. Thus, the 2% FNC treatment did not impact (either positively or negatively) the appearance of iceberg and romaine lettuce compared to a water wash control. Methyl paraben is commonly used as a preservative in food products and cosmetics, and any

extension in shelf life may affect labeling (i.e. a preservative label on lettuce washed with FNC), which is not necessary based upon appearance data. Iceberg lettuce remained salable until day six where it fell below the overall quality rating of "4", per the Karlruher Schema scale (Appendix B) (*26*). Pinking was the most obvious defect for 0% and 2% samples, which began on day three and became worse over time. Romaine lettuce remained salable throughout the study, as it stayed above an overall quality rating of "5" (Appendix B) (9). Pictures were captured using fluorescent lighting with a black background and can be found in Appendix D.

Lettuce quality throughout the duration of a shelf life study has many factors that affect the length of salability. Past studies have shown antimicrobials, such as chlorine dioxide and sodium hypochlorite, can extend the shelf life of fresh-cut lettuce compared to a water washed control (*12*). In the present study, the 2% FNC wash did not extend the shelf life of fresh-cut iceberg and romaine lettuce beyond that of the 0% (water wash control).

Microbiological Sampling

Coliforms and generic *E. coli* were not detected on any romaine or iceberg lettuce samples at any point throughout the shelf life study. The limit of detection for this assay is ~0.5 $\log_{10}$ CFU/g; therefore, coliform and *E. coli* populations were <0.5 $\log_{10}$ CFU/g (data not shown).

On day zero, FNC wash concentration was not a significant variable for iceberg ($P=0.5974$) or romaine ($P=0.8917$) lettuce with regards to aerobic microorganism populations. This indicates that washing lettuce with pure water is as effective at reducing aerobic plate count populations as washing with FNC at 1%, 2% or 3% concentrations on day zero (Figures 4-8 and 4-9).

Iceberg and romaine washed with 0% and 2% were analyzed for total aerobic plate counts throughout the shelf life. FNC concentration was nearly a significant variable (P=0.0819) for iceberg lettuce (Figure 4-10). As Figure 4-11 depicts, a significant difference (P=0.0203) in concentration was observed for romaine, with the 2% FNC wash demonstrating improved efficacy at reducing total aerobic plate counts in comparison to pure water.

Sampling day was a significant variable (P=0.0305) for iceberg lettuce throughout the shelf life. Because a sampling day x concentration effect was not observed (P=0.7208), data for each concentration are not shown across the sampling days. Thus, when a sampling day effect is observed, all data collected on each day for both 0% and 2% FNC washes are averaged together into one data point. Figure 4-12 illustrates an initial decline in total aerobic populations on day 0; however, these reductions gradually decline, which indicates an increase in total aerobic microorganisms throughout the shelf life of iceberg lettuce. On days five and seven, the negative log reductions indicate that total aerobic populations increased above the population that was observed for the unwashed iceberg lettuce sample on day 0. Steady growth of microorganisms on produce subjected to antimicrobial washes has been previously reported (*9*, *18*).

Sampling day (P=0.2540) and the sampling day x concentration interaction (P=0.1766) were not statistically significant for romaine lettuce. Figure 4-13 illustrates all 0% and 2% FNC data averaged into a single data point value for each sampling day throughout the romaine shelf life. In general, these data demonstrate that washing with 0% or 2% FNC reduces total aerobic bacteria populations on romaine lettuce, with population reductions still observed on day seven (reductions calculated from the day zero unwashed sample population). The fact that a negative log reduction was not observed on any sampling day indicates that the total aerobic plate count

populations on romaine washed with 0% or 2% FNC were, on average, less than the population that was recorded for the day zero unwashed control sample.

4.3.2 Residue Analysis

A 5 ppm methyl paraben standard prepared using the lettuce extraction supernatant as the diluent yielded a distinct peak as shown in Figure 4-14. Lower concentrations of methyl paraben (e.g. 2.5 ppm) were evaluated; however, no clear, intense peaks were generated. Therefore, it was determined that 5 ppm is the limit of detection for methyl paraben when analyzing samples as described above. As Figure 4-15 shows, injecting the extracted lettuce matrix (sans benzalkonium chloride or methyl paraben) yielded no peak clarity or intensity. Based on the y-axis, Figure 4-15 is zoomed in on background, which is presumably representative of lettuce debris. Importantly, even a low concentration of methyl paraben (5 ppm) in the lettuce matrix generates a clear peak for residue analysis, which indicates that unknown samples with clear peaks are informative of methyl paraben concentrations equal to or greater than 5 ppm. As shown in Figure 4-16, lettuce subjected to a 3% FNC wash (the highest concentration) followed by a potable water rinse and dewatering, yielded a chromatogram with no distinct peaks. In fact, the chromatogram resembles that of Figure 4-15, which suggests that the amount of methyl paraben on lettuce, when subjected to a 3% FNC wash, is near zero ppm. Because all of the methyl paraben chromatograms from FNC washed lettuce highly resembled that of Figure 4-16, it was concluded that the residue for all concentrations was <5 ppm.

The residue of benzalkonium chloride on both romaine and iceberg lettuce washed with various concentrations of FNC, followed by potable water rinsing and dewatering, was found to be <10 ppm. This conclusion is based off of the chromatogram of lettuce washed with 3% FNC (Figure 4-18), which resembled that of the extracted lettuce matrix (Figure 4-15) that did not

contain benzalkonium chloride. All benzalkonium chloride chromatograms of FNC washed lettuce highly resembled Figure 4-18; thus, it was concluded that <10 ppm benzalkonium chloride residue remained on all FNC washed lettuce samples. The limit of detection was determined to be 10 ppm, as lower concentrations lacked peak clarity or intensity.

Residue data collected from the shelf life samples indicate methyl paraben concentrations are <5 ppm on romaine and iceberg lettuce after washing with a FNC solution up to 3%. This is not surprising, as FNC washed lettuce was subjected to a second potable water wash that likely removed residual methyl paraben and benzalkonium chloride. For lettuce washed with the industry standard 150 ppm chlorine, a secondary potable water wash has been shown to remove residual chlorine (*6, 30*). The use of a salad spinner further removes water and decreases the likelihood of residue, as well. Methyl paraben is Generally Recognized As Safe (GRAS) by the FDA, and according to CFR Chapter 21 §184.1490, methyl paraben must not exceed 0.01% in a foodstuff (*4*). The data generated indicate that the methyl paraben residues on romaine and iceberg lettuce, when washed as described herein, satisfy these requirements. Benzalkonium chloride has also been evaluated for GRAS status and, according to a report by the Burdock Consulting Group dated September 23, 2013, benzalkonium chloride did earn GRAS status, although the FDA does not currently list it as GRAS (*5*).

As the lettuce extraction method was based on a benzalkonium chloride method by Diez et al. (*13*), some concern about whether or not the same method can be used for methyl paraben extraction may exist. The primary concern would be how soluble methyl paraben is in acetonitrile and if it would be removed during extraction from the lettuce tissue. Benzalkonium chloride is highly soluble in water, and thus acetonitrile, so it should readily be removed (*5*, *14*). Little research has been done on the maximum solubility of methyl paraben in acetonitrile, but its

solubility in water may provide insight, as both solvents are polar. The maximum solubility of methyl paraben in water is ~25 g/L, an amount less than the 100 g/L maximum solubility of benzalkonium chloride in water (*15*). It is for this reason that substantial preliminary research efforts were dedicated to measuring methyl paraben recovery from lettuce tissue to demonstrate that methyl paraben that using the methods as described for benzalkonium chloride extraction were also effective for methyl paraben.

Methyl paraben and benzalkonium chloride residues were not detected as a residual on iceberg and romaine lettuce; thus, a higher concentration of FNC (> 3%) would have to be evaluated to determine the concentration at which residues do remain. A higher concentration of the antimicrobial may achieve better bacterial kill on the surface of plant tissues and in the water used for washing. However, higher concentrations of FNC must be within the allowable limits for use and additional residue analyses would be required to ensure compliance with FDA standards.

4.3.3 Pathogen Reduction Study

Concentrations of benzalkonium chloride and methyl paraben in all FNC wash waters are located in Tables 4-3 and 4-4.

Wash water

Regardless of whether the FNC wash water samples were plated on selective media or the injury recovery media, concentration was a highly significant variable for all pathogens investigated ($P \leq 0.05$). As Figures 4-19 – 4-24 illustrate, 1%, 2%, and 3% FNC significantly reduced pathogen populations in the wash waters in comparison to the 0% FNC wash water. It is also important to note that a statistical difference was not observed among the 1%, 2%, or 3% concentrations, which suggests that preparing wash waters with 2% or 3% FNC provides no

added benefit over the 1% solution. There was a >2.0 log reduction (99% reduction) observed for each of these foodborne pathogens regardless of media type used for enumeration. As TSA is a nonselective, nutritive agar, the purpose of it is to recover potentially injured cells. Injured cells may not be able to immediately grow on selective agar alone. Therefore, using TSA with a selective media overlay is more representative of total viable pathogen populations, as it accounts for injured cell populations. Injured cells do pose a food safety risk, so their presence needs to be evaluated (*14*). These data indicate that 1%, 2%, or 3% FNC solutions are effective at significantly reducing *L. monocytogenes, E. coli* O157:H7, and *Salmonella* populations in water. Recognizing that controlling pathogen populations in wash water is the primary goal of using chlorinated water in the produce industry, it is worth noting that FNC may also be an effective and feasible pathogen control treatment for wash water.

In the present study, 1%, 2%, and 3% FNC significantly ($P \leq 0.05$) reduced pathogens in wash water by nearly 3 logs when plated on selective media, regardless of washing time, as contact time was not a significant variable. This ~3 log reduction of pathogens indicates that FNC is effective at reducing pathogen populations in wash water. Research by Beuchat and Ryu (*9*) used a 200 ppm chlorine solution (higher than what is typically used with lettuce) and found populations of *L. monocytogenes* to be reduced by approximately 2.0 $\log_{10}$ CFU/g after a ten second exposure. A similar reduction was observed using a *Salmonella* cocktail. Although not analyzed with a 10 second exposure, the current study demonstrated that FNC was able to reduce three prevalent foodborne pathogens to a similar degree as the chlorine-based sanitizer. Future research may warrant investigating FNC and chlorine in the same study in order to make valid comparisons.

Salmonella Recovery on Plant Tissue

When plated on XLD, FNC concentration (P=0.0009) and contact time (P=0.0127) effects were observed. Based on figure 4-25, both 2% and 3% FNC solutions significantly reduced *Salmonella* compared to 0% FNC treatment on romaine lettuce. However, no significant difference in *Salmonella* populations was observed between 1% and 3% FNC. Furthermore, no significant difference was found between 0% and 1% FNC. Figure 4-26 shows a 5 minute contact time significantly reduced populations on romaine lettuce compared to a 1 minute contact time. Although statistically significant, the difference in log reductions between the two contact times was 0.14 $\log_{10}$ CFU/g, which is considered negligible from a biological sense. When plated on XLD, all log reductions on inoculated cut romaine lettuce were less than 1.0 $\log_{10}$ CFU/g, and pure water alone (0% FNC) achieved ~0.5 $\log_{10}$ CFU/g reduction in *Salmonella* populations (Figure 4-25). This, coupled with the fact that significant differences were not observed for FNC concentration when *Salmonella* was plated on TSA with an XLD overlay (Figure 4-27), demonstrates that there is little to no advantage gained by washing with FNC in lieu of pure water. This suggests the TSA overlay was able to revive injured *Salmonella* cells that would not have otherwise survived on selective XLD. This is important, as research shows injured cells, otherwise known as viable but nonculturable cells, pose a food safety risk, as they can still be pathogenic (*14*) following recovery. Figures 4-28 – 4-32 illustrate all insignificant variables associated with *Salmonella* reductions on fresh-cut romaine and iceberg lettuce.

Salmonella spp. has been studied in post-harvest produce wash studies; however, much of the research is for cantaloupes and honeydew. Beuchat et al. (*9*) compared a 200 ppm chlorine to a 2,000 ppm solution in killing a *Salmonella* spp. cocktail on lettuce. The authors reported a 2,000 ppm chlorine solution was effective at reducing *Salmonella* spp. by 2.30 $\log_{10}$ CFU/g.

While this reduction is notable, it is important to consider that such a high concentration is likely not realistic for a postharvest processing setting, as a large chlorine residue is likely to occur (*9*). Other research found that soaking cantaloupes for 60 seconds in 200 ppm chlorine solution was only able to achieve a 0.7 $\log_{10}$ CFU/g reduction in *Salmonella* Typhimurium (*25*). An additional reduction was found when scrubbing the cantaloupe but that is not a feasible mechanism for delicate romaine and iceberg plant tissue. Like the present study, previous research has demonstrated limited success with regards to reducing *Salmonella* on lettuce and other produce products.

Listeria monocytogenes Recovery on Plant Tissue

As shown in Figure 4-33, plating on selective MOX agar revealed that the 3% FNC wash significantly ($P \leq 0.05$) improved *L. monocytogenes* population reductions on fresh-cut iceberg lettuce in comparison to the 0% and 1% FNC wash solutions. The *L. monocytogenes* population reductions achieved by the 3% FNC wash performed the best; however, this wash achieved a 1.26 $\log_{10}$ CFU/g reduction, which was only 0.65 $\log_{10}$ better than the 0% water control wash. The 2% FNC wash also significantly reduced populations of *L. monocytogenes* compared to the 0%; however, no difference was detected for 2% and 1% FNC concentrations. No statistical difference was detected between 0% and 1% FNC wash solutions at reducing *L. monocytogenes* populations on inoculated cut iceberg lettuce.

As shown in Figure 4-34, plating on TSA with a MOX overlay demonstrated that 1%, 2%, and 3% FNC wash solutions significantly ($P=0.0013$) reduced *L. monocytogenes* on cut romaine lettuce compared to the 0% FNC wash. While significant, the reduction of *L. monocytogenes* on cut inoculated iceberg lettuce was less than 0.5 $\log_{10}$ CFU/g greater than the reductions observed for the 0% wash. These data suggest that washing lettuce in 1%, 2% or 3%

FNC provides little added benefit beyond washing with water (0%). Figures 4-35 – 4-40 illustrate all insignificant variables associated with *L. monocytogenes* reductions on fresh-cut romaine and iceberg lettuce.

Research by Zhang and Farber (*33*) evaluated post-harvest washes to reduce populations of *L. monocytogenes* on fresh-cut lettuce. The authors subjected fresh-cut lettuce to 5 ppm chlorine dioxide, 200 ppm chlorine, 1% lactic acid, 1% acetic acid, or trisodium phosphate for either a five or ten minute wash. Their results showed a 200 ppm chlorine solution (at 22°C) was able to reduce *L. monocytogenes* by 1.7 $\log_{10}$ CFU/g when washed for ten minutes. The chlorine dioxide, lactic acid, and acetic acid solutions were similar with a ~1.0 $\log_{10}$ CFU/g reduction (regardless of contact time) while trisodium phosphate did not impact populations (*33*). A 1.7 $\log_{10}$ CFU/g reduction of *L. monocytogenes* is noteworthy; however, it must be questioned if a ten minute wash is feasible in a commercial setting. Similarly, the present study demonstrated limited efficacy when evaluating FNC as a more feasible 1 minute or 5 minute wash.

<u>*E. coli* O157:H7 Recovery on Plant Tissue</u>

When plated on $SMAC_{rif}$, contact time was a significant variable, with a five-minute contact time of FNC significantly reducing *E. coli* O157:H7 populations on both iceberg (Figure 4-41; P=0.0493) and romaine (Figure 4-42; P=0.0293) lettuce compared to a one minute contact time. Although significantly different, the difference in population reductions was well below 0.5 $\log_{10}$ CFU/g for contact time on both romaine and iceberg lettuce. It is also noteworthy that when plated on TSA+$SMAC_{rif}$, these significant differences in contact time were no longer detected (P>0.05). Therefore, the greater reductions achieved with a five minute contact time are negligible.

A concentration effect was also observed for iceberg (P=0.0097) and romaine (P=0.0257) lettuce plated on $SMAC_{rif}$. Figure 4-43 shows the log_{10} CFU/g reductions found on iceberg lettuce plated on $SMAC_{rif}$. A 3% FNC solution was significantly better at reducing *E. coli* O157:H7 populations when compared to 0% and 1% solutions; however no significant difference was observed between the 3% and 2% FNC solutions. Figure 4-44 shows 2% and 3% FNC solutions are significantly better at reducing *E. coli* O157:H7 populations compared to 0% FNC, on romaine lettuce. However, the 3% and 2% FNC washes were not significantly different compared to the 1% wash. All population reductions were well below 1.0 log_{10} CFU/g, which suggests there is little to no benefit of washing with FNC in comparison to pure water.

Regardless of significant differences in FNC concentration on $SMAC_{rif}$, *E. coli* O157:H7 population reductions on inoculated cut lettuce were less than 1.0 log_{10} CFU/g for all wash concentrations and the difference in log reductions achieved by the 1%, 2%, or 3% FNC washes in comparison to the 0% wash was less than 0.5 log_{10} CFU/g. These data indicate that little to no advantage exists for washing fresh-cut lettuce with FNC in lieu of pure water relative to reducing *E. coli* O157:H7 populations on the lettuce itself.

Figure 4-45 shows a significant difference in log_{10} CFU/g reductions of *E. coli* O157:H7 when TSA with a $SMAC_{rif}$ overlay was used to recover injured *E. coli* O157:H7 cells on romaine lettuce. A 3% FNC solution significantly reduced populations compared to 0%, 1%, and 2% solutions. A marginal reduction was observed, with 3% FNC reducing *E. coli* O157:H7 populations by 0.66 log_{10} CFU/g, 0.37 log_{10} CFU/g, and 0.41 log_{10} CFU/g more than the 0%, 1%, and 2% washes, respectively. The 1% FNC wash was significantly better than the 0% wash; however, no difference was detected between 0% and 2% FNC washes. Figures 4-46 – 4-48

illustrate all insignificant variables associated with *E. coli* O157:H7 reductions on fresh-cut romaine and iceberg lettuce.

Data collected in this study indicate that FNC demonstrated similar efficacy against *E. coli* O157:H7 as other interventions previously investigated against this pathogen. A study by Keskinen et al. (2009) evaluated the efficacy of various antimicrobial post-harvest washes in reducing *E. coli* O157:H7 on lettuce. The research team artificially inoculated fresh-cut romaine and iceberg lettuce with a high population (untreated, inoculated control was ~7 $\log_{10}$ CFU/g of) of *E. coli* O157:H7 and subjected them to either 200 ppm chlorine, acidic electrolyzed water with chlorine dioxide, or chlorous acid. The greatest reduction of *E. coli* O157:H7 on lettuce tissue was observed using 200 ppm of chlorous acid, at pH 8.0, but the reduction was only 1.45 $\log_{10}$ CFU/g. Other treatments were not able to reduce *E. coli* O157:H7 populations by more than one log. The industry standard 200 ppm chlorine wash was able to achieve a mere 0.65 $\log_{10}$ CFU/g reduction, which provides further evidence that the objective of current postharvest washes is not to significantly reduce pathogenic microbes on plant tissue; rather, to reduce populations in free water. This study also concluded that the length of time *E. coli* O157:H7 attached to the cut lettuce tissue had no impact on efficacy of washes (*17*). This study evaluated the total *E. coli* O157:H7 population on the surface and interior of fresh-cut lettuce leaves. Another study by Beuchat et al. (1998) showed similar results of chlorines ability to reduce *E. coli* O157:H7 on whole lettuce leaves (*9*).

Summary of Pathogen Results

Overall, FNC concentrations of 1%, 2%, and 3% significantly reduced ($P \leq 0.05$) populations of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* in the wash water used for inoculated romaine and iceberg lettuce. However, the FNC concentrations evaluated in this

study did not consistently reduce the three foodborne pathogens on inoculated cut romaine or iceberg lettuce tissue. The largest reduction observed was 1.75 $\log_{10}$ CFU/g in *E. coli* O157:H7 on cut romaine lettuce. However, this wash performed a mere 0.66 $\log_{10}$ CFU/g better at reducing *E. coli* O157:H7 populations than washing with 0% FNC (pure water).

Overall, no post-harvest wash has been successful enough to change the market standard of chlorine. As in this study, some postharvest washes exhibit efficacy in killing foodborne pathogens in free wash water; however, exhibiting that same effect on produce tissue has been a challenge. Even removing 99% (2.0 $\log_{10}$ CFU/g) of pathogens is hardly worth noting as some foodborne pathogens have infectious doses of less than 100 cells (*18*). The difficulty found with post-harvest antimicrobial washes may explain the FDA's emphasis on prevention as a means of reducing foodborne illness associated with and leafy greens and other produce.

Table 4-1. Methyl paraben concentrations in 1%, 2%, and 3% washes used during shelf life and residue study. Wash samples were taken pre- and post-washing. All values are in parts per million.

Methyl Paraben Concentrations					
	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1% FNC Wash Pre Wash	95.45	101.18	90.41	95.68	5.39
1% FNC Wash Post Wash	94.27	100.07	88.45	94.26	5.81
2% FNC Wash Pre Wash	183.19	165.22	160.79	169.73	11.86
2% FNC Wash Post Wash	158.91	164.35	154.31	159.19	5.03
3% FNC Wash Pre Wash	276.51	286.34	289.57	284.14	6.80
3% FNC Wash Post Wash	233.22	275.02	232.93	247.06	24.22

Table 4-2. Benzalkonium chloride concentrations in 1%, 2%, and 3% washes used during shelf life and residue study. All values are in parts per million.

Benzalkonium Chloride Concentrations					
	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1% Wash Pre Wash	61.71	63.45	64.97	63.38	1.63
1% Wash Post Wash	59.99	54.78	58.53	57.77	2.69
2% Wash Pre Wash	103.47	103.48	110.08	105.68	3.81
2% Wash Post Wash	101.51	98.47	108.24	102.74	5.00
3% Wash Pre Wash	169.73	164.19	171.13	168.35	3.67
3% Wash Post Wash	166.48	157.01	159.21	160.90	4.96

Table 4-3. Methyl paraben concentrations (ppm) in 1%, 2%, and 3% FNC washes used during the Pathogen Reduction Study. Wash solution samples were taken pre- and post-washing of cut lettuce.

Methyl Paraben Concentrations					
	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1% Wash Pre Wash	45.95	66.93	77.11	63.33	15.89
1% Wash Post Wash	67.81	61.80	71.83	67.15	5.05
2% Wash Pre Wash	127.99	135.35	136.37	133.24	4.57
2% Wash Post Wash	114.84	114.62	130.21	119.89	8.94
3% Wash Pre Wash	189.55	182.04	229.00	200.20	25.23
3% Wash Post Wash	186.77	170.66	201.77	186.40	15.56

Table 4-4. Benzalkonium chloride concentrations in 1%, 2%, and 3% washes used during the Pathogen Reduction Study. All values are in parts per million.

Benzalkonium Chloride Concentrations					
	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1% Wash Pre Wash	48.07	50.29	58.00	52.12	5.21
1% Wash Post Wash	51.48	47.38	50.34	49.73	2.12
2% Wash Pre Wash	82.32	84.51	90.67	85.83	4.33
2% Wash Post Wash	77.04	79.37	84.83	80.41	4.00
3% Wash Pre Wash	131.96	120.23	133.60	128.60	7.29
3% Wash Post Wash	117.81	118.98	131.35	122.71	7.50

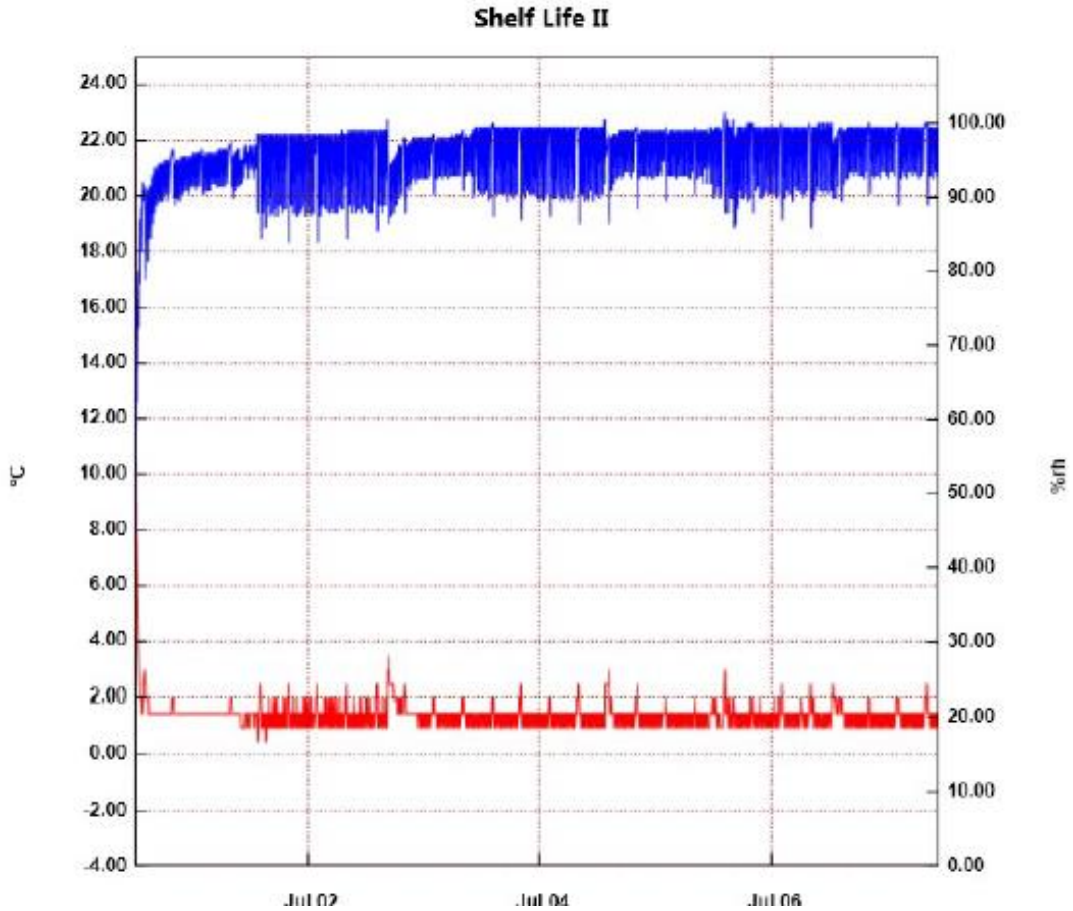
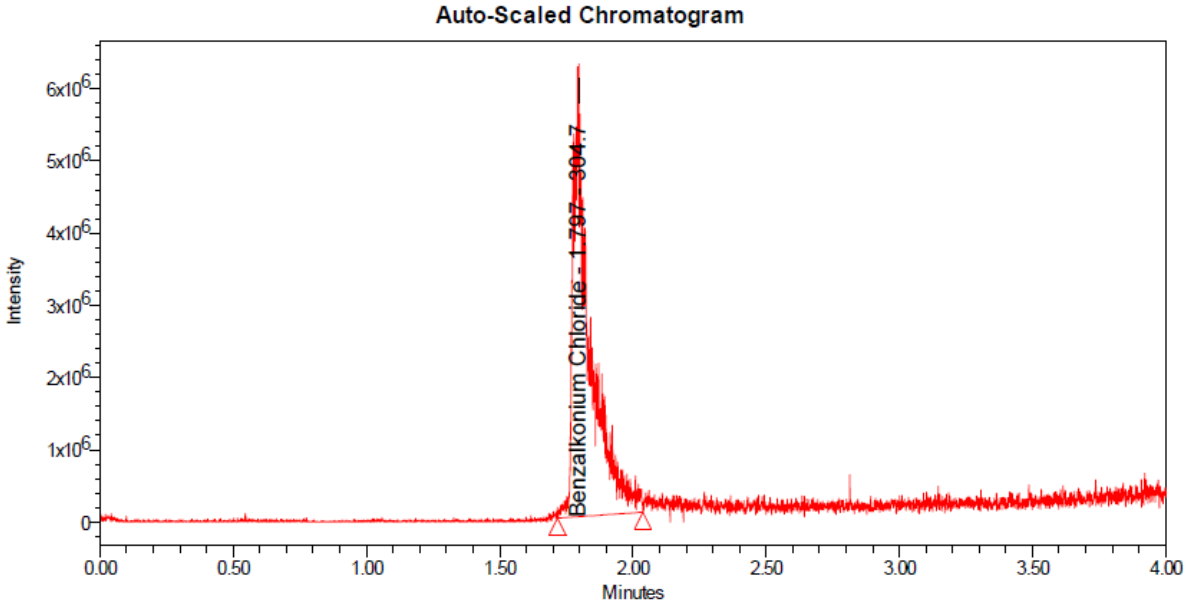


Figure 4-1. Temperature and relative humidity (RH) of the cooler throughout the shelf life study. The red line depicts the temperature (°C) and the blue line relative humidity (%rh).

SAMPLE INFORMATION

Sample Name:	R3 2%FNC pre wash	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	2% BAC washwater
Vial:	2:F,7	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC wash water
Injection Volume:	1.00 ul	Channel Name:	305.7Da
Run Time:	4.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 8:11:54 PM CST		
Date Processed:	12/2/2016 8:32:29 PM CST		



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Benzalkonium Chloride	1.797	26195354	5776758	81.745	ppm

Figure 4-2. Example chromatogram of benzalkonium chloride in FNC wash solutions.

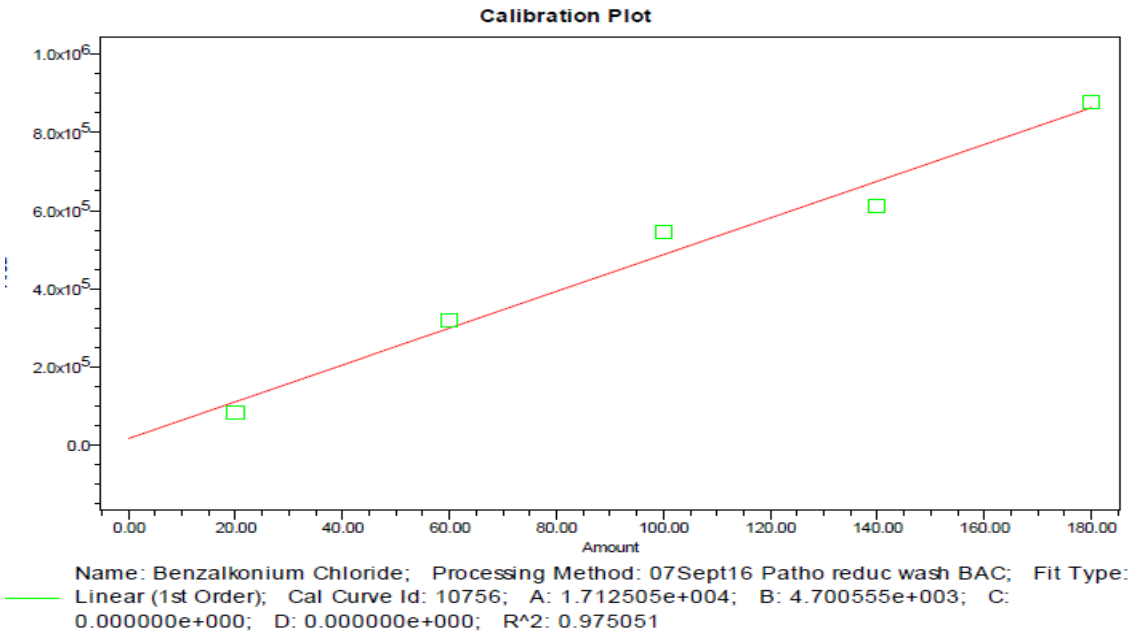


Figure 4-3. The standard curve used to analyze the amount of benzalkonium chloride in FNC wash solutions.

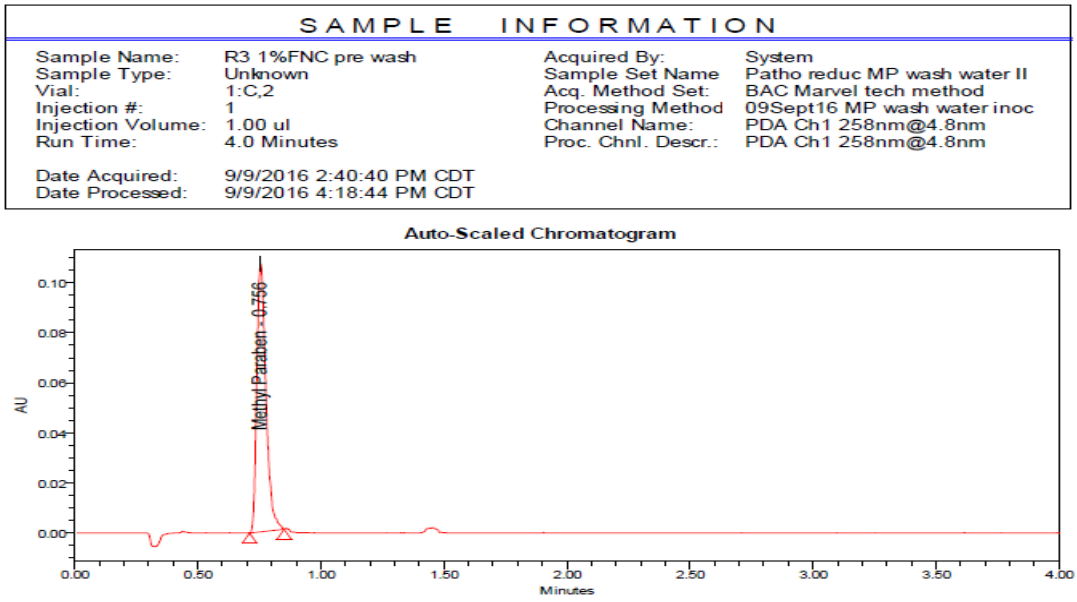


Figure 4-4. Example methyl paraben peak in FNC wash solutions.

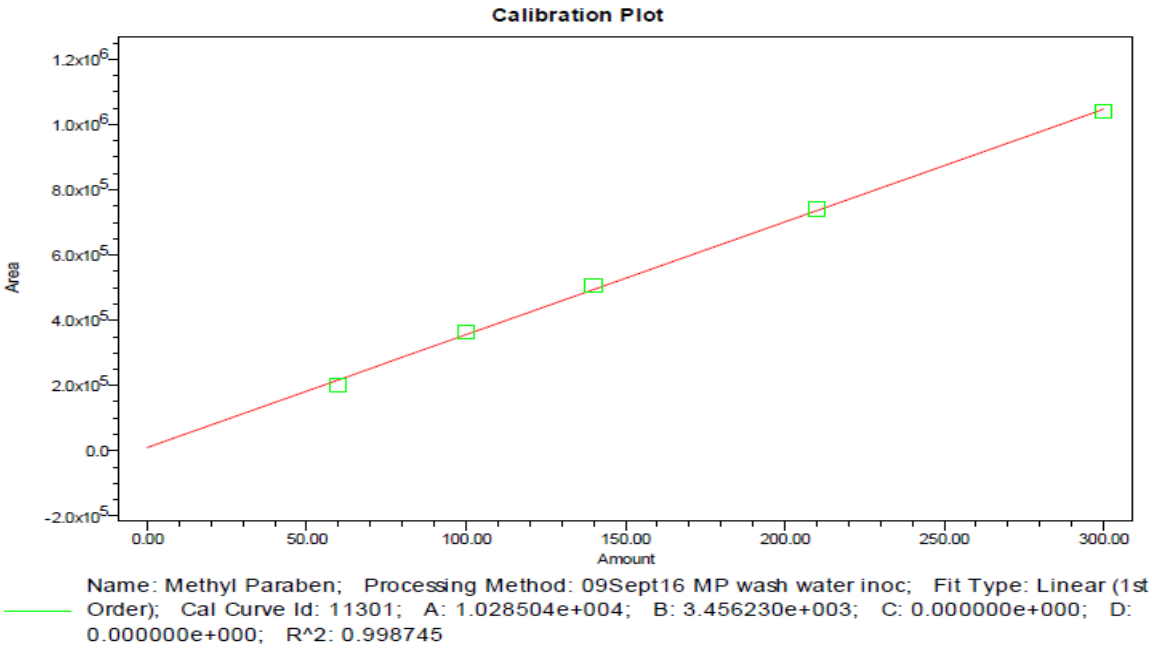


Figure 4-5. Standard curve used to analyze the amount of methyl paraben in FNC wash solutions.

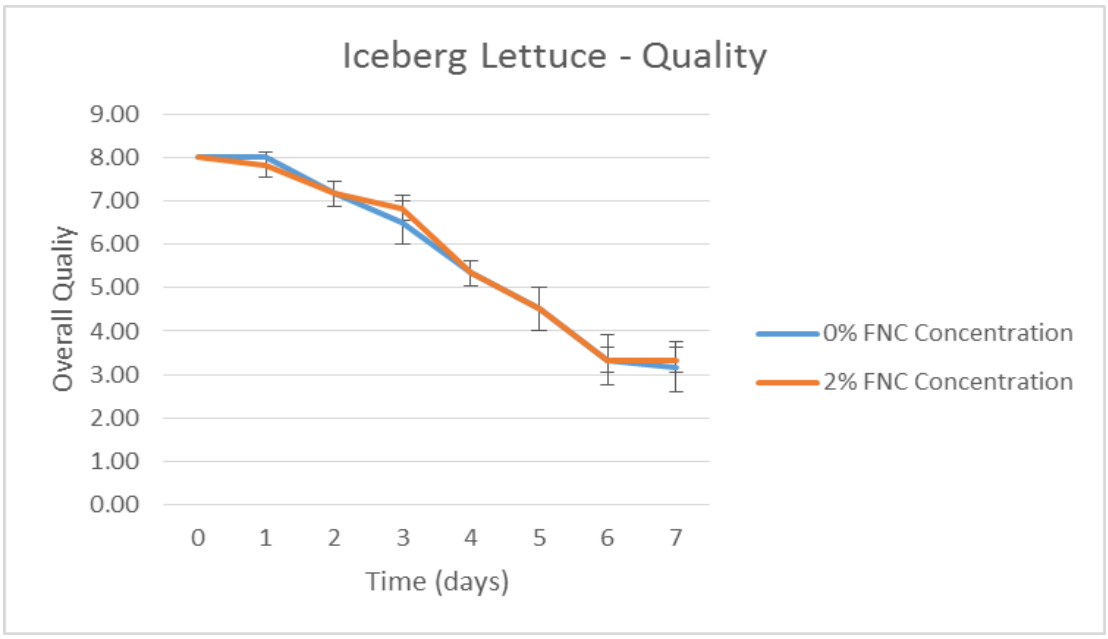


Figure 4-6. Overall appearance scoring results throughout seven shelf life study for iceberg lettuce stored at ~4°C. No significant difference was observed (P>0.05).

Error bars represent one standard deviation from the mean.

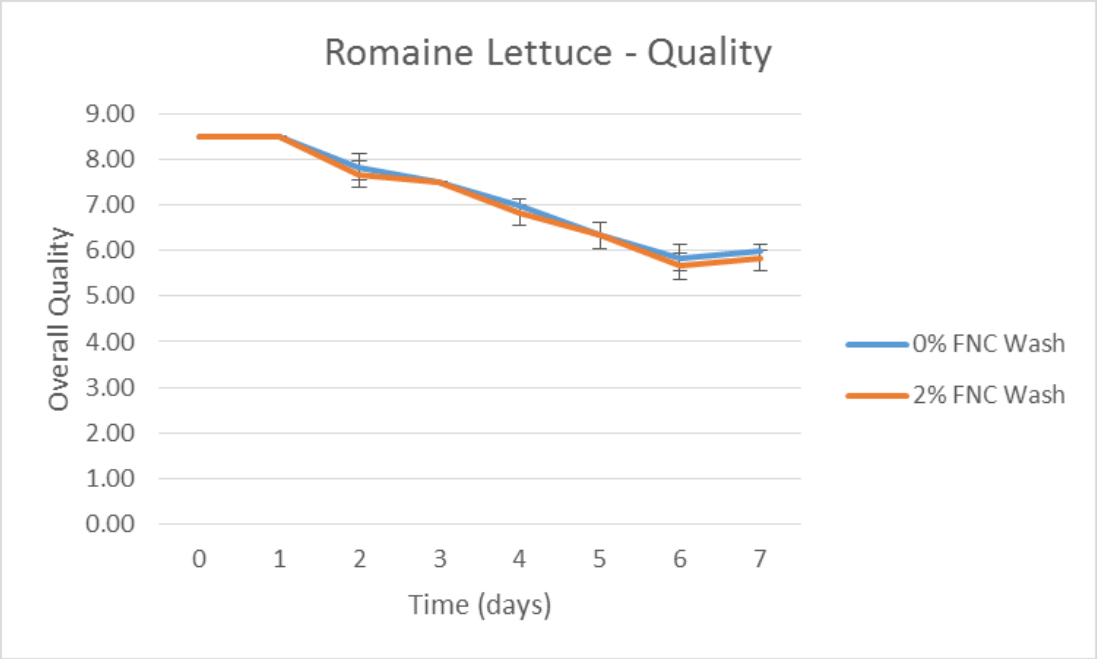


Figure 4-7. Overall appearance results from seven day shelf life study for romaine lettuce stored at ~4°C. No significant difference was observed (P>0.05).

Error bars represent one standard deviation from the mean.

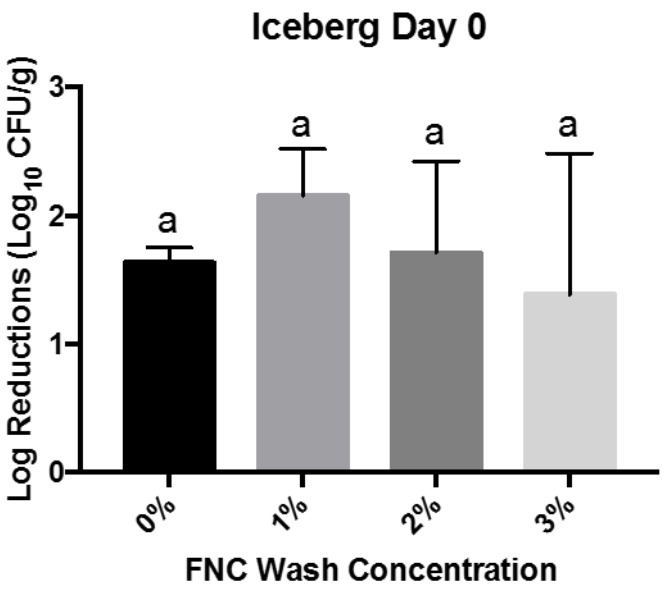


Figure 4-8. Log reduction of total aerobic bacteria compared to unwashed iceberg lettuce on day zero.

Error bars represent one standard deviation from the mean.

[a] indicates wash concentrations did not differ statistically (P=0.5974)

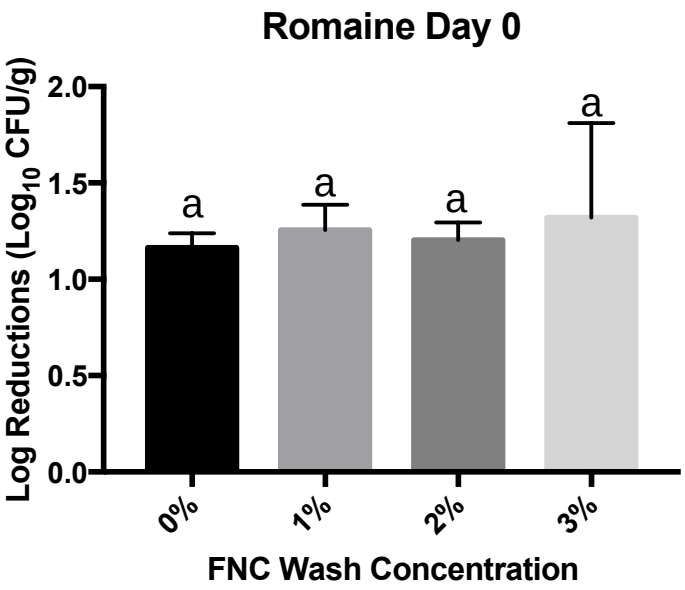


Figure 4-9. Log reductions of total aerobic bacteria compared to unwashed romaine lettuce on day zero.

Error bars represent one standard deviation from the mean.

[a] indicates wash concentrations did not differ statistically (P=0.8917)

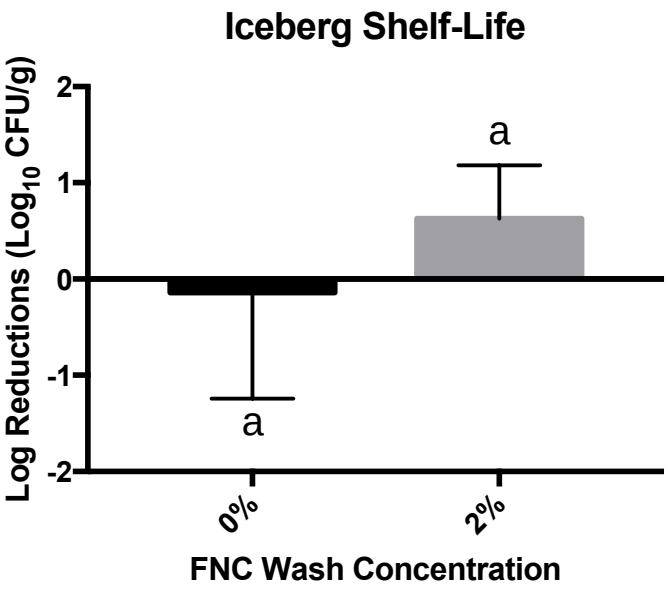


Figure 4-10. Log reductions in total aerobic bacteria on iceberg lettuce achieved by 0% and 2% FNC when compared to unwashed iceberg lettuce on day zero.

Error bars represent one standard deviation from the mean.

[a] indicates wash concentrations did not differ statistically (P=0.0819)

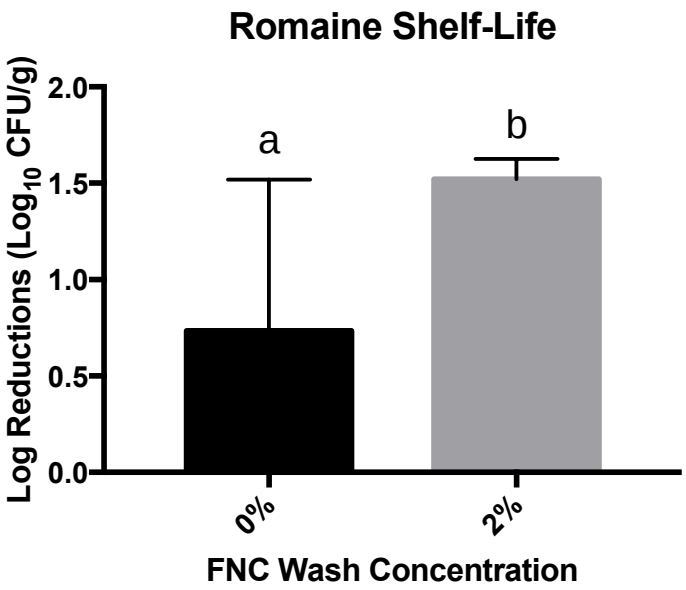


Figure 4-11. Log reductions of total aerobic bacteria population on romaine lettuce achieved by 0% and 2% FNC when compared to the day zero unwashed control sample.

Error bars represent one standard deviation from the mean.

[a, b] indicates FNC wash concentrations that differ statistically (P=0.0203).

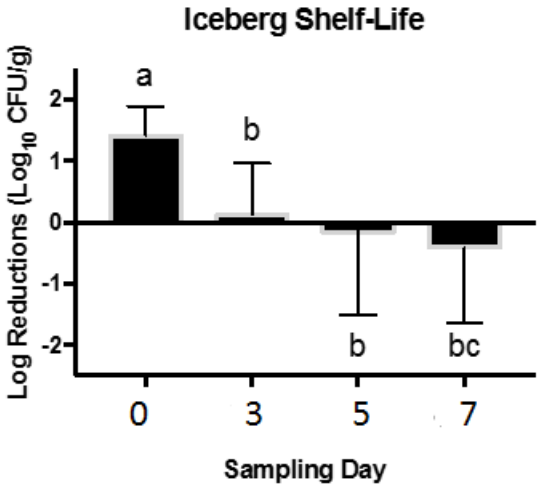


Figure 4-12. Log reductions in total aerobic bacteria on iceberg throughout the shelf life when compared to the day zero unwashed control sample.

Error bars represent one standard deviation from the mean.

[a,b,c] indicates sampling days that differ significantly (P=0.0305)

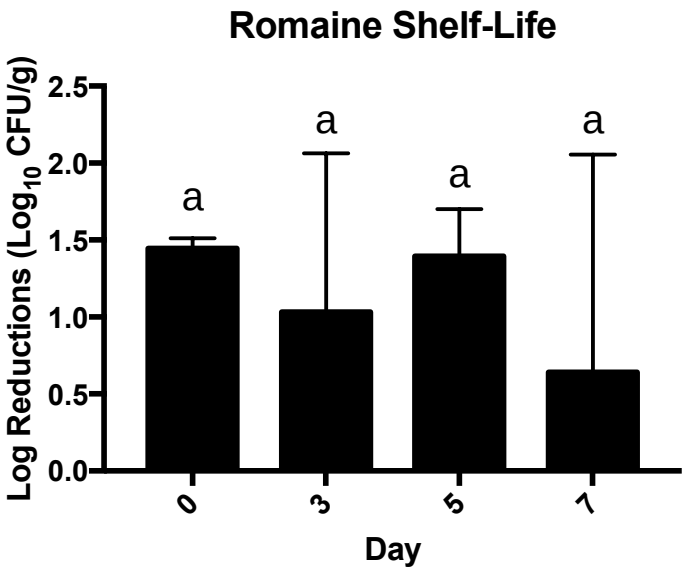


Figure 4-13. Log reductions in total aerobic bacteria on romaine throughout the shelf life when compared to the day zero unwashed control sample.
Error bars represent one standard deviation from the mean.

[a] indicates day did not differ statistically (P=0.2540)

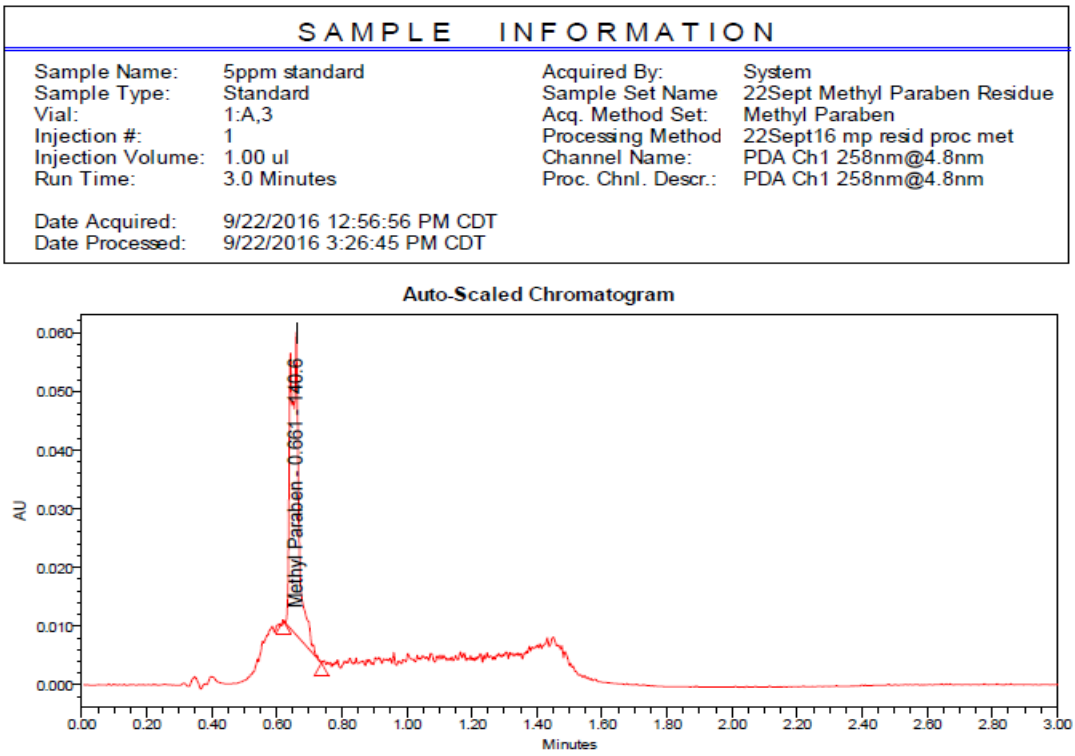


Figure 4-14. Five parts per million methyl paraben standard in an extracted lettuce supernatant.

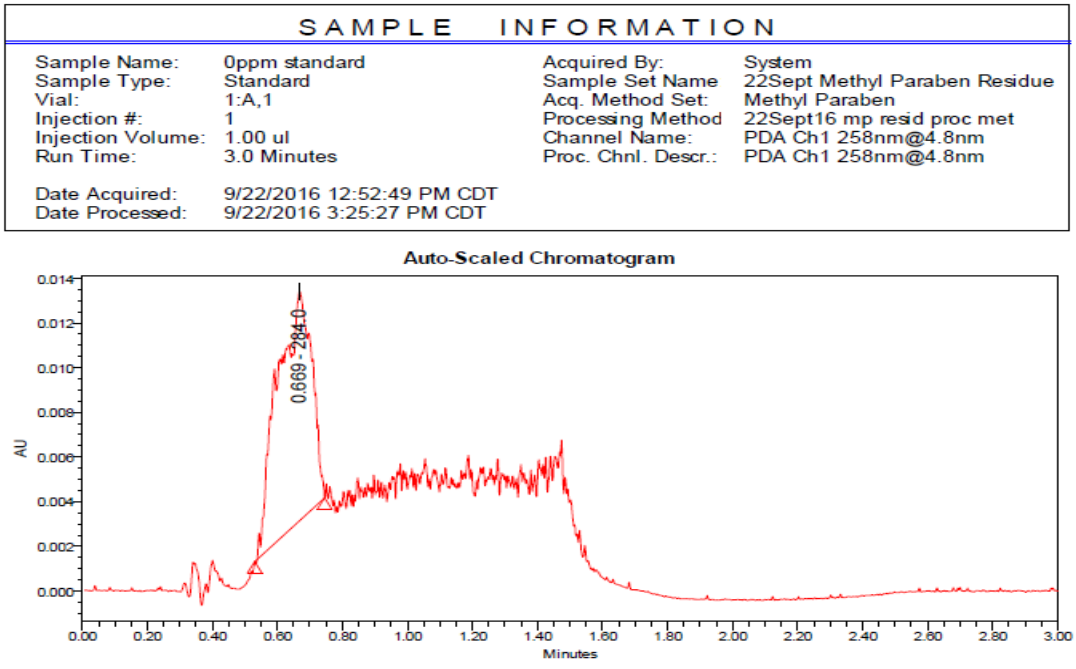


Figure 4-15. Injected extracted lettuce matrix for methyl paraben determination.

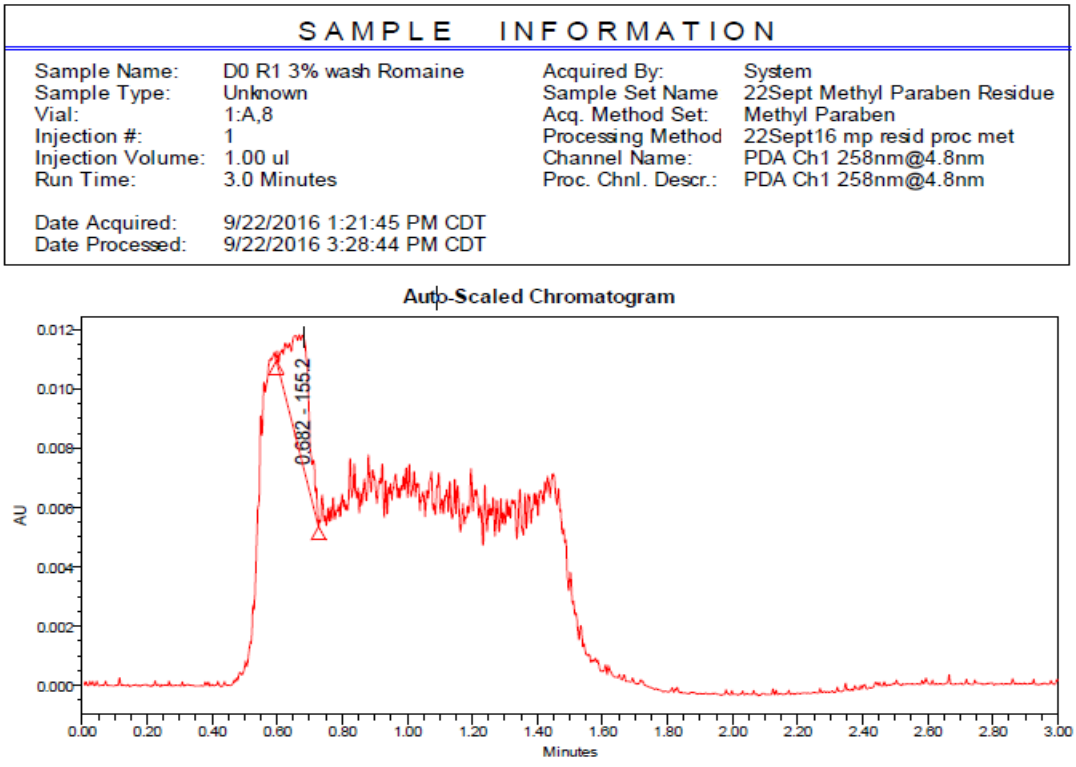


Figure 4-16. An example chromatogram from a methyl paraben extraction. This fresh-cut lettuce extraction had been subjected to a 3% FNC wash.

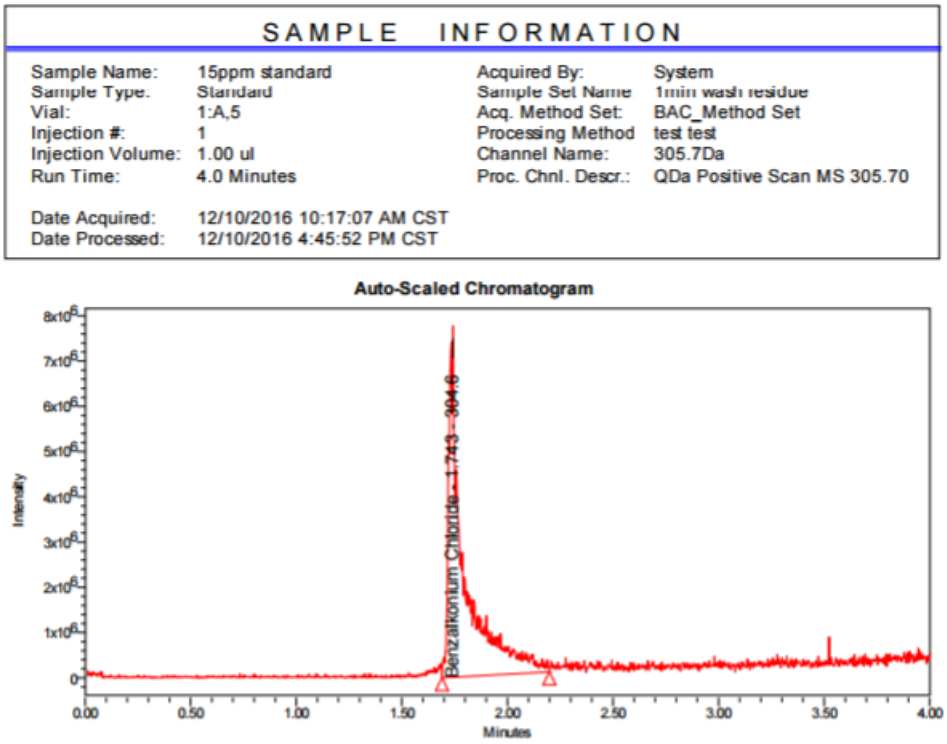


Figure 4-17. Fifteen parts per million benzalkonium chloride standard in extracted lettuce supernatant.

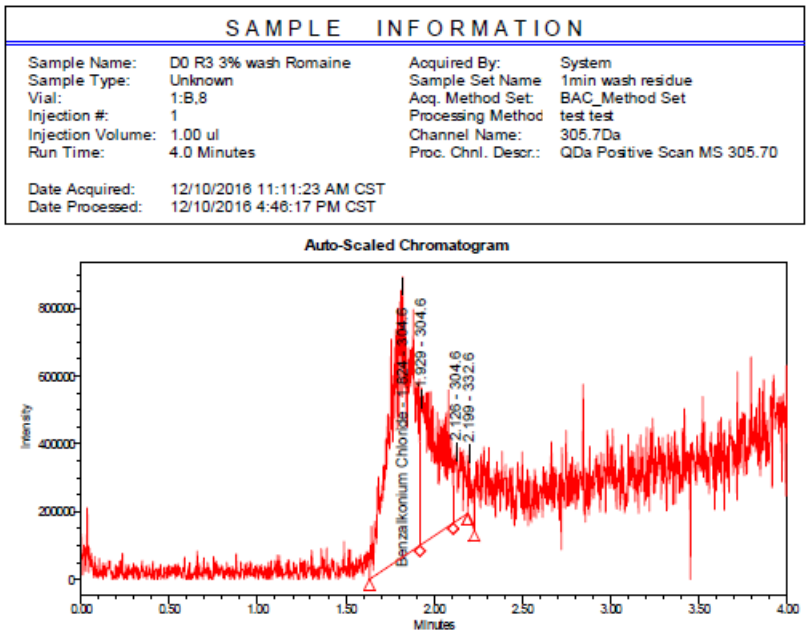


Figure 4-18. An example chromatogram from a benzalkonium chloride extraction. This lettuce extracted had been subjected to a 3% FNC wash.

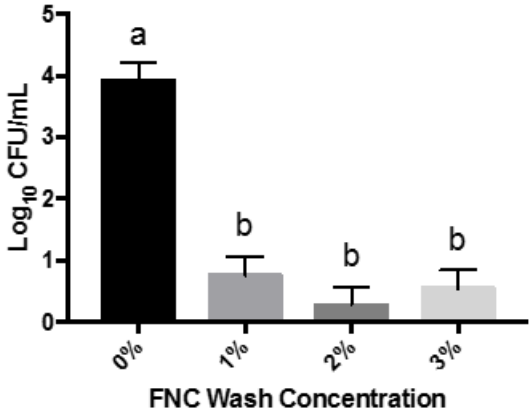


Figure 4-19. *Salmonella* detected in FNC wash water post-washing of inoculated cut lettuce plated on XLD agar.
Error bars represent standard error of the mean.
[ab] indicates concentrations that differ statistically ($P<0.0001$).

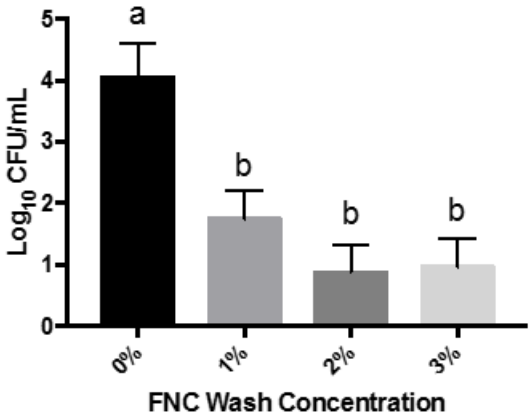


Figure 4-20. *Salmonella* detected in FNC wash water post-washing of inoculated cut lettuce, plated on TSA with an XLD overlay.
Error bars represent standard error of the mean.
[ab] indicates concentrations that differ statistically ($P<0.0001$).

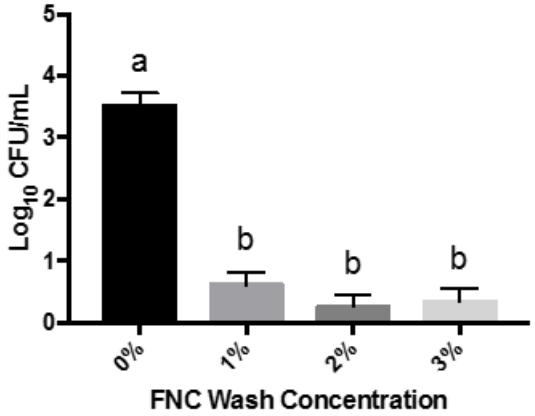


Figure 4-21. *Listeria monocytogenes* detected in FNC wash water post-washing of inoculated cut lettuce, plated on MOX.
Error bars represent standard error of the mean.
[ab] indicates concentrations that differ statistically ($P<0.0001$).

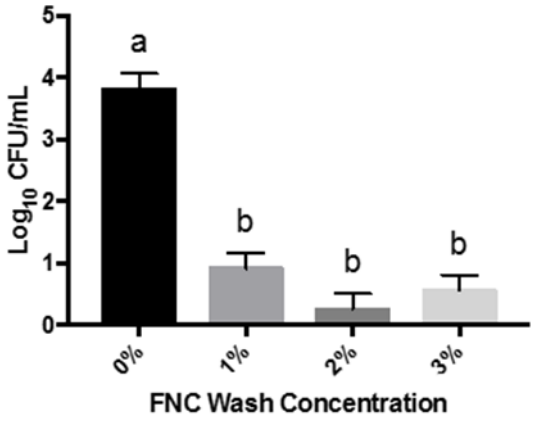


Figure 4-22. *Listeria monocytogenes* detected in FNC wash water post-washing of inoculated cut lettuce, plated on TSA with a MOX overlay.
Error bars represent standard error of the mean.
[ab] indicates concentrations that differ statistically ($P<0.0001$).

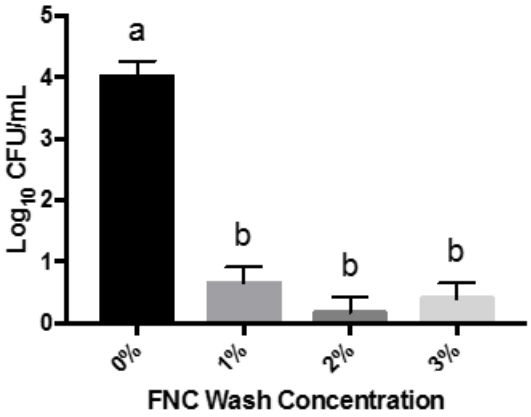


Figure 4-23. *Escherichia coli* O157:H7 detected in FNC wash water post-washing of inoculated cut lettuce, plated on SMAC.
Error bars represent standard error of the mean.
[ab]indicates concentrations that differ statistically ($P<0.0001$).

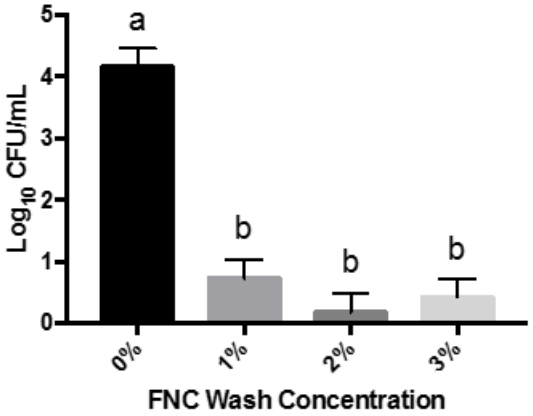


Figure 4-24. *Escherichia coli* O157:H7 detected in FNC wash water post-washing of inoculated cut lettuce plated on TSA with a SMAC overlay.
Error bars represent standard error of the mean.
[a] indicates concentrations that differ statistically ($P<0.0001$).

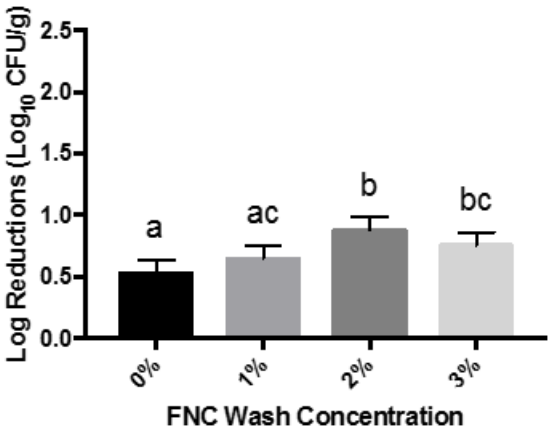


Figure 4-25. *Salmonella* population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with increasing concentrations of FNC solutions. *Salmonella* populations determined by plating on selective XLD agar.
Error bars represent standard error of mean
[abc] indicates concentrations that differ significantly (P=0.0009)

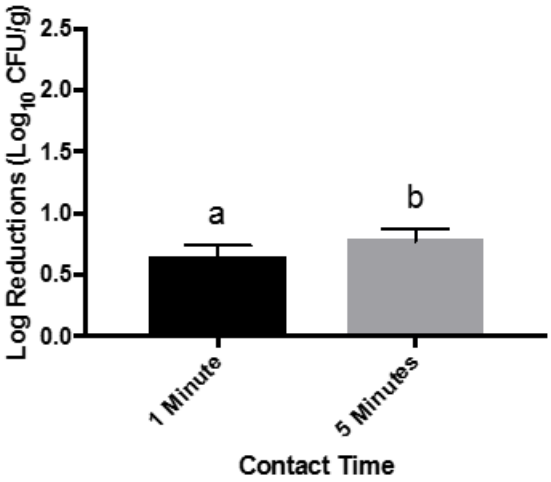


Figure 4-26. *Salmonella* population reductions on inoculated cut romaine lettuce based on FNC contact time, as determined by plating on selective XLD agar.
Error bars represent standard error of mean
[a] indicates a statistical difference (P=0.0127)

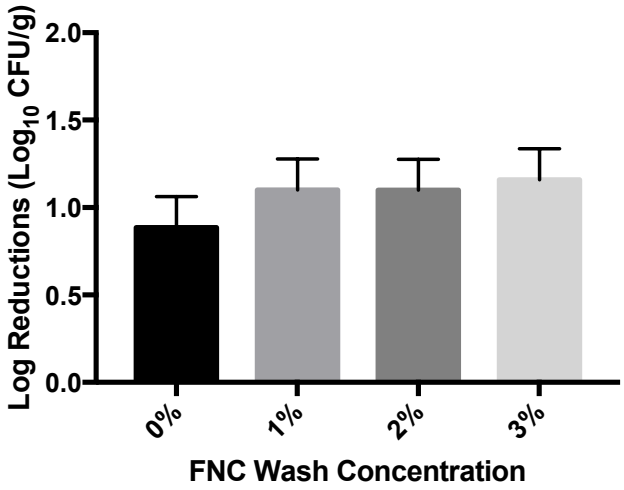


Figure 4-27. *Salmonella* population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying concentrations of FNC solutions. *Salmonella* populations determined by plating on TSA with an XLD overlay.

Error bars represent standard error of mean

Concentrations do not differ statistically (P=0.2601)

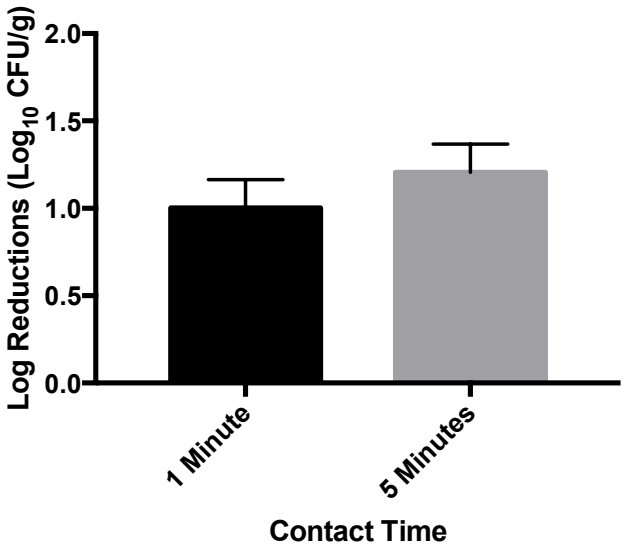


Figure 4-28. *Salmonella* population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Salmonella* populations determined by plating on TSA with an XLD overlay.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.2501)

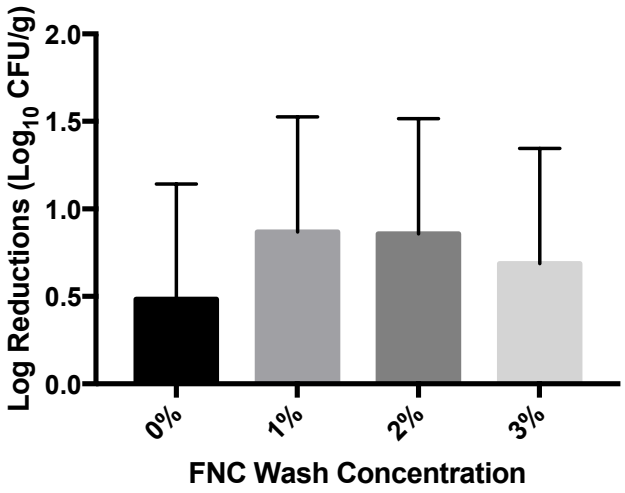


Figure 4-29. *Salmonella* population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying concentrations of FNC solutions. *Salmonella* populations determined by plating on TSA with an XLD overlay.

Error bars represent standard error of mean

Concentrations do not differ statistically (P=0.3851)

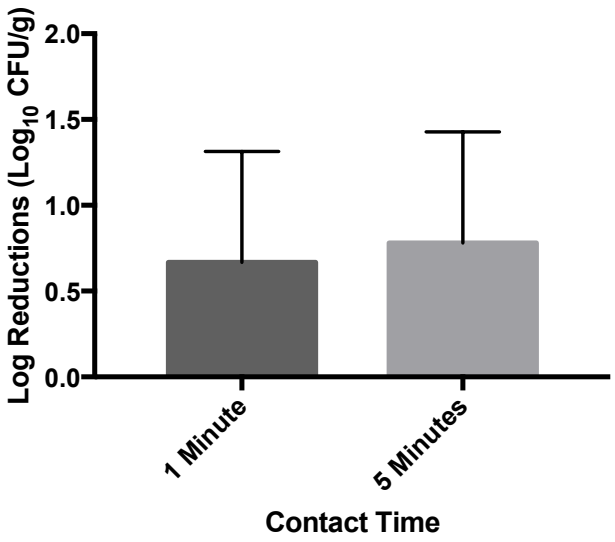


Figure 4-30. *Salmonella* population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Salmonella* populations determined by plating on TSA with an XLD overlay.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.5201)

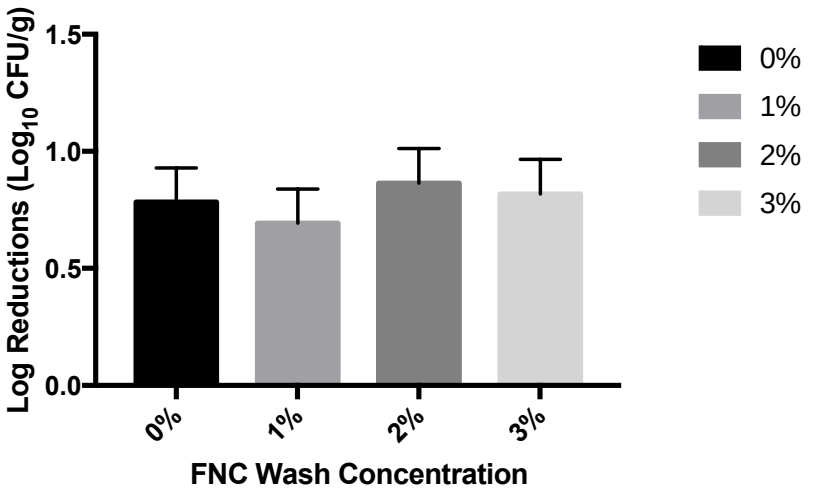


Figure 4-31. *Salmonella* population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying concentrations of FNC solutions. *Salmonella* populations determined by plating on selective XLD agar.

Error bars represent standard error of mean

Concentrations do not differ statistically (P=0.5725)

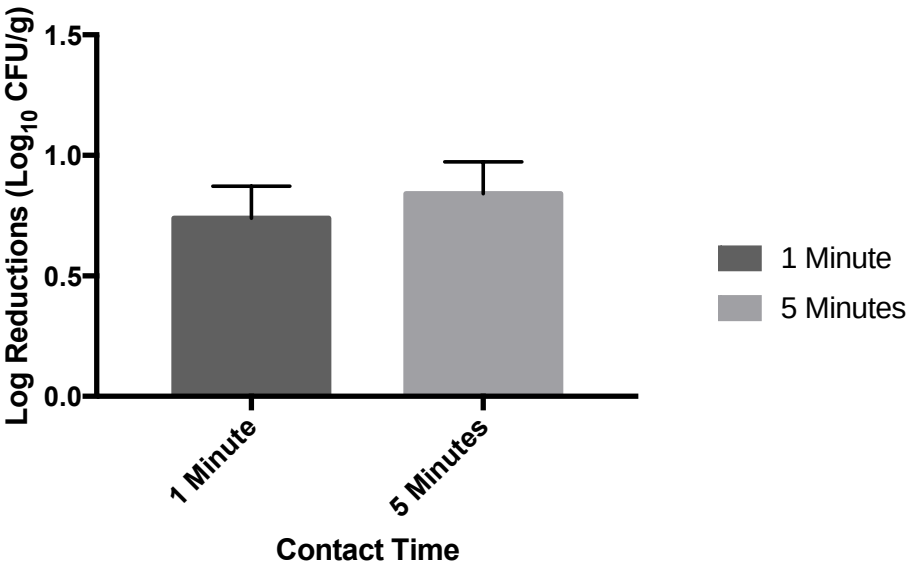


Figure 4-32. *Salmonella* population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Salmonella* populations determined by plating on selective XLD agar.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.2667)

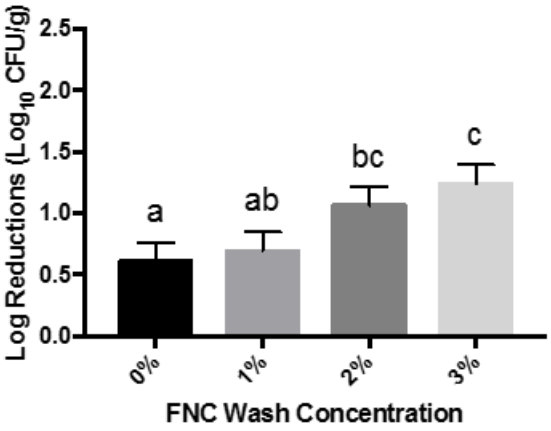


Figure 4-33. *Listeria monocytogenes* population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with increasing concentrations of FNC solutions. *Listeria monocytogenes* populations determined by plating on selective MOX agar.

Error bars represent standard error of mean
[abc] indicates concentrations differ significantly (P=0.0125)

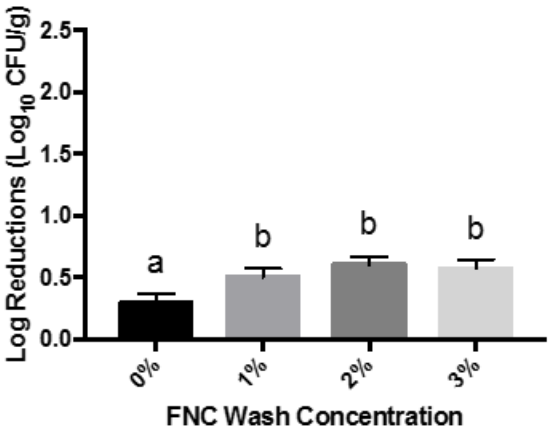


Figure 4-34. *Listeria monocytogenes* population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with increasing concentrations of FNC solutions. *Listeria monocytogenes* populations determined by plating on TSA with a MOX overlay.

Error bars represent standard error of mean
[ab] indicates concentrations differ significantly (P=0.0013)

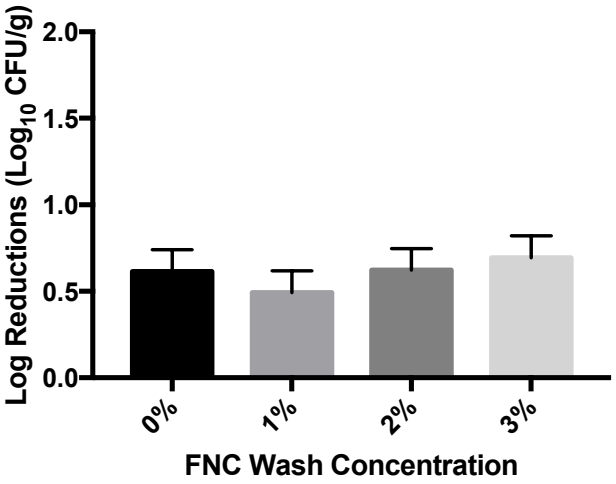


Figure 4-35. *Listeria monocytogenes* population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying concentrations of FNC solutions. *Listeria monocytogenes* populations determined by plating on TSA with a MOX overlay.

Error bars represent standard error of mean

Concentrations do not differ statistically (P=0.1712)

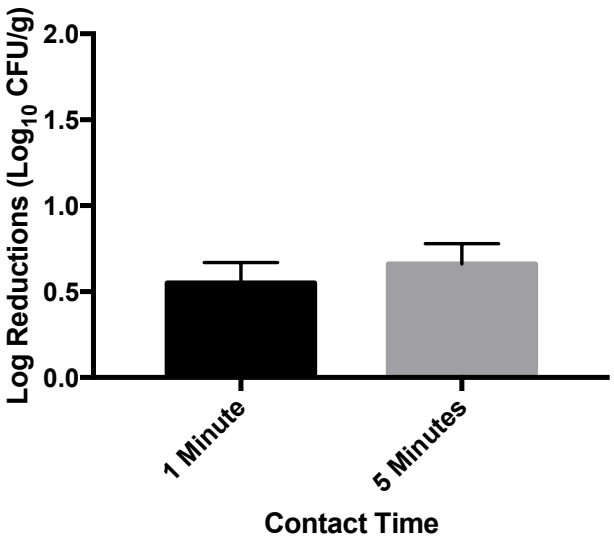


Figure 4-36. *Listeria monocytogenes* population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Listeria monocytogenes* populations determined by plating on TSA with a MOX overlay.

Error bars represent standard error of mean.

Contact times do not differ statistically (P=0.0879)

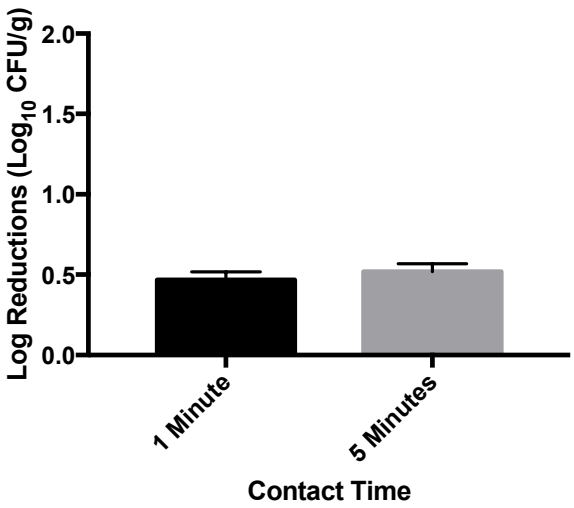


Figure 4-37. *Listeria monocytogenes* population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Listeria monocytogenes* populations determined by plating on TSA with a MOX overlay.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.4656)

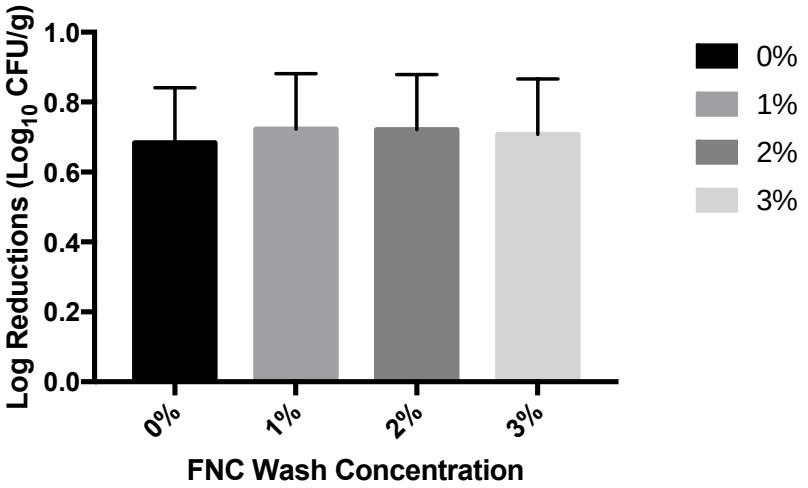


Figure 4-38. *Listeria monocytogenes* population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying concentrations of FNC solutions. *Listeria monocytogenes* populations determined by plating on selective MOX agar.

Error bars represent standard error of mean

Concentrations do not differ statistically (P=0.9937)

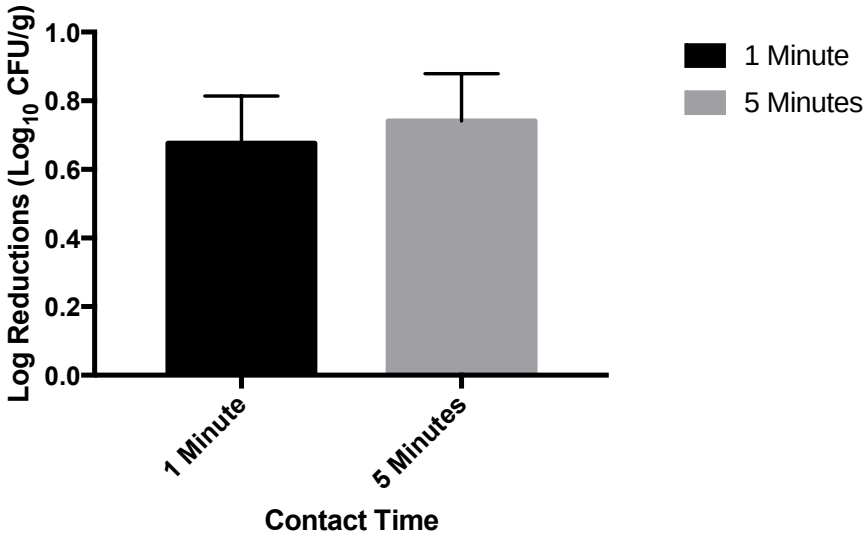


Figure 4-39. *Listeria monocytogenes* population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Listeria monocytogenes* populations determined by plating on selective MOX agar.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.5648)

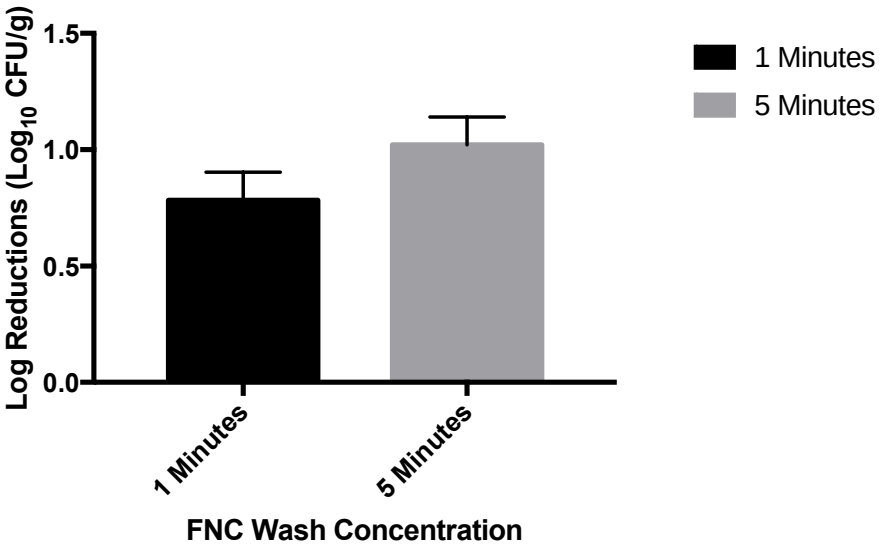


Figure 4-40. *Listeria monocytogenes* population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Listeria monocytogenes* populations determined by plating on selective MOX agar.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.0956)

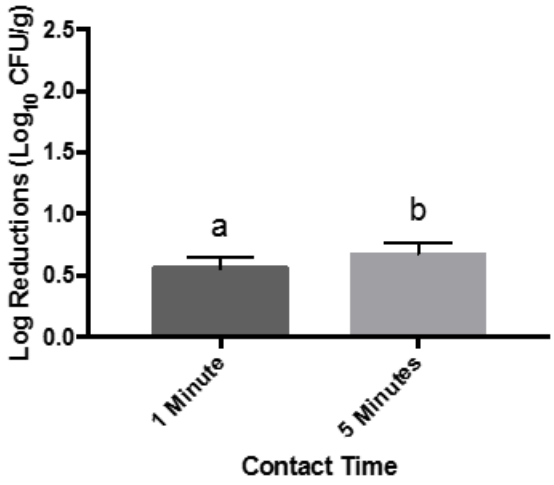


Figure 4-41. *Escherichia* coli O157:H7 population reductions on inoculated cut iceberg lettuce based on FNC contact time, as determined by plating on selective SMAC agar. All FNC concentrations (0-3%) were pooled to get these data.

Error bars represent standard error of mean
[ab] indicates a statistical difference (P=0.0493)

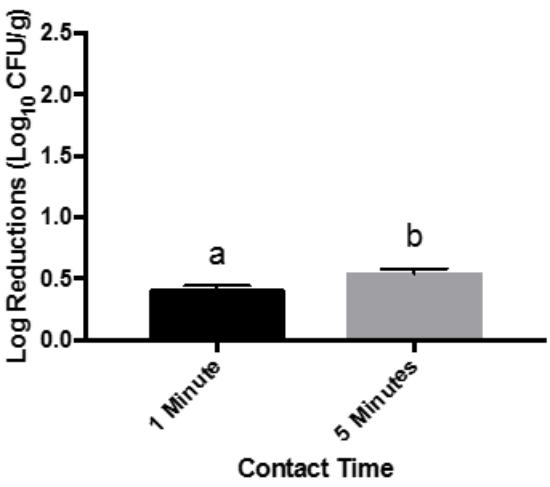


Figure 4-42. *Escherichia coli* O157:H7 population reductions on inoculated cut romaine lettuce based on FNC contact time, as determined by plating on selective SMAC agar. All FNC concentrations (0-3%) were pooled to get these data.

Error bars represent standard error of mean
[ab] indicates a statistical difference (P=0.0293)

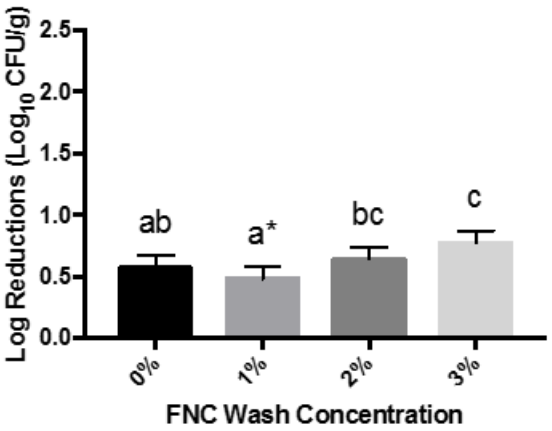


Figure 4-43. *Escherichia coli* O157:H7 population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with increasing concentrations of FNC solutions. *Escherichia* coli O157:H7 populations determined by plating on selective SMAC agar. Error bars represent standard error of mean
[abc] indicates concentrations differ significantly (P=0.0097)

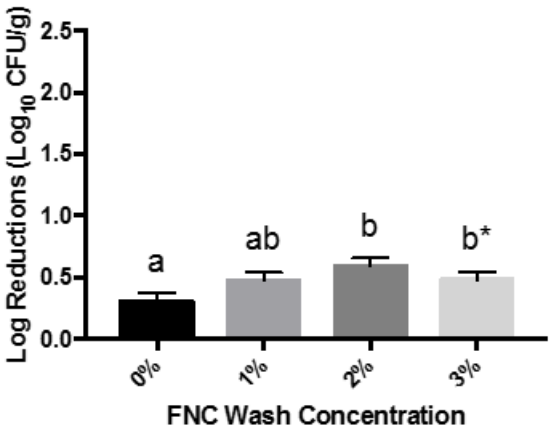


Figure 4-44. *Escherichia coli* O157:H7 population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with increasing concentrations of FNC solutions. *Escherichia* coli O157:H7 populations determined by plating on selective SMAC agar.
Error bars represent standard error of mean
[ab] indicates concentrations differ significantly (P=0.0257**)**

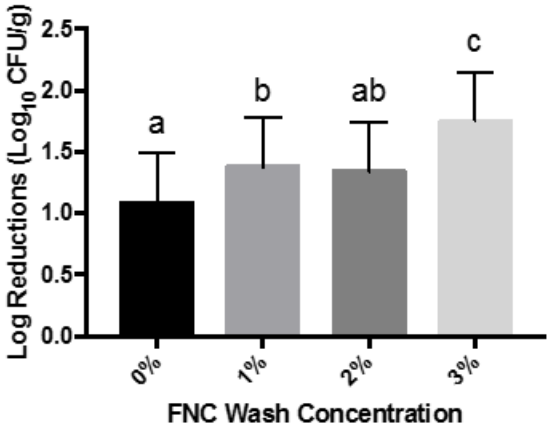


Figure 4-45. *Escherichia* coli O157:H7 population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with increasing concentrations of FNC solutions. *Escherichia* coli O157:H7 populations determined by plating on TSA with a SMAC overlay.
Error bars represent standard error of mean
[abc] indicates concentrations differ significantly (P=0.0013)

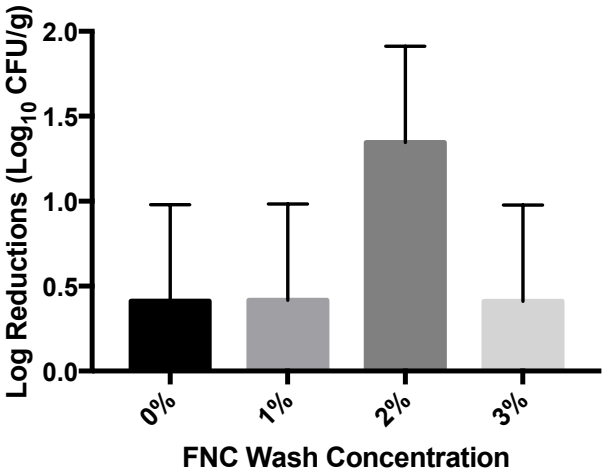


Figure 4-46. *Escherichia coli* O157:H7 population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying concentrations of FNC solutions. *Escherichia coli* O157:H7 populations determined by plating on TSA with a SMAC overlay.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.5792)

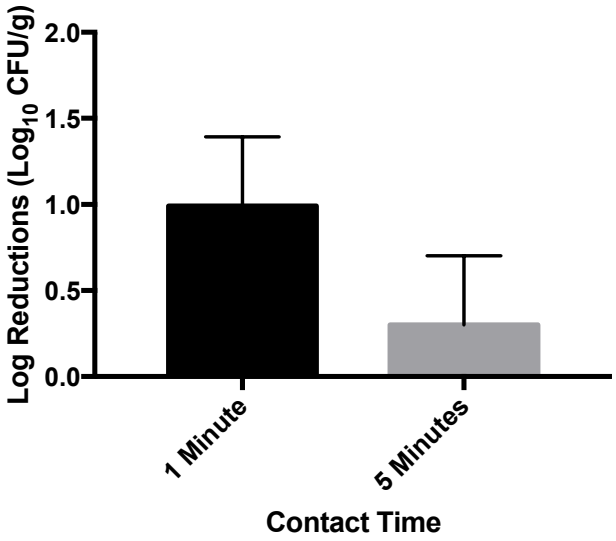


Figure 4-47. *Escherichia coli* O157:H7 population reductions achieved on inoculated cut iceberg lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Escherichia coli* O157:H7 populations determined by plating on TSA with a SMAC overlay.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.2409)

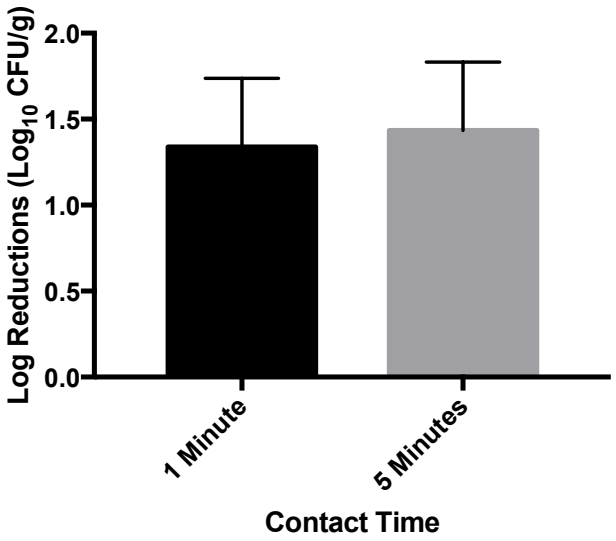


Figure 4-48. *Escherichia coli* O157:H7 population reductions achieved on inoculated cut romaine lettuce (inoculated at ~7.0 $\log_{10}$ CFU/g) after washing with varying contact times. *Escherichia coli* O157:H7 populations determined by plating on TSA with a SMAC overlay.

Error bars represent standard error of mean

Contact times do not differ statistically (P=0.3383)

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Chapter 5 – Summary and Conclusions

Antimicrobials were applied both pre- and postharvest in order to investigate novel approaches to reducing microbial populations on romaine and iceberg lettuce. While controlling microbial populations is important, a number of considerations are critical for assessing intervention efficacy, including product quality and shelf life. Therefore, microbiological and post-treatment quality assessments were performed in Objectives I and II. Applying an antimicrobial pre-harvest to lettuce is a somewhat novel approach to controlling microbial populations on lettuce and potassium bisulfate in particular had not been investigated for this purpose. Therefore, Objective I was designed as a preliminary study to evaluate efficacy of this compound as a novel pre-harvest intervention.

Objective I evaluated the efficacy of a 0.25% solution of potassium bisulfate at reducing previously inoculated populations of either nonpathogenic *E. coli* or *L. innocua* on lettuce pre-harvest. The impact of treatment on overall quality of the lettuce products was also investigated. Reductions in surrogate microorganism populations were compared to an inoculated, untreated control as well as inoculated lettuce treated with water. Comparing the mean populations of *E. coli* at harvest showed that the only significant reduction of *E. coli* populations occurred when potassium bisulfate was applied one week before harvest. Interestingly, when potassium bisulfate was applied one week and two days before harvest, *E. coli* populations were statistically similar to that of the untreated, inoculated control. This indicates that the additional potassium bisulfate application two days before harvest may have negatively impacted the ability of potassium bisulfate to at reduce previous inoculated populations of *E. coli* on lettuce. It is possible surviving *E. coli* populations either became more acid tolerant or became less susceptible to potassium bisulfate prior to the application of the second treatment. However, it is also possible

this observed phenomenon is random and due to variations in attachment. *Listeria innocua* data showed a statistically significant decrease in populations when 0.25% potassium bisulfate was applied both one week and two days before harvest, which suggests that multiple applications impacts efficacy. Results from the post-treatment overall quality analysis revealed brown speckling on various leaves of lettuce subjected to potassium bisulfate following a single application. No significant difference in overall quality was detected, which presumably was because brown speckling was not on every leaf, and the defects were small when present. However, efforts to reduce or eliminate this leaf burning should be emphasized in the future, particularly if potassium bisulfate will be applied more than once pre-harvest.

Data from this objective indicates potential use of potassium bisulfate as a pre-harvest antimicrobial to control either foodborne pathogens like *Escherichia coli* O157:H7 or, potentially, *L. monocytogenes*. It should be noted that surrogate microorganisms were used in this study; therefore, all data presented can only provide a general indication as to how *Escherichia coli* O157:H7 and *L. monocytogenes* may behave under similar conditions. Unfortunately, no single treatment was able to control both foodborne pathogen surrogates making optimization a likely necessity. For example, including a surfactant with the treatment, varying the spray application, or combining with another antimicrobial may improve the success already shown. The addition of a surfactant may also reduce treatment burning, as it would more evenly spread the solution across the hydrophobic leaf cuticle upon application. As potassium bisulfate was used in favor of the more researched antimicrobial sodium bisulfate (due to potassium being a key plant nutrient), further studies may be warranted to determine if application of potassium bisulfate applied more than seven days before harvest would impact plant health.

Objective II was a two-part study evaluating the use of benzalkonium chloride, methyl paraben, and acetic acid as a postharvest lettuce wash. As with Objective I, lettuce was evaluated to elucidate the impact of this wash on microbial populations and product quality. The first part of the study evaluated the efficacy of this postharvest wash at reducing previously inoculated populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in the wash water and on the tissue of lettuce (fresh-cut romaine and iceberg). Data suggest that the blend of benzalkonium chloride, methyl paraben, and acetic acid significantly reduced populations of each pathogen by 2-3 $\log_{10}$ CFU/mL (99-99.9% reduction) in the wash water. Such a reduction in pathogens within five minutes surpasses the industry standard of 150 ppm chlorine, which has been shown in previous research to reduce the same foodborne pathogens in wash water by ~97.8% (nearly 2 logs) during a 3 minute contact time. This would make a blend of benzalkonium chloride, methyl paraben, and acetic acid a viable alternative to chlorine-based sanitation.

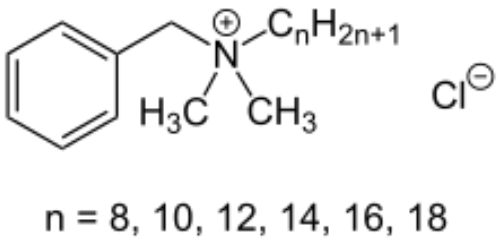
The ability of this postharvest wash to reduce *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in lettuce tissue revealed results similar to other postharvest washes. In general, statistical differences were most evident at the highest antimicrobial concentration evaluated, particularly when plated on selective media. However, no single concentration significantly reduced pathogen populations on both iceberg and romaine lettuce. When considering the more conservative injury recovery medium, pathogen reductions were less than 0.5 $\log_{10}$ CFU/g greater than the water control. Therefore, washing with even the highest FNC concentration provided little to no added benefit. In general, there was little to no added benefit to washing with any of the FNC concentrations for five minutes rather than 1 minute

The second part of Objective II evaluated the shelf life of lettuce and residues of benzalkonium chloride and methyl paraben post-washing. This was accomplished by sampling

random lettuce samples immediately after washing and subjecting them to a previously developed method for extracting benzalkonium chloride from lettuce. The results in this study demonstrate that concentrations of methyl paraben and benzalkonium chloride on iceberg and romaine lettuce post-washing were below the limit of detection (<5 ppm for methyl paraben and <10 ppm for benzalkonium chloride). No difference in shelf life was observed for romaine and iceberg lettuce subjected to 0% and 2% FNC washes. On day zero, total aerobic populations on romaine and iceberg lettuce were significantly reduced by 0%, 1%, 2%, and 3% FNC washes in comparison to an unwashed control sample. With exception of romaine, the 2% wash did not significantly improve reductions in total aerobic populations in comparison to the 0% water wash during the shelf life. In general, 0% and 2% FNC washes inconsistently reduced populations throughout the shelf life of iceberg and romaine lettuce. This suggests a preservative effect is lacking, which would mean that additional labeling in a retail setting would not be required if lettuce was subjected to the antimicrobial blend of benzalkonium chloride, methyl paraben, and acetic acid.

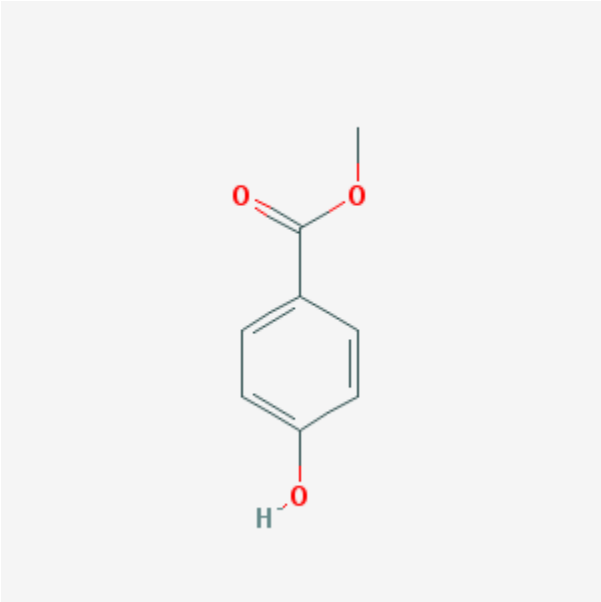
APPENDIX A

MOLECULAR STRUCTURES



Benzalkonium chloride molecule courtesy of: http://www.sigmaaldrich.com/catalog/product/aldrich/234427?lang=en&region=US

Figure A-1. An example benzalkonium chloride molecule



Methyl Paraben molecule courtesy of: https://pubchem.ncbi.nlm.nih.gov/compound/Methyl_4-hydroxybenzoate#section=Top

Figure A-2. An example methyl paraben molecule

APPENDIX B

QUALITY SCALES

Romaine Lettuce Quality Rating Scale

Rating	Definition
9	Fresh appearance with bright green color; crisp texture; no discoloration or decay.
7	Slight loss of green color; no russeting; slight water loss not objectionable; slight butt-end discoloration.
5	Noticeable loss of green color; noticeable water loss on outer leaves; moderate discoloration of butt-end.
3	More yellow than green; outer leaves limp and need trimming; severe discoloration; decay may be noticeable. May have bitter flavor.
1	Yellow. Severe wilting and extreme discoloration; soft rot or fungal mycelium on outer leaves.

Jeff Brecht

Figure B-1. Quality scale used to evaluate romaine lettuce (*Brecht, 2012*)

Iceberg Quality Scale

Karlruher Schema (Rating description for sensorical check on edible materials)

	Appearance		*Odour/Smell*	*Taste*	*Texture/Consistency*
	Colour	*Shape*			
9- Excellent	Exceptionally appealing, bright, natural colour, typical for the sample.	Perfect preservation of shape, firm, undamaged shape.	Exceptional delicate, distinct, characteristic odour.	Exceptional delicate, distinct, characteristic taste.	Exceptional good, characteristic texture, eg. firm, succulent tissue, very tender.
8- Very good	Bright natural colour, typical for the sample, single slightly discoloured specimen allowed.	Very well preserved shape, single slightly changed specimen.	Full, strong, characteristic odour.	Full strong, characteristic taste	Very good, characteristic texture, eg. firm tissue, tender
7- Good	Natural colours, typical for sample, a little too pale or too dark, a few slightly discoloured specimen allowed.	Well preserved shape, a few slightly or single strongly changed specimen.	Good characteristic odour.	Good characteristic taste.	Good characteristic texture, eg. still tender.
6- Satisfactory	Slight reduction of typical colour image, eg. irregular light or dark.	Still good preserved shape, a few slightly or occasional strongly damaged specimen.	Normal, slightly reduced odour, eg. slightly flat, not rounded.	Normal, slightly reduced taste, eg. slightly flat, not rounded.	Normal, slightly reduced texture, slight deviation e.g. largly still tender.
5- Mediocre	Impairment of typical colour, eg. slightly bleached, unbalanced.	Impairment of natural shape, eg. slight loss of shape, shrunken.	Impairment in natural odour, still acceptable, eg. quite flat, tangy, perfumed, slightly haylike.	Impairment in natural taste, still acceptable, eg. quite flat, tangy, slightly perfumed, slightly haylike.	Impairment of texture, still acceptable, eg. partly irregular, slightly too soft or too hard.
4- Borderline	Discoloration of sample surface, eg. with stripes of other colour nuances, not yet unpleasant.	General loss of shape, shrunken, still not unpleasant.	Clearly reduced odour, eg. stale, perfumed, haylike, slightly musty.	Clearly reduced taste, eg. stale, perfumed, haylike, slightly musty.	Clearly reduced texture, eg. irregular, too soft, too hard, too watery.
3- Poor	Strong discolouration of sample, eg. blue or greyish.	Generally severe loss of shape, shrunken.	Altered odour, eg. completely stale, slightly rancid or fermented, no longer appealing.	Altered taste, eg. completely stale, slightly rancid or fermented, no longer appealing	Altered texture, eg. very irregular, too soft, too hard, slightly leatherlike, slightly tough.
2- Bad	Complete discolouration of sample surface, original colour no longer perceptible.	Severely changed shape. Still not repulsive, advanced disintegration.	Unpleasantly changed odour, still not repulsive, eg. rancid, fishy or strongly haylike.	Unpleasantly changed taste, still not repulsive, eg. rancid, fishy or strongly haylike.	Unpleasantly changed texture, fully paste-like, extremely hard.
1- Very bad	Complete discolouration of sample surface towards repulsive colours.	Shape of sample has fully disintegrated.	Repulsive, strange odour, eg. foul, fermented, spoiled.	Repulsive, strange taste, eg. foul, fermented, spoiled.	Repulsive texture.

Annex 3 Karlruher scheme (Rating description for sensorial check on edible materials)

Figure B-2. Quality scale used to evaluate iceberg lettuce. (*Paulus et al., 1969*).

APPENDIX C

OBJECTIVE II: THEORETICAL VS ACTUAL WASH WATER CONCNETRATIONS

Table C-1. Concentrations of benzalkonium chloride in solutions made in various volumes from 100% FNC made in one gallon. To values for each volume/concentration combination show same solution injected twice.

	Made from 1 gallon stock FNC								
Volume of solution made	1%		Mean	2%		Mean	3%		Mean
100mL	44.81	46.65	45.73	87.28	92.98	90.13	151.10	155.05	153.08
250mL	30.23	32.07	31.15	90.366	92.63	91.50	153.57	148.8	151.19
1000mL	43.18	43.9	43.54	96.68	97.87	97.28	156.75	158.81	157.78
2000mL	44.16	46.47	45.32	86.66	91.52	89.09	158.24	158.60	158.42

Table C-2. Concentrations of benzalkonium chloride in solutions made in various volumes from 100% FNC made in 250 mL.

	Made from 250mL stock FNC (made 31Aug16)								
Volume of solution made	1%		Mean	2%		Mean	3%		Mean
100mL	23.19	24.37	23.78	51.71	52.12	51.92	80.80	80.17	80.49
250mL	21.56	22.02	21.79	47.72	50.59	49.16	77.55	77.93	77.74
1000mL	23.28	22.62	22.95	44.07	45.41	44.74	76.22	75.51	75.87
2000mL	18.43	19.89	19.16	48.57	48.56	48.57	85.46	87.11	86.29

APPENDIX D

ADDITIONAL OBJECTIVE II FIGURES AND PICTURES

Figure D-1. Day three shelf life study picture of iceberg lettuce washed in 2% FNC.

Figure D-2. By day five, pinking became noticeable for all iceberg lettuce specimens. This negatively impacted the appearance score.

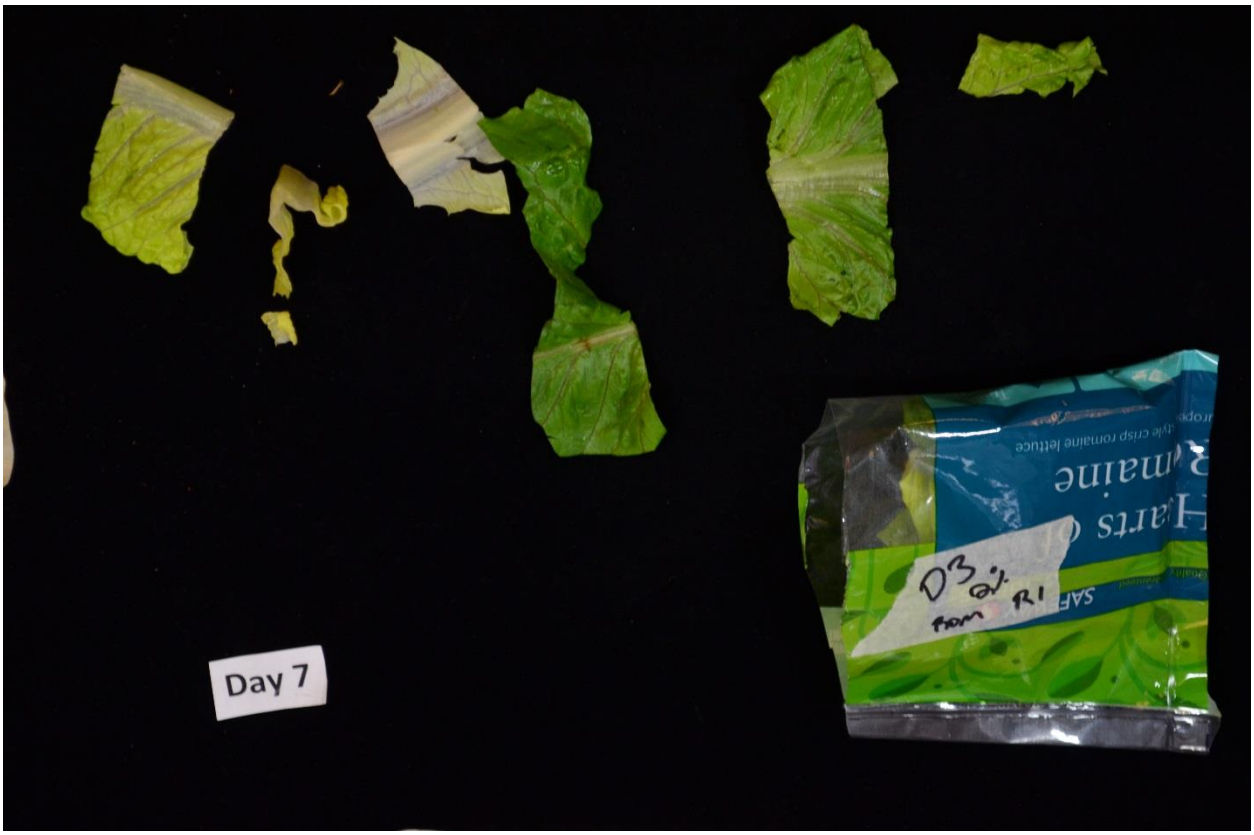


Figure D-3. By day seven, romaine lettuce washed in 2% FNC still had high enough salable quality.

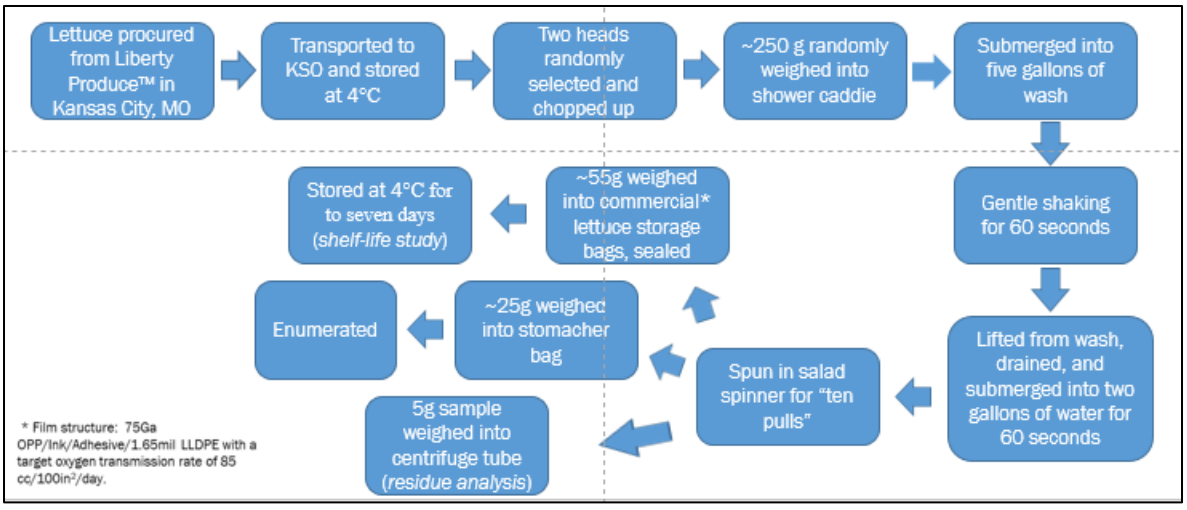


Figure D-4. The step-by-step process of washing fresh-cut romaine and iceberg lettuce in FNC.