EVALUATION OF OZONE, ELECTROLYZED WATER, AND BACTERIOPHAGE AS ANTIMICROBIAL INTERVENTIONS FOR RAW BEEF.

By

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Bachelor of Science in Biological Sciences

Oklahoma State University

Stillwater, OK

2011

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2013

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ACKNOWLEDGEMENTS

Members of the Committee, thank you for your willingness to give of your time and your talent to be a part of this committee. It takes a village…

Dr. Peter M. Muriana- my advisor throughout the past 2 years at OSU. Thank you for making sure there is a program that allows individuals to bring themselves to their own growth and development. Your support and understanding in the final leg of this race enabled me to cross the finish line. I want to be a developer just like you when I grow up. Without your encouragement I never would have started this journey. Without your guidance, support, and investment in me, I never would have finished.

To my colleagues and those that eventually became my friends, thank you for all of your support. I couldn't have completed this program without you.

Name: CHRISTIAN S. MONTALVO

Date of Degree: JULY, 2013

Title of Study: EVALUATION OF OZONE, ELECTROLYZED WATER, AND BACTERIOPHAGE AS ANTIMICROBIAL INTERVENTIONS FOR RAW BEEF.

Major Field: FOOD SCIENCE

Escherichia coli O157:H7 and *Salmonella* are frontrunners in many foodborne outbreaks, specifically those involving raw beef. Usually an easy way to prevent illness from occurring is essentially by cooking foods to the proper temperature, which would kill any lingering pathogens. Unfortunately a lot of processing methods are starting to use applications such as needle tenderization, maceration, and even brine injections to produce increasingly palatable products. This allows any bacterial or potential pathogens that would just be found on the outer pieces of the meat to be relocated into the sterile inner tissues (i.e., 'translocated'), which presents a serious issue when a consumer requests a cut of meat not to be cooked all the way through. The USDA-FSIS refers to such products as 'non-intact' (U.S. Department of Agriculture, 1999). Due to the increase of foodborne illnesses there has been much research towards innovative antimicrobials and disinfection methods for foods and surfaces in processing areas (Guentzel et al., 2008). The objective of these studies was to measure the effectiveness of three popular antimicrobial solutions (ozone, electrolyzed water, and bacteriophage) as potential interventions against *E. coli* O157:H7 and/or *Salmonella* spp. on carcasses and meat surfaces that would be subsequently subjected to blade tenderization. Our research suggests that both ozone and electrolyzed water will not serve as an effective antimicrobial if directly applied to beef, whether for carcass application or non-intact beef cuts. This is because the studies we performed indicate that both ozone and electrolyzed water are rendered ineffective by organic material (i.e. beef). Our research also proposes that although bacteriophage demonstrated modest reductions in our trials, they may not be widely applicable due to the difference of sensitivity to various strains.

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CHAPTER I

INTRODUCTION

Food safety has become a continuing concern around the globe, affecting even many first world countries (Mahmoud, 2007). Foodborne pathogens cost the United States economy 6.5 billion dollars per year (Pimentel et al., 2001) and it is estimated that these same pathogens lead to more than 9 million infections and nearly 9,000 deaths annually (CDC, 2013; Mead et al., 1999). Food processors, food safety researchers, and regulatory agencies have responded to this challenge by increasing their efforts towards preventing food and facility contamination by pathogens such as *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* (Murphy et al., 2004). With the greatest risk of foodborne infection coming from minimally or unprocessed fresh foods (Cheigh et al., 2011), a lot of attention is focused on raw meat.

Escherichia coli O157:H7 is the frontrunner in many foodborne outbreaks, specifically in raw beef. Over a 6-year time span (2002-2008), 11,921 samples of meat were collected and tested for *Escherichia coli*, an indicator of fecal contamination. The samples consisted of chicken breast, ground turkey, ground beef, and pork chops and were gathered from four different states. Of the samples tested during this period, 69.5% were positive for *E. coli*. In 2007 and 2008 a

large number of recalls occurred in the beef industry due to *E. coli* O157:H7 contamination which is considered an 'adulterant' by USDA-FSIS on beef carcasses or raw ground beef. This greatly affects not only consumer health but also results in a financial struggle for the industry as it tries to implement various programs designed to reduce the presence of pathogens on raw meats (Laster et al., 2012).

Salmonella is another pathogen that has caused great stress to the food processing industry (i.e., all *Salmonella* spp./serotypes are considered human pathogens). Around 40,000 cases of non-typhoid salmonellosis are reported annually to the CDC (Guenther et al., 2012). *Salmonella* has the ability to flourish and produce outbreaks in a variety of food items including beef and chicken products, eggs, and even seafood. As with *Escherichia coli*, *Salmonella* has the ability to cause socioeconomic stress due to illness, medical costs, loss of worker productivity, disability, mortality, and financial strain from recalls and litigation (Echeverry et al., 2010).

These two pathogens are highly capable of causing many foodborne infections due to the consumption of contaminated food products such as meats (Echeverry et al., 2010). These illnesses can lead to long-term health effects such as hemolytic uremic syndrome (caused by *E. coli* O157:H7) or even death. An easy way to prevent illness from occurring is essentially by cooking foods to the proper temperature, which would kill any lingering pathogens. Any residual bacteria on the exterior portions of the meat is eliminated during the cooking process regardless of the internal temperature requested by consumers. This holds true as long as the meat were intact. Even though many consumers prefer meats that are cooked at temperatures such as 'rare' or 'medium', a lot of processing methods are starting to use applications such as needle tenderization, maceration, and even brine injections to produce increasingly palatable products. This allows any bacterial or potential pathogens that would be found on the outer pieces of the meat to be translocated into the sterile inner tissues, which presents a serious issue when a consumer requests a cut of meat not to be cooked all the way through. The USDA-FSIS refers to

such products as 'non-intact' (U.S. Department of Agriculture, 1999). Mechanically-tenderized beef, needle-injected (i.e., enhanced), and vacuum marinated beef are considered non-intact. Over the last decade, many foodborne outbreaks have been associated with mechanically tenderized meats (Echeverry et al., 2010). Recently, USDA-FSIS has proposed new labeling requirements of meats that have been mechanically tenderized (U. S. Department of Agriculture, 2013).

Due to the increase of foodborne illnesses, research aimed towards innovative antimicrobials and disinfection methods for foods and surfaces in processing areas has increased (Guentzel et al., 2008). The development of antimicrobial interventions to prevent the prevalence of foodborne pathogens has been encouraged by the USDA (Yoder et al., 2012). Although the much of the focus in meat processing has been to control contamination from hide to carcass, additional attention was also needed for steps before and after fabrication (Pittman et al., 2012). Specific organic acids, chlorinated compounds, hot water, and steam have received GRAS (Generally Recognized As Safe) approval and have been used by meat processing industries for years. Although accepted and approved by FDA/USDA, many chemical sanitizers may damage the quality of foods, leach nutrients, and cause detrimental effects to processing equipment in addition to negative effects of the overall environment. There is a continuous need for novel methods and solutions to reduce and abolish bacteria that may cause foodborne illness.

An oxidative antimicrobial allowed for use on foods is aqueous ozone solutions (Trinidade et al., 2012). Ozone (O_3) is produced by first splitting O_2 with high voltage electrical energy. The then two separate O- radical molecules join remaining unbroken O_2 molecules resulting in the formation of O_3 (Khadre et al., 2001). Ozone has proven to possess the ability to inactivate a large number of microorganisms such as bacteria, fungi, yeast, parasites, and viruses (Chawla et al., 2008). When ozone comes into contact with bacteria, it has the ability to alter intracellular enzymes, nucleic material, and constituents of the cell envelope resulting in the

deactivation of the bacteria (Khadre et al., 2001). Ozonated water was given GRAS approval in 1997 and has since been examined for use in processing produce and red meat (Fabrizio et al., 2002). Use of ozone in a processing environment is seen as an attractive method due its ability to dissipate quickly into non-toxic remains (Pryor and Rice 1999), as well as leaving behind almost no residue. Since chlorine compounds have been known to posses carcinogenic properties, ozone has received praise as a non-chlorinated alternative in the food processing environment (Crowe et al., 2012). Unfortunately, when ozone comes into contact with organic materials such as beef it tends to dissipate (i.e., become reduced) too quickly resulting in little to no antimicrobial effect. In Yonder et al. (2012), the effect of ozone was said to be no more substantial than treating the samples with tap water. When beef carcass surfaces were treated with direct ozone in comparison with water to reduce inoculated pathogens, there was no significant difference between the results of both treatments (Bosilevac et al., 2005).

Electrolyzed water (i.e., hypochlorous acid) is a solution that has received GRAS approval for the antimicrobial use in food processing plants (Bosilevac et al., 2005). It is created when salt solution (NaCl) is passed through a bipolar membrane. Two solutions are produced at the various electrodes, one at the anode with either a low or neutral pH (hypochlorous acid, or 'anolyte') and one at the cathode with a high pH (i.e., 'catholyte') (Fabrizio & Cutter, 2004). Chlorine-containing oxidizing compounds (such as electrolyzed water) have been used for years in the food industry as antimicrobial agents. They have proven to be useful due their availability, cost, and prevalence in eliminating a variety of harmful pathogens (Rahman et al., 2012). When bacterial cell walls come into contact with hypochlorous acid, an oxidative effect leads to the loss of enzyme activity or DNA cleavage (Elano et al., 2010). Although electrolyzed water at very low pH has been known to have detrimental effects on equipment as well as humans who may come into direct contact with it regularly, neutral pH electrolyzed water tends to have less corrosive effects on processing surfaces as well as lowered human health risks because Cl_2 off-

gassing is reduced (Guetzel et al.,). Unfortunately, electrolyzed water shoes greater reduction on inorganic rather than organic materials (Fabrizio and Cutter, 2004) and the product is increasingly sensitive to storage conditions (Rahman et al, 2012). It has been indicated that longer exposure time of products to the electrolyzed water may result in higher reductions. Fabrizio et al. (2002) showed that electrolyzed water could be effective at treatments time of around 40 minutes and may still not result in a significant reduction that would be relevant to the food processing industry.

The FDA has recently allowed the use of biological antimicrobials, such as bacteriophage, to be used as antimicrobial interventions in foods (Code of Federal Regulations, 2012). Bacteriophage are naturally occurring, highly specific bacterial viruses that can be an effective biocontrol of pathogens and spoilage organisms in foods (Guenther et al., 2012). Where many promising sanitizers can be corrosive, toxic, and have the ability to break down foods and surfaces they come into contact with, bacteriophage do not posses any of these issues. Phage can be used as a natural antimicrobial to reduce bacterial pathogens from the food supply (Viazis et al., 2011). They are not hazardous to humans, foods, or equipment, yet can be aggressive when in the presence of foodborne pathogens. Lytic bacteriophage, which are available commercially, infect the bacteria and replicate creating more phage DNA within the bacterial cell. This eventually causes the cell to burst, releasing bacteriophage that can continue to infect more bacterial cells. Guenther et al, found that *Salmonella* phage gave up to a 5-log reduction effect on select food samples. Bacteriophage are also found to be very safe if consumed, in which studies with human volunteers showed no significant negative effects after being fed high doses of bacteriophage (Hagens and Loessner, 2010). However, for a decently high microbial reduction to occur, a high number of phage is required to be applied. Basically, the number of phage used in any application must be sufficiently high to ensure that rapid contact of susceptible bacterium and phage can occur (Hagens and Loessner, 2010). This can be increasingly challenging when phage

are applied to food surfaces where movement of the bacterial virus (which is necessary for it's infection of pathogens to occur) isn't as easy as it would be in just an aqueous solution. The bacteria will continue to remain present as well as even potentially proliferate if the phage do not attach and infect the host cells (Hagens and Loessner, 2010).

There is still a need for new methods of reducing and eliminating food-borne pathogens that could lead to serious illness, death, and detrimental effects to the food industry economy. With mechanically tenderized meat becoming increasingly popular by consumers, the development of a potent yet safe antimicrobial intervention is becoming progressively crucial considering the regulatory stance of mechanical tenderization as a possible health risk. The objective of these studies was to measure the effectiveness of three popular antimicrobial solutions (ozone, electrolyzed water, and bacteriophage) as potential interventions against *E. coli* O157:H7 and/or *Salmonella* spp. on carcasses and meat surfaces that would be subsequently subjected to blade tenderization.

CHAPTER II

REVIEW OF LITERATURE

Foodborne diseases

Food safety, just as important as any other public health concern (Guetzel et al., 2008). Foodborne illnesses are consistently a major threat all over the globe, even in the first world countries (Mahmoud, 2007). The Center for Disease Control (CDC) states that the incidence of two or more similar illnesses caused by consumption of a common contaminated food source can be defined as a foodborne disease outbreak (CDC, 2013). Foodborne pathogens repeatedly cause an estimation of >9 million infections and nearly 9,000 fatalities annually (CDC, 2013; Mead et al., 1999). Approximately 1,527 foodborne illness outbreaks occurred during 2009-2010 in the U.S., District of Colombia, and Puerto Rico combined. This resulted in 29,444 reported illnesses, 1,184 hospitalizations, and 23 deaths of those infected (CDC, 2013). More specifically, bacterial pathogens cause 60% of the hospitalized foodborne diseases and make up almost two thirds of the calculated number of foodborne pathogen-related deaths (Koohmaraie et al., 2005). Between the years 1988 and 1992, 79% of the outbreaks and 90% of overall foodborne illness in the U.S were caused by bacterial pathogens (Koohmaraie et al., 2005).

There has been a collective concern amongst food processors, food safety researchers, and regulatory agencies due to the increasing outbreaks of foodborne sickness caused by pathogens such as *Escherichia coli* O157:H7, *Salmonella,* and *Listeria monocytogenes* (Murphy et al., 2004). As if loss to human life and serious illness weren't enough, these microbial pathogens are estimated to cost the US economy around 6.5 billion dollars per year (Pimentel et al., 2001).

Foodborne diseases are encountered from various products worldwide. The greatest risk of foodborne illness comes from minimally – or unprocessed fresh foods (Cheigh et al., 2011), with meat products, and even more specifically beef products serving as extremely prominent causes of foodborne sickness. In 2009 and 2010 the CDC was able to assign a single "food vehicle commodity" to 299 individual outbreaks, of these the most implicated "commodity" was beef (13%) (CDC, 2013).

In order to properly progress the urgency of improving food safety in products such as fresh beef, innovative examining tools were necessary to assist researchers in their quest to isolate, categorize, and track the source of such bacterial contaminations (Koohmaraie et al, 2005). Many factors such as handling, improper sanitization, and temperature of fresh products has been found to be the most crucial environmental consideration that can either progress or halt bacterial growth in foods (Ding et al., 2009).

Applications including trimming, washing, vacuuming, and even spraying with various antimicrobial solutions as well as pasteurizing meat surfaces with steam or hot water have all been developed and concluded as substantially effective methods for removing bacteria from meat (Dorsa, 1997). Researchers have also discovered that even more efficient methods consist of the combination of these treatments. This has lead to more substantial reduction and safer products for consumers. The food industry, and more specifically, the beef industry, has unrelentingly continued their search for ways to increase the safety of meat. The ability to

continuously reduce contamination of *E. coli 0157:H7* and other pathogens is a task that is endlessly researched (Laster et al., 2012). To date, the most proficient way to reduce and even eliminate pathogens is to consider their sources, vectors, and frequency in all environments related to food production. For example, the shelf life of a food product should be determined by taking understanding and recognizing the probable development of a specific pathogen throughout storage (Mataragas et al., 2010).

Due to current U. S. regulations, any processor or business that produces beef or any meat product must reevaluate their written HACCP plan annually, allowing for constant changes and improvements (Echeverry, et al., 2010). Persistent research and cooperation improves microbiological quality of raw beef significantly from where it once was (Koohmaraie et al., 2005).

E. coli O157:H7 : Foodborne pathogen

Escherichia coli is a gram negative bacterium from a heterogeneous group, which is generally nonpathogenic and occurs naturally in the intestinal microflora of human beings and animals (Gyles, 2006). This specific species has evolved to the point of being able to cause disease in humans. For the past 20 years, *Escherichia coli* O157:H7 has been a pathogen that has been a serious concern to the meat processing industry (Bosilevac et al., 2005). Today, *E. coli* O157:H7 is perhaps the most prevalent serotype, with an estimated 63,153 U.S. cases of foodborne illness due to this specific pathogen (Scallan et al., 2011). A widespread and frequently occurring pathogen, E. *coli* O157:H7 is seen as a risk mainly because of its low infectious dose (Viazis et al., 2011 and Gyles, 2006).

A major "reservoir" of shiga toxin-producing *Eschericia coli* is dairy and beef cattle with which humans come into direct contact. Human infection is usually paired with consumption of

food and water tainted with cattle manure (Gyles, 2006). The occurrence of this pathogen in beef products or processing environment is considered an adulterant with a zero tolerance policy for its presence (Kannan et al., 2010).

Escherichia coli have a wide range of hosts and are a commensal bacterium in humans and animals. Although frequently present in the environment, they are a known indicator of fecal contamination when found in food and water. Due to its pervasiveness in humans and animals, as well as its role as a commensal and pathogenic microorganism, *E. coli* possess the ability to obtain, preserve, and transfer resistance genes from other organisms in its environment. This capability has contributed to *E.coli* becoming one of the most common microorganisms that are resistant to antimicrobials (Zhao et al., 2012).

The beef industry took a prevalent hit from this foodborne pathogen during the years of 2007 and 2008 consisting of a multitude of recalls that were not only detrimental to consumer health but financially to the industry (Laster et al., 2012). In 2001 to 2003, 10 retail market survey study was performed, in which *E. coli* O157:H7 contamination rates were compiled together throughout the area of Minneapolis - St. Paul, MN. It was discovered that 92% of poultry and 69% of beef and pork were affected (Zhao et al., 2012). 11,921 samples of meat were collected from four different states (GA, MD, OR, and TN) between the years of 2002 and 2008. The samples were made up of chicken breast, ground turkey, ground beef, and pork chops. 69.5% of these samples tested positive for the presence of *Escherichia coli* (Zhao et al., 2012).

An initial source of *E. coli* O157:H7 and other pathogenic contamination in commercial beef processing can be found on the hides of cattle during pre-evisceration (Bosilevac et al., 2005). *E. coli* outbreaks have not just occurred in meat products, but a significant amount of outbreaks have been linked to milk, cheese, yogurt, water, salad dressings, and vegetables. The O157:H7 serotype has even had the ability to live in slightly acidic environments such as apple

juice. This situation can potentially allow the pathogen to build a tolerance towards other acidic surroundings (Gyles 2006).

A majority of illnesses occur in the warmer months of the year and have been associated with eating undercooked, contaminated ground beef (commonly, undercooked hamburgers); however, contaminated fruits and vegetables are increasingly becoming sources of *E.* coli O157:H7 infections (Abuladze et al., 2008). The most prevalent path of transmission for shiga-toxin producing *E. coli* (STEC) infections in humans is consumption of raw and even undercooked foods such as meat and milk (Martin and Beutin, 2011).

Although *E. coli* seldom generates clinical disease in animals, human infections caused by the pathogen can be lead to hemolytic uremic syndrome, thrombotic-thrombocytopenic purpura, and hemorrhagic colitis (Murpy et al., 2004). Whenever there is a severe *E. coli* outbreak, the internal process of the disease is comprised of the bacteria incapacitating the human's natural defense mechanisms (such as acid resistance) leading to colonization in the intestine and injury from toxin secretion, which is typically due to the O157:H7 serotype. This serotype possesses the ability to inhabit the large intestine by a distinctive attachment and "effacing lesion". The lesion occurs when the bacteria is able to adhere by secreting a type of "effector proteins" which infuse to the epithelial cells of the intestine (Gyles, 2006). *E. coli* O157:H7 is able to discharge large amounts of shiga toxins that can cause severe damage to the intestinal lining in humans (Koohmaraie et al., 2005). Once consumed, the internalized *E. coli* cells may endure resulting in serious illness, ranging from slight bloody diarrhea to serious and life-threatening hemolytic-uremic syndrome (Belongia et al., 1991).

Individuals such as children and the elderly who tend to have lower immune systems are the most at risk to severe situations such as hemolytic uremic syndrome. Even though the

kidneys are a common "target", *E. coli* infections can affect many other organs in the human body including the central nervous system. (Gyles, 2006).

In the name *E. coli* O157:H7, the "O" and "H" actually refer to specific antigens. The O (ohne) antigen is determined by the bacterium cell wall polysaccharide portion and the H (Hauch) antigen is based on the flagella of the cell. *E. coli* isolates can occur in a number of combinations with O antigens numbered 1 to 181 and H occurring in 53 different types (Gyles, 2006).

Due to the prevalence of O157:H7 in foodborne infections, *E. coli* strains are commonly labeled as either O157 or nonO157 (Gyles, 2006). Although this does not mean that nonO157 are to be ignored or that infections caused by other serotypes can be any less devastating. Germany experienced an eye-opening outbreak during 2011 in which sprout seeds contaminated with the STEC serotype O104:H4 lead to over 800 cases of hemolytic uremic syndrome and more tragically to the death of 53 individuals. Although this outbreak wasn't specifically E. coli O157:H7, the continuous study of this more commonly known pathogen can help lead to potential control of other STEC serotypes (Bielaszewska et al., 2011).

***Salmonella:* Foodborne pathogen**

Salmonella is another worldwide pathogen capable of causing economic and health-related distress. Responsible for disease related outbreaks in both animals and humans, *Salmonella* are zoonotic enterobacteria (Murphy et al., 2004). Along with *Escherichia coli* O157:H7, these two pathogens are highly proficient in triggering large amounts of infections that could potentially lead to mortality in humans. Majority of *Salmonella* infections are associated to the ingestion of contaminated foods, such as meat and meat products (Echeverry et al., 2010). Pathogens such as Salmonella can lead to a negative 'socioeconomic' effect due to recall, litigation, illness, medical costs, disability, and even death (Echeverry et al., 2010).

Salmonellosis is one of the most common foodborne illnesses (if not the most) in the Unites States (Koohmaraie et al., 2005). The Centers for Disease Control has around 40,000 cases of non-typhoid salmonellosis reported annually in the U.S. (Guenther et al,. 2012). In 2009 and 2010 combined, the CDC (2013) reported foodborne *Salmonella* infections as the second most prevalent single-etiology outbreaks in the U.S., making up 30% of total outbreaks and 36% of total illnesses. From the 29,444 reported outbreak-related illnesses in 2009 and 2010, 4% resulted in hospitalization. *Salmonella* infections lead to 49% of these 1,184 hospitalizations (CDC, 2013).

Salmonellosis can be transmitted in a variety of ways, mostly from humans consuming contaminated food items (Murphy et al., 2004). *Salmonella* has been discovered in a multitude of foods, which has lead to disease outbreaks from a variety of products, including several different meats. *Salmonella* has also been known to contaminate seafood from harvest to consumption and is the leading pathogen in seafood-associated bacterial outbreaks all over the world (Rajkowski, 2012). *Salmonella* is so prevalent that is has been found in higher rates on the hides of beef cattle than in feces (Koohmaraie et al., 2005).

Antimicrobial resistance

Over the past 60 years, increased usage of antibiotics to reduce bacterial infections has actually lead to greater resistance of bacterial strains to these very products. Antibiotic resistant bacteria are continuously developing and proliferating in environments, especially ones related to food processing (Duffy et al., 2006). Unfortunately majority of life-threatening pathogenic bacteria are resistant to many antibiotics that were at one point a crucial tool in medicine. This has become a severe issue in today's medicine especially due to the upsurge of immunosuppressed patients (Sulakvelidze, 2001). Inspection of bacteria resistance to antibiotics

has become continuously important for immunosuppressed individuals (Hauser et al., 2013). The use of antimicrobials in food animals and their role in stimulating resistant food-borne bacteria has developed into a critical public health issue. In order to properly assess the standard resistance rates as well as the impression of specific interventions, a continuous supervision of antibiotic applications is absolutely essential (Zhao et al., 2012).

Experiments and literature propose that antibiotic resistant bacteria may exhibit altered 'growth kinetics' in laboratory media in addition to distinctive resistance to other applications such as acid and heat (Duffy et al., 2006). The level of antimicrobial resistance in *E. coli* can be used as a gauge of resistance distribution for other bacterial populations while also displaying the selective pressure created by the antimicrobials used in treatment of food animals and humans. Due to its easy of accessibility as well as natural presence in the gut of many animals, *E. coli* has been selected as an indicator organism in programs and studies that investigate antimicrobial resistance worldwide (Zhao et al., 2012).

In Wang et al (2013), *L. monocytogenes* isolates were gathered from various food samples for analysis of antimicrobial susceptibility. In this study they found that many of the found isolates were resistant to multiple antibiotics, which can be an impending public health threat. Since resistance ability varies based on use of antimicrobial as well as geographical location, it has now become an essential worldwide occurrence to supervise antibiotic vulnerability of *L. monocytogenes* (Wang et al., 2013). The amount of *Listeria* strains resistant to one or more antibiotics has dramatically increased over time (Granier et al., 2011).

Increased consumer demand for a safe, pathogen-free meat supply increases the need for antimicrobial application in the manufacturing process (Stivarius et al., 2002). In order to better understand and prevent antimicrobial resistance in bacteria, initial attention needs to be given to antimicrobial use in humans and animals. In order to halt multi-drug resistant strains, steps need

to be taken to change the patterns of resistance as well as increase knowledge on how to implement preventative measures in the future (Harakeh et al., 2005).

Antimicrobial intervention

Food safety is an important issue for both consumers and food industry (Mahmoud, 2007). The proliferation of foodborne illnesses and food safety problems has resulted in the continual progress of innovative antimicrobials and methods to disinfect foods and surfaces in food processing areas (Guentzel et al., 2008). The USDA advocates antimicrobial interventions to decrease the occurrence of harmful microorganisms on raw food products (Yoder et al., 2012). Developing an effective method to reduce or eliminate foodborne pathogens is crucial to food safety and human health (Rahman et al., 2012).

Decontaminating fruits, vegetables, and ground meat presents considerable challenges. The two most common strategies used to limit the growth of bacteria on fruits and vegetables are washing with water and washing with solutions of various antibacterial chemicals (Abuladze et al., 2008). However, working with decontaminating agents on meat products can be much more complicated. Concern about the contamination of beef with enteric pathogens has led to extensive investigation of treatments for reducing the numbers of bacteria on dressed beef carcasses (Gill & Landers, 2003). Research indicates the best opportunity to prevent further contamination after slaughter is a final wash of the carcass surface occurs prior to chilling (Yoder et al., 2012). Contamination ultimately can cause consumer illness if the processor or the consumer does not appropriately handle the products (Harris et al., 2012). Customarily, the concentration of antimicrobial intervention has focused solely on controlling hide and carcass contamination, but the steps during and after fabrication are just as crucial and need to be given sufficient attention as well (Pittman et al., 2012). It was suggested as early as 1976 that whenever

beef carcasses are fabricated into retail cuts, any newly exposed carcass surface could be subject to microbial contamination (Stivarius et al., 2002).

The goal of a decontaminating agent is to posses the ability to alter any microbial cell structures and inactivate enzymes necessary for metabolism and growth. Many essential cellular processes are dependent on the integrity of the cell membranes and cell walls. Inactivation of metabolic enzymes would also mean the cessation of many biological reactions within the cell that could eventually lead to cell death (Alonzo, 2012).

Obviously a large concern is the change in palatability of food products (especially meat) when treated with antimicrobial interventions. In a recent study by Harris et al (2012), antimicrobial interventions such as acetic acid, lactic acid, acidified sodium chlorite, and sterile water were applied to ground beef. All antimicrobial interventions statistically reduced the pathogen load of *E. coli* O157:H7 and *Salmonella typhimurium* up to 0.5 and 0.6 log respectively, within 24 hours of treatment. Untrained panelists could not detect differences between control and the antimicrobial treated samples when presented in triangle tests, reaffirming consumer perception of ground beef palatability is not significantly altered by antimicrobial treatments (Harris et al., 2012).

Along with the concern of palatability, efficacy of solutions over a long period of time is another issue that needs to be constantly monitored in order to protect company's financial investment into a product. When understanding product shelf life, exact considerations need to be made in order to properly evaluate the danger of possible pathogenic growth as well as comprehend the risk and properties of any plausible intervention methods or solutions (Mataragas et al., 2010). Moreover, many of the current available chemical sanitizers that have sufficient shelf lives may damage foods, as well as harmfully affect the environment. This is an issue that

tends to mostly effect the decontamination of ground beef and other beef products (Abuladze et al., 2008).

Many solutions such as organic acids, chlorinated compounds, hot water, heat, steam, have GRAS approval and have been utilized by the meat and poultry industries for years. Although these methods are able to reduce foodborne pathogens in a food-processing environment almost immediately when utilized, researchers continuously investigate other solutions, agents, and methods that can be used in an antimicrobial fashion. This is to further be ahead of the game to keep all processing and prevention of spoilage organisms to be continuously economically effective (Fabrizio et al., 2002).

Single application interventions can be proficient in reducing bacterial contamination, but there has been recent emphasis on performing multiple intervention treatments in a sequential order to eliminate even more microbial populations. Combinations of hot water washes, trimming, chemical sprays, and even steam applications are viewed as having an increased detrimental effect on foodborne pathogens (Pittman et al., 2012).

There are several antimicrobial interventions that are highly proficient in reducing detrimental microorganisms that can be found on all foods and their processing environments, but finding specific ones that are cost-effective, safe, and easily accessible is where the real need lies (Yoder et al., 2012).

Blade and injection tenderization concerns

The muscle of a healthy animal starts out as essentially free of bacteria, but unfortunately, even when worked with under the most 'stringent conditions', animal muscle can easily become contaminated during processing. Starting at harvest, contamination can occur from the

environment, hide, or even from accidental contact with the contents of the intestinal tract. Once exposed to the atmosphere during post-slaughter processing, such as fabrication, microbial contamination can occur from the external environment. When mechanical tenderization methods were initiated in the 1970s, there was concern from the beginning on whether the processes could increase the chances of contamination.

Non-intact beef products are comprised of any cut of beef that may be subjected to grinding, mechanically tenderizing with needles, restructured, or even injected with various solutions that would increase flavor and/or tenderness (Yoon et al., 2009). Consumers believe tenderness, flavor, and juiciness are the most imperative characteristics accompanying beef 'palatability' (George et al., 2000). Less delectable cuts of beef that are measured as 'tough' can be tenderized when subjected to processes such as needle-blade tenderization, moisture-brine enhancement, or even a combination of these applications. Which increases tenderness and juiciness of the final product (Echeverry et al., 2010). In needle tenderization or solution enhancement, pieces of meat are subjected to penetration of very sharp blades or injected with brines that increase the juiciness and palatability of the final product. This allows the meat to be altered without stretching or tearing apart the actual muscle fibers of the product (Echeverry et al., 2010). These processes have been practiced for years and at least 18% of beef products in the U. S. available at the retail level have been mechanically tenderized or injected with solutions for improvement of flavor and/or tenderness (Yoon et al., 2011).

Customers unknowingly may request a mechanically tenderized piece of meat from a restaurant which they could assume to be a "whole cut of meat" (Echeverry et al., 2010). The main issue occurs when these customers request for their meat to be cooked as 'rare' or 'medium'. In recent studies, data indicated that cooking a piece of non-intact beef to 65°C (Gill et al., 2009) or greater will radically eliminate a substantial number of potential pathogens, but many consumers prefer their beef (especially non-intact steaks) to be undercooked (Yoon et al.,

2009). Rare and medium meat is not cooked to the appropriate temperature, which prevents the killing of any existing pathogens.

When a piece of meat is regularly fabricated and processed, any microbial contamination will reside on the surface of the meat. As long as the outside is cooked to an appropriate temperature a customer could eat a cut of beef almost as rare with very little chance of infection due to contamination. Microbiological risk occurs when injection applications of tenderization and marinating solutions are put into beef muscle cuts. These processes may lead to internalization of *E. coli* O157:H7, or other food-borne pathogens, (that would normally just be on the surface) into the sterile deep tissues (Hajmeer et al., 2000).

Studies have shown that 3-4% of *E. coli* O157:H7 cells on the surface of beef subprimals can be internalized into the tissue by methods such as blade tenderization (Yoon et al., 2011). Smith et al. (2013), calculated that non-intact beef cuts had a risk of 11 times higher than intact beef cuts when compared for overall probability of contamination and infection. Over the last decade, many foodborne outbreaks have been connected to contaminated mechanically tenderized meat due to translocation of pathogens such as *E. coli* O157:H7 and *Salmonella* from surfaces of beef and pork to the internal muscle (Echeverry et al., 2010). This may seem to be an issue pertaining only to individuals who order their steak 'medium' or 'rare', but chemicals in flavor-enhancing solutions may prevent thermal inactivation of the bacteria or increase their resistance to heat during the cooking process (Sofos et al., 2008). Due to several outbreaks and recalls, the microbiological associated risks with these tenderization methods has led to the inclusion of *E. coli* O157: H7 being recognized as an adulterant in non-intact beef products (USDA-FSIS, 1999).

Ozone

Ozone is an oxidant that has found applications in the food industry as 'ozonated water'. It is generated by splitting oxygen (O_2) into individual oxygen radical molecules (O·) with high voltage electrical energy. Once split, the two separate O· molecules latch onto other unbroken O_2 molecules forming Ozone (O_3). This process occurs frequently in nature such as when lightning strikes (Khadre et al., 2001) and is an important part of the atmosphere shielding us from the harmful effects of ultraviolet light from the sun.

As an oxidant, ozone has found applications as a sanitizing solution in the food processing industry, either as a sanitizer for equipment surfaces (food contact surfaces) or for rinse treatment of various foods. Once an interaction has occurred between bacteria and molecular ozone, the products from the broke-down ozone have the ability to "inactivate" bacteria and other microorganisms quickly by altering intracellular enzymes, nucleic material and constituents of the cell envelope (Khadre et al., 2001). Two deactivation methods of microorganisms by ozone are known. First, ozone rapidly oxidizes the sulfhydryl groups, which are often important components of enzymes. It also has the ability to oxidize the amino acids of enzymes, peptides and proteins. The second process is very common in Gram-negative bacteria; ozone has the ability to oxidize polyunsaturated fatty acids into peroxy acids. This causes the lipoproteins and lipopolysaccharides to break down resulting in cell breakage and eventually rupture (Trindade et al., 2012).

Ozone has displayed the ability to inactivate a large number of organisms, including bacteria, fungi, yeast, parasites, and viruses (Chawla et al., 2008). Even more so, it has also been recognized as a safe (GRAS) for reducing bacterial populations on particular surfaces found in processing environments, such as stainless steel. Systems used to generate ozonated water have become very popular and are readily available (Chawla et al., 2008). Companies have been able

to commercially produce ozone with machines that inject high voltage electricity into oxygen-enriched streams.

Due to an increase in outbreaks of food contamination by various microorganisms, there has been increse in recent years for the need of a potent, yet safe antimicrobial technique. A potential antimicrobial that could be used is aqueous ozone (Trinidade et al., 2012). The FDA and USDA revised food additive regulations to allow use of gaseous and aqueous forms of ozone as an antimicrobial on foods in processing environments (Chawla et al., 2008). In 1997, Ozonated water was generally recognized as safe (GRAS) and its use has been investigated in the processing of fresh produce and red meat (Fabrizio et al., 2002). Since the 1940s, ozone has been used in the process of disinfecting drinking water. The use of ozone is performed at many municipal water treatment plants around the world, with the majority of commercially-available bottled water having been treated with ozone since the 1980s (Bosilevac et al., 2005).

Due to its many appealing characteristics such as its ability to dissipate quickly after treatment into non-toxic remnants (i.e., oxygen and water), ozone is considered a process rather than an additive (Pryor and Rice, 1999). Because ozone leaves almost no residue on treated food, it has also received approval by the National Organic Program for processed foods, allowing products that are treated with ozone to be labeled as “organic” or “made with organic” (Calder et al., 2011). Ozone is preferred over chlorinated oxidants, such as hypochlorite or hypochlorous acid, which are prone to producing potentially carcinogenic compounds when coming in contact with organic material, and therefore is preferred as a non-chlorinated substitute in the food processing industry (Crowe et al., 2012).

Despite the fact that ozone seems like a promising answer to many food processing issues, it has displayed much more potential when reducing microorganisms on surfaces than in environments with a high organic burden. Ozone alone has shown limited effectiveness when

inhibiting microbial growth on organic material likely due to its high oxidizing capacity (Calder et al., 2011).

Although it can be very effective, ozone has some restraints. Ozone is unstable, has a short half-life, and breaks down under normal storage conditions into oxygen and water. Its short half-life is considered a serious limitation and requires a generator to be situated nearby the actual point of application; even pumping ozone for a considerable distance across a plant facility may cause it to lose half its oxidizing capacity. It can also be hazardous to human health as it may 'off-gas' ozone gas into the working environment (Kim et al., 1999). The product also remains to be scrutinized in various research and investigations due to the fact that it has a strong oxidizing potential that is created by the tri-atomic ozone molecule/radicals (hydroxyl, hydroperoxy, and superoxide radicals) that are produced during its disintegration (Crowe et al., 2012).

Direct applications of ozonated water to numerous foodstuffs have not always resulted in significant reductions. There has been "variable success" when used in a variety of seafood immersion experiments (Crowe et al., 2012). Yoder et al (2012) found ozone to be the least effective antimicrobial when beef pieces were treated with various solutions. The reduction of pathogens from ozone in their study was said to be no more substantial than when treated with tap water (Yoder et al., 2012). When beef carcass surfaces were treated directly with ozone, in comparison to water, to reduce populations of inoculated pathogens, the results displayed no significant difference between the two treatments (Bosilevac et al., 2005).

There are many physical or mechanical factors that can affect the already highly unstable ozone solution during application by spray or dipping (i.e., a fine nozzle spray applicator may cause more off-gassing of ozone gas than large droplet nozzles). The understanding of ozone limitations may be the key to finding how it may be applied successfully. If factors such as temperature, source of water, application time, pH, material being treated, and concentration

(ppm) are adequate, ozonated water can be useful to diminish spoilage bacteria and further advance quality of a product. In many studies, higher ozone concentrations and length of the exposure times studied were proven more effective for decreasing levels of spoilage microorganisms (Chawla et al., 2008). Trindade et al. (2012) found that improved microbial reduction was observed when freshly slaughtered chicken carcasses were immersed in low-level chilled chlorine or ozone solution for 45 minutes. Significant reductions with ozone were comparable to that obtained with chlorine solution (Trindade et al., 2012). In many experiments, ozone has proven more effective when a "pretreatment" precedes the application. Bosilevac et al. (2005) found that the efficacy of ozone treatment is increased when competing organic particles are removed and when a mechanical means such as high-pressure application is used to dislodge bacteria. In Crowe et al. (2012), when salmon filets inoculated with *Listeria spp*. were treated under a spray system, the filets that were sprayed three times had higher reductions (although not extremely significant) than the ones sprayed only once and twice. This was probably due to the "friction and shear generated" along with the "microbial oxidation" from the spray systems. It seems that the antimicrobial potential of ozone not only is heavily influenced by chosen application mechanism but also by the type and amount of microbial population present (Crowe et al., 2012). In order to further the use of ozone in food processing environments, it is essential to evaluate the "tolerance" of select foods to specific quantities and contact times (Trindade et al., 2012).

Electrolyzed water

The hydrolysis of saline solutions produces what is often referred to as 'electrolyzed water', i.e., hypochlorous acid, an oxidant that contains chlorine. Electrolyzed water has attracted a lot of recent considerations as a high-performance, new technique for prospective use in the

food industry (Mahmoud, 2007). Electrolyzed water is accepted as a GRAS substance, so it is therefore an approved novel antimicrobial that can be utilized inside of a processing plant at specifically permitted levels (Bosilevac et al., 2005). It is an "environmentally friendly" sanitizing method that has shown potential to eliminate a wide-ranging scale of foodborne pathogens (Guentzel et al., 2008).

Chlorine-containing compounds have for a long time commonly been used throughout the food industry as sanitizers and antimicrobial agents in food processing due to their obtainability, cost effectiveness, and ability to kill a variety of pathogens (Rahman et al., 2012). Acidic electrolyzed water (pH of 3.0 or less), which is produced by the electrolysis of a diluted NaCl solution, has been able to act as an antimicrobial and reduce foodborne pathogens such as *E. coli* O157:H7, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *and Listeria monocytogenes* (Liao et al., 2007). This is because it contains a high oxidation-reduction potential as well as available chlorine (Cui et al., 2009). Chlorine has been accepted as a disinfecting agent in the following three forms: chlorine gas, calcium hypochlorite, and sodium hypochlorite (NaClO) (Elano et al., 2010). When compounds dissolve in water, hypochlorous acids are produced resulting in an oxidative effect inside the cell wall of most bacteria; this then produces the loss of enzyme activity or results in DNA cleavage (Elano et al., 2010). Hypochlorous acid is known for having a low pH and containing active chlorine, this allows it to have a strong oxidative-reduction potential (ORP) similar to that of ozone (Bosilevac et al., 2005). Bacteria require a neutral pH to reproduce. When a microorganism's membrane is introduced to electrolyzed water's low pH solution, it becomes flooded with hydrogen ions, which affects the permeability of the cell disabling it's ability to reproduce (Fabrizio et al., 2002). Chlorine is said to be a more effective antimicrobial when used in preventing cross-contamination by treating food- and non-food contact surfaces (James et al., 1992).

Both electrolyzed water and ozone have a sufficient oxidation-reduction potential, but what makes electrolyzed water unique is that it is comprised of free chlorine, which is a further bactericide (Bosilevac et al., 2005). When using electrolyzed water, the antimicrobial activity of the actual chlorine-compounds varies on the quantity of free chlorine available in the solution, the pH, the temperature, and the amount of organic matter in the sample it is being used against (Matthews, 2006).

Electrolyzed water solutions are produced when electrolysis of a 0.1% concentration of NaCl solution occurs in deionized water. It is created when a weak salt solution of NaCl and water is passed through a bipolar membrane, this produces two very different solutions: one that is acidic (low pH), high ORP solution, and the other that is a basic solution (usually a high pH) with low ORP and is comprised of free chlorine (Fabrizio & Cutter, 2004). During electrolysis, NaCl dissociates into chlorine (Cl-, which is negatively charged) and sodium (Na+, which is positively charged). At the same time hydroxyl (OH-) ion and hydrogen (H+) ion are formed. Chlorine and hydroxyl ions are focused in the anode section where they are able to produce hypochlorous acid (HOCl). This is all happening while the positively charged sodium ions obtain electrons and convert into sodium molecules found in the cathode section. The sodium molecules are then able to form sodium hydroxide (NaOH) after being able to attach with the water molecules. Generally, the voltage is maintained between 11 and 12 V of direct current and after electrolysis occurs, the anodic solution will achieve a pH of 2.2-2.7 with 20-100 ppm of available chlorine. Solution from the anode is often referred to as 'anolyte' (most often, anolyte is produced at ~ pH 6.0-6.5 because the chlorine gas can more readily off-gas as Cl_2 at very low pH). The cathodic solution will often achieve a pH of 11-12 (Mahmoud, 2007) and solution from the cathode side of electrolysis is referred to as 'catholyte'. Electrolyzed water application has the ability to expose organisms to two ranges of pH (alkaline at pH 11-12 and acid pH 2-6) this creates strain and injury to the cells being treated. The effect of applying both catholyte and

anolyte usually has a greater effect than what has been observed using ozonated water (Bosilevac et al., 2005).

The enhanced antimicrobial effect of anolyte solution at low pH has limitations and drawbacks. The low pH causes dissolved free chlorine to be off-gassed as volatile Cl_2, which can reduce the solutions' bactericidal ability and therefore reduce its capability for long-term function and antimicrobial effectiveness (Guentzel et al., 2008). There is also a widespread concern for the deleterious consequence that electrolyzed water may cause on the environment and health of humans working in the vicinity, plus the high acidity of solutions may lead to deterioration of processing equipment.

Since the low pH found in the anodic solution has such a negative reputation, there have been applications of neutral/near-neutral electrolyzed water to produce a less acidic pH (5.0-6.5). This process tends to be less expensive as well as more effective and convenient. There is no need for a membrane and in addition to using diluted NaCl, it can also electrolytically utilize dilute HCl solutions (Gomez-Lopez et al., 2007). The near-neutral solution produced by these generators is made up of 95% hypochlorous acid, which tends to serve as an aggressive antimicrobial (Cui et al., 2009). Despite its strong potential, neutral electrolyzed water is not as harsh on processing equipment and is much safer to use by workers than acidic electrolyzed water (Abadias et al., 2008). Guetzel et al (2008), found 100% reduction when treating pure cultures of *Escherichia coli, Salmonella* Typhimurium, *Listeria monocytogenes,* as well as numerous other pathogens with different concentrations of the neutral electrolyzed oxidizing water. They also discovered when spraying various types of produce with the neutral electrolyzed water at concentrations of 250-300 ppm that there was a 79-100% reduction, and when dipping spinach leaves into the solution for 10 minutes at 100-120 ppm they achieved a 4 to 5-log reduction. However they discovered that when dipping lettuce for 10 minutes at 100-120 ppm there was only a 0.25 log reduction, this was thought to be because of the grooves found in lettuce that can

allow pathogen protection from dip treatment solutions. This suggests that each type of food item may have its own process designed especially for its peculiar circumstances in allowing microbial reductions during sanitary treatments.

Although there have been studies done where electrolyzed water has shown adequate antimicrobial effects, there have been many studies where little to no effect was observed. It is known that electrolyzed water has the ability to decrease foodborne pathogens when placed in cell suspensions (Fabrizio et al., 2002). Kalchayanand et al. (2008) found that electrolyzed oxidizing water and ozonated water reduced *E. coli* O157:H7 less than 0.5 logs CFU/cm^2. This study showed that both electrolyzed oxidizing water and ozonated water were not effective sanitization approaches to a dramatic reduction of *E. coli* O157:H7 (Kalchayanand et al., 2008). In Fabrizio and Cutter (2005), frankfurters inoculated with *L. monocytogenes* were dipped in acidic electrolyzed water to observe reduction. In this experiment they found that at the most there was a 1.5 log reduction when dipped for 15 minutes. However when a shelf life study was performed immediately after dipping, the reduction was maintained for up to 7 days until the reduction diminished and counts began to increase (days 14 and 21). In the same experiment a spray treatment with both acidic and basic forms of electrolyzed water (performed without analogous controls with just water), demonstrated only a 0.6 log reduction (Fabrizio and Cutter, 2005).

Because of its components, electrolyzed water does not seem to work well in the presence of large amounts of organic material. In recent studies, it has been implied that when electrolyzed water comes into contact with matter rich in amino acids, peptides, and amines, the active chlorine component that is generated by electrolyzed water may be transformed to N-chlorate compounds (due to the amino acids and proteins), causing less free chlorine to be available. Free chlorine is the key ingredient needed to deactivate bacterial cells (Kalchayanand et al., 2008). It is also known free chlorine binds to organic material, causing the creation of

chloramines to occur. This can produce a prolonged antimicrobial effect on the organic material therefore further injuring cells and continuing the efficacy of the leftover antimicrobial, however this is also considered toxic for human consumption (Fabrizio et al., 2002).

This issue has also caused chlorine dioxide to gather notice due to its antimicrobial prevalence and manifestation when surrounded by high levels of organic matter. Chlorine dioxide, another chlorine-containing antimicrobial, tends to be more soluble in water than regular chlorine and does not create chlorinated organic compounds the way chlorine does. When utilized, acidified sodium chlorite yields active chlorine dioxide, which demonstrates tremendous bactericidal action in combination with acidity (Elano et al., 2010).

It was also observed that when using a spray treatment, electrolyzed water performed more efficiently when introduced to organic material if exposure time is extended. Fabrizio and Cutter (2004) showed the effects of various solutions when sprayed onto pork, including electrolyzed water, lactic acid, distilled water, and chlorine. It was stated that the absence of antimicrobial activity on the pork from the electrolyzed water solution was probably due to "insufficient contact time". In other studies, King et al. (2001) found that electrolyzed water seemed to work effectively on biofilms when the material was treated for approximately 300 seconds. Fabrizio et al. (2002) found that electrolyzed water can be effective at treatment times of up to 40 minutes. Although when treatment time was increased, it doesn't always show a significant reduction in microbial viability. Fabrizio and Cutter (2005) inoculated frankfurters and ham surfaces with *Listeria monocytogenes* and treated them for up to 30 minutes with acidic electrolyzed oxidizing water. Although a decrease in the pathogen did occur, within almost every combination of treatment performed, there was less than a 1-log reduction, which is not considered a substantial reduction for RTE foods, especially RTE meats. It is also possible that some portion of these small reductions were due to the physical removal of the cells being washed away by the spray treatment (i.e., spray dislodgement) rather than being killed by solution

lethality. The study also pointed out that although increasing the treatment time resulted in a slightly greater reduction, that increased processing time is considered expensive (i.e., "time is money"). Processors may have minutes, and more likely only seconds (for commercial conditions) to treat RTE meat surfaces with an antimicrobial prior to packaging (Fabrizio and Cutter, 2005).

Storage conditions can also adversely disturb the chemical and physical properties of electrolyzed water. Rahman et al. (2012) found that when electrolyzed water was stored open, agitated, and in diffused light conditions, it resulted in an increasingly detrimental loss of chlorine. Cui et al. (2009) observed storage conditions of neutral and acidic electrolyzed water were closely and monitored four different storage conditions for 30 days. They found that although pH values of every sample waivered minimally the oxidizing reduction potential for the neutral electrolyzed water increased significantly for both open and closed treatments being tested. They also found that although the oxidizing reduction potential of the acidic electrolyzed water retained in closed containers didn't change over the 30 days, the samples with open storage decreased by 22% and showed no bactericidal activity (available chlorine actually dissipated after 6 days). Closed containers should be used when working with electrolyzed water, since this can influence the physicochemical properties of the solution and help to minimize chlorine loss and lengthen the lifespan of the antimicrobial solutions (Cui et al., 2009).

Fabrizio and Cutter (2005) also observed a "bleaching" reaction on the surface of meats using electrolyzed water. This is due to high ORP level solution that oxidizes the pigments on the surface of meat, therefore changing its color (Fabrizio and Cutter, 2005). Electrolyzed water at a near-neutral pH did not reduce meat color as much and was much more safe due to its ability to minimize corrosion on equipment and surfaces, as well as human health risks were lessened since the concern of Cl_2 off-gassing is also reduced (Guentzel et al., 2008).

In the same study it was discovered that low concentration electrolyzed water appears to have the beneficial potential of retaining adequate antimicrobial activity while containing low available chlorine. They also discovered that low concentration electrolyzed water has a moderately unwavering shelf life in closed storage conditions as well as showed abundant bactericidal activity against *E. coli* O157:H7 and *L. monocytogenes*. More neutral, low concentration electrolyzed water (pH 6.8-7.4) has demonstrated sufficient antimicrobial effects against microorganisms while in cell suspensions as well as pathogens and other spoilage organisms found on vegetables, poultry, and meat (Rahman et al., 2012).

There are many advantages of electrolyzed water, two of these include the fact that it can be effortlessly produced prior to use while also being inexpensive since it consists of water and 0.1% NaCl (Mahmoud, 2007).

Bacteriophage

Bacteriophage are essentially 'bacterial viruses', which obtain the ability to infect specific, susceptible strains. Generally, bacteriophage attach to specific 'phage receptors' on the cell surface and inject their phage DNA into the host cell. Bacteriophage infection of a bacterial cell can result in one of three infection scenarios: aborted infection, lysogenic infection, or lytic infection (Fig. 1). Aborted infection is when the host restriction modification genes identify the infected bacteriophage DNA as foreign and digest it. Lysogenic infection occurs when the bacteriophage DNA is incorporated into the chromosome of the host cell, which is then propagated along and carried within the host chromosome. In the lytic infection scenario, the bacteriophage DNA replicates after infection, therefore creating more phage DNA that becomes encapsulated into new bacteriophage particles, and then the eventual host cell death due to the lytic release of the phage particles. In this infection, 'burst size' can be measured by the amount

of bacteriophage released into the (ranging from just a few phage to as many as 20-40). This significant measurement is due to the fact that freshly released bacteriophage from the lytic infection process possess the ability to move on and re-infect other neighboring host cells. The rate at which phage are able to reproduce and re-infect cells can easily outrun the rate it takes bacterial cells to replicate.

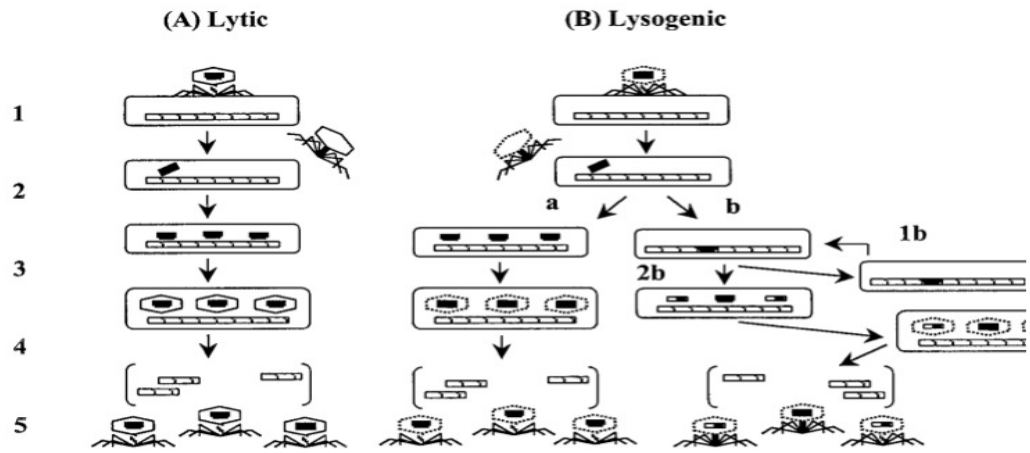

Figure 1. Lytic and Lysogenic Bacteriophage Cycle (Sulakvelidze et al., 2001).

Lytic bacteriophage were discovered separately in 1915 and 1917 by Frederick Twort and Felix d'Herelle (Duckworth, 1976). Research of bacteriophage ignited during the early 1920s, and it was believed before the use of antibiotics that bacterial diseases and infections could be healed by the use of phage treatment; this became known as the "Twort-d'Herelle phenomenon" or the "bacteriophage phenomenon". Development of therapeutic phage for human use occurred commercially in the United States until the widespread use of antibiotics become more convenient (Sulakvelidze, 2001). When this occurred, use of phage as a protective treatment was somewhat forgotten in the U.S. and Western Europe while the practice continued in other places such as the former Soviet Union and in Eastern Europe (Sulakvelidze, 2005).

As the occurrence of more and more antibiotic-resistant bacteria ensued, an interest in phage research resurfaced. With the help of innovative technology that wasn't available when bacteriophage were first discovered, scientists have been able to improve their understanding of the bacterial virus' properties and mechanisms (Sulakvelidze, 2005). Bacteriophage may offer highly specific and effective biocontrol of pathogens. Large-scale pharmaceutical companies have started focusing a majority of their research towards the development of treatments such as bacteriophage that may serve as alternatives to antibiotics (Sulakvelidze, 2005).

The concept of using phage applications against spoilage bacteria and pathogens in foods has received increasing interest during the last years (Guenther et al., 2012). Many chemical sanitizers are corrosive to equipment surfaces and/or affect the aesthetic appearance of the food product, and therefore are unacceptable for treating foods or surfaces that come into direct contact with food. New approaches are needed to aid the prevention of diseases caused by natural or intentional spreading of pathogenic bacteria and lytic bacteriophage may provide one such approach (Abuladze et al., 2008).

Lytic bacteriophage are now available commercially for both '*in vivo* and *in vitro* antipathogenic interventions' (Greer, 2005; Sulakvelidze, 2001; Gross, 2011). Due to the recent heightened interest there is now the option for biocontrol of unwanted pathogens with the use of phage or phage products in food production (Hagens and Loessner, 2010). Companies such as Intralytx Inc. (Baltimore, MD), have developed technology that takes advantage of lytic bacteriophage in food safety, but one of the big issues is the ability of bacteriophage to work on solid food surfaces where their mobility maybe limited.

Phage can be used as a natural antimicrobial method to reduce bacterial pathogens from the food supply (Viazis et al., 2011). A mixed *Listeria* phage preparation became approved for food additive production in ready-to-eat meats and poultry while also a phage preparation

comprised of a virulent single *Listeria* phage received "GRAS" (generally recognized as safe) status for its use in all products (CFR, 2012). There are also phage preparations that are active against *E. coli* with approval of being sprayed, showered, or "nebulized" on cattle and chickens prior to slaughter (Hagens and Loessner, 2010). Guenther et al. (2012), found that *Salmonella* phage were able to reduce counts in foods at a storage temperature of 15°C by up to 5 log units. The phage can also suppress *Salmonella* counts below the detection limit in food stored at 8°C. The use of bacteriophage in combination with other antimicrobial methods has also started to be researched as well. Viazis et al. found data that suggests *E. coli* O157:H7 specific phage combined with TC (*Tran*-cinnamaldehyde, an essential oil) *can* be a potential intervention against foodborne pathogens (Viazis et al., 2011).

In phage applications, it is essential to use virulent, non-integrating, lytic, non-transducing bacteriophage for biocontrol of pathogens (Hagens and Loessner, 2010). Most research performed over phage infection has been done in liquid form, with dense pure cultures of highly permissive host bacteria. Critical host cell concentration threshold is approximately 10^5 cells per ml, but even a very small initial number of phage can cause complete "lysis" of the bacterial culture in a relatively short time frame as long as those particular phage come into direct contact with the bacterial culture (Hagens and Loessner, 2010).

When phage are used against pathogens in food matrices, a completely different set of premises must be taken under consideration. A sufficiently high number of phage is required to hit and infect the few bacterial target cells present, therefore a low number of bacteria are unlikely to be affected by low numbers of phage, this is because phage and bacteria are unlikely to meet. Hagens and Loessner (2010) use the comparison of an apple-sized phage encountering a human who is scuba diving very deeply in Loch Ness of Scotland (which has a volume of 7 km^3) to proficiently describe how long it could take an individual phage to find an individual bacterium in 1 ml of fluid. Basically, the number of phage used in any application must be sufficiently high

to ensure that rapid contact of the bacterium and phage can actually occur (Hagens and Loessner, 2010).

Occasionally in a food-related application, the issue is if the doubling time of bacteria replication is shorter than the time necessary to achieve an infection and kill a bacterium, then the number of bacteria will initially increase in spite of phage presence. The bacteria will continue to remain present if the phage are not allowed to reach a critical number in order to exponentially replicate. Therefore, a sizeable number of phage is necessary to achieve both infection and a fast/significant drop in bacterial viable counts (Hagens and Loessner, 2010).

Phage application must be designed to follow the simple rules of distribution and diffusion in order to be effective. Factors such as pH greatly impact the strength of the phage, a low pH may deleteriously affect the ability of some phage to persist and exert their antibacterial activity in some foods (Abuladze et al., 2008). It has been discovered that the optimal time point of application is likely at (or very close to) the moment bacterial contaminants enter the "food matrix", thus, phage application would be best used by food processors (Hagens and Loessner, 2010). Mechanism of application is also going to result in various efficacies. Due to the fact that phage survival isn't proficient in inconstant environments, dipping or washing treatments may not be the best route of application. This could actually result in rapid deterioration of the phage and therefore halting any further antimicrobial activity (Hagens and Loessner, 2010).

A large concern among consumers has been where bacteriophage actually come from, and if they are really safe for human use, especially in regards to consumption. Our environment naturally holds a large abundance of phage particles. For example, aquatic environments hold the most with 10^9 phage per milliliter in freshwater environments and 10^7 phage per milliliter in marine surface systems. Fermented foods have also been known to have exceptionally high numbers of phage as well as fresh vegetables (Hagens and Loessner, 2010). *E. coli*

bacteriophage have been recovered from sewage, waste water, polluted rivers, fecal samples of humans and animals (Viazis et al., 2011). They have also been discovered in fresh chicken, pork, ground beef, mushrooms, and other foods with counts as high as 10^4 phage per gram (Hagens and Lessner, 2007). In regards to consumption safety of the phage, there have been studies, where both animals and human volunteers were used, the results showed no significant negative effects on subjects after being fed high doses of bacteriophage (Hagens and Loessner, 2010). Another amazing aspect of the study is that *E. coli* phage fed to subjects seemed to have little effect on the *E. coli* occurring in the natural gut ecological systems. This seemed to occur because the commensal *E. coli* populations live in "niches" not easily accessible by the phage. This refers back to the fact that bacteriophage occur naturally in our environment including on the foods that we eat. It is sometimes hard to believe that we consume large amounts of phage every day, even if our diets consist of unspoiled and fresh foods (Hagens and Loessner, 2010). Abuladze et al. (2008), found that the consumption of *E. coli* specific phage (by eating foods on which it has been applied) is unlikely to alter the microbial balance of the gastrointestinal tract.

In conclusion, bacteriophage offer a number of desirable properties in regards to food safety. They are designed to kill their host cells, are usually highly specific, they do not cross species or genus barriers, they are self-replicating and self-limiting, and they are ubiquitously distributed in nature (Guenther et al., 2012). Phage can play an important role in biocontrol of pathogens found in food, and with the use of increased research and experimentation, they could be considered ideal antibacterial agents for food use. More importantly, the approach of using bacteriophage to reduce contamination of foods by bacterial pathogens may be one of the most environmentally friendly and natural approaches for reducing the incidence of food-borne disease. Phage may also be useful for decontaminating food processing plants and other buildings and facilities naturally or intentionally contaminated by pathogenic bacteria (Abuladze

et al., 2008). It is expected that additional phage products will arise and continue to be in the market in the near future (Hagens & Loessner, 2010).

Phage resistance

One of the most frequent questions regarding phage and their safety towards the environment is the concern of phage resistance (Hagens and Loessner, 2010). The mechanisms of potential bacterial resistance against phage differs from their resistance towards antibiotics. This allows phage use to not affect the vulnerability of bacteria towards antibiotics while also preventing the unlikely selection for phage resistance in 'untargeted species' (Viazis et al., 2011). The combination of both antibiotics and phage can actually help with struggle towards eliminating antibiotic-resistant bacterial pathogens in clinical applications (Sulakvelidze, 2005).

If phage-resistant bacterial mutants were to arise, the only way they would present a threat is if they were able to take refuge in an environmental niche. If this were to happen in a food application environment, the number of bacterial cells per weight unit would need to be extremely high for such a mutation to actually develop into a significant issue. This is very unlikely (Hagens and Loessner, 2010).

Additionally, application of phage directly to a batch of food may cause further dilution and potentially lead to a rise in bacterial resistance, but consistent decontamination of equipment in the areas where phage is constantly used can actually prevent the development of any kind of resistance (Hagens and Loessner, 2010). When food alone is treated resistance is actually not as much of a problem. Usually processing environments have taken extra care to ensure that phage pressure is minimal in potential niches and reservoirs. The use of potent chemical sanitizers, although not appropriate for use on foods, can be a highly efficient and affordable method when disinfecting the areas where phage resistant bacteria may reside (Hagens and Loessner, 2010).

Effectiveness of a phage treatment is not likely to be subjective to any potential phage-resistant bacteria (Hagens and Loessner, 2010).

It is important to understand that although the rise of phage-resistant bacteria is very unlikely, phage used should still have an adequately extensive variety of hosts, and the rotation of different phage is crucial when wanting to prevent any selection of potential phage-resistant strains for the future (Hagens and Lessner, 2010). Specific bacteria phage susceptibility should be closely supervised as well as consistent renovation of phage to prevent any potential resistance (Sulakvelidze, 2005). Abuladze et al., states that the emergence of phage-resistant mutants is not perceived as a serious problem for practical applications in sanitization of food processing areas.

CHAPTER III

METHODOLOGY

Bacterial strains.

For the following types of experiments, mixed 'cocktails' of either *E. coli* O157:H7 or *Salmonella* were used:

Experiments involving ozone and electrolyzed water.

A mixture of four different *E. coli* O157:H7 strains were used: ATCC 43890 (California outbreak isolate from human feces), ATCC 43894 (Michigan outbreak isolate from human feces), ATCC 43895 (hamburger isolate implicated in human outbreak), and ATCC 35150 (human feces, clinical isolate).

Experiments involving E. coli O157-specific bacteriophage (i.e., EcoShield^{Tm}).

A mixture of *E. coli* strains ATCC 43894, 229 Na1 R/25, 230 Na1 R/25, and 231 Na1 R/25 were used.

Experiments involving Salmonella-specific bacteriophage (i.e., SalmoFresh^{Tm}).

A mixture of six different *Salmonella* serotypes were used: *S. enterica* ser. Thompson 120, *S. enterica* ser. Heidelberg F5038BG1, *S. enterica* ser. Montevideo FSIS 051, *S. enterica* ser. Hadar MF60404, *S. enterica* ser. Enteritidis H3527, *S. enterica* ser. Typhimurium H3380.

All cultures were transferred at a 1:100 dilution from thawed frozen stocks at -20°C into 9 ml of sterile Tryptic Soy Broth (TSB). After inoculation, the tubes were incubated at 30°C for 24 hours, and then transferred a second time before use. All *E. coli* strains used in the following experiments were resistant to rifamycin SV (10 µg/ml; MP Biomedicals LLC, Solon, OH) and gentamycin (10 µg/ml; Amresco, Solon, OH). *Salmonella* strains used in this study were resistant to spectinomycin (10 µg/ml; Enzo Life Sciences, Inc., Farmingdale, NY) and novobiocin (100 µg/ml; Sigma Chemical Co., St. Louis, MO). The use of antibiotic resistant bacteria and the use of antibiotics in plating media allowed the enumeration of inoculated strains on food products that were not sterile and excluded the enumeration of indigenous bacteria.

Washed versus unwashed cells.

In experiments with ozone, bacterial samples were washed prior to inoculation of meat to compare with trials of cells that remained suspended in growth media. Washed cultures were not used when working with bacteriophage or electrolyzed water. Cultures were 'washed' by centrifuging them in a Sorvall RC 5C plus centrifuge for 15 min at 14,000 RPM (SS-34 rotor), decanted, and re-suspended in sterile water. This process was repeated three times.

Meat preparation and inoculation.

For ozone and electrolyzed water experiments, beef roasts were purchased fresh from a local Wal-Mart beef case (Stillwater, OK). The roasts were sliced into sample discs of 20.25 cm^2 (2-inch diameter) using a 8512 Univex- Max slicer (Univex Corp., Salem, NH.) and a 2-inch diameter stainless steel coring cylinder. For bacteriophage experiments, beef and fat pieces were hand cut from "trim meat" obtained from the FAPC meat pilot plant into samples of similar size. Meat samples were sliced, stored frozen, and then defrosted prior to use for experiments. Sample discs were inoculated with 100 µl of washed, or unwashed cells, of freshly grown overnight culture (no dilution), which was spread evenly across the surface of each piece with a sterile

gloved finger. After inoculation, samples were allowed to sit at 5°C for 30 min to allow for bacterial attachment.

Spray systems.

Five types of spray systems were used in our experiments. The first was with the Ross Industries blade tenderizer with integrated antimicrobial spray intervention system applied at 40 psi (Fig. 2). This spray system was partially used for experiments involving only ozone. It utilized stainless steel and plastic kynar nozzles.

Figure 2. Ross industries spray system. Stainless steel and kynar spray nozzles.

The second type of spray nozzle was a Delta showerhead (Fig. 3) that could be attached directly to the ozone machine for a pressurized spray (in house water pressure) or by attaching a funnel, which would allow a non-pressurized gravity drip for application of various solutions.

Figure 3. Delta showerhead connected directly to ozone generator (left) and Delta showerhead gravity deluge (right).

The third type was connected to an air-assisted, automatically timed, fine mist spray system in which the treatment solution was supplied by a pump and reservoir (Fig. 4) while being expelled by a pressurized air source (40 psi) for any designated time entered into the digital controller at a time.

Figure 4. Pump and reservoir utilized in air-assisted automated spray system. Air-assisted spray nozzle.

The fourth type was a single-nozzled sprayer (Fig. 5) in which the treatment as supplied by the same reservoir and pump setup, but without additional air assistance and solutions were again sprayed at 40 psi.

Figure 5. Single nozzle spray utilized in pressurized EW application.

The third and the fourth spray systems produce a mist-like spray over a larger surface area. The third system used a stainless steel fine mist pressurized air-assisted nozzle (Fig. 4) while the fourth system used a plastic spray nozzles (Fig. 5). The plastic nozzle was an attempt to reduce potential off-gassing of chlorine from the the electrolyzed water solution during antimicrobial spray treatments because it released a coarser spray.

The fifth type of spray system involved manual, hand-held spray bottles (Fig. 6) that were purchased from the local Wal-Mart (Stillwater, OK). The use of the reservoir-pump-manifold system to spray bacteriophage required too much bacteriophage solution that much was 'wasted' due to the volume of solution required to fill the reservoir and to purge the system after a water wash. We therefore resorted to the use of handheld spray treatments to minimize waste of our bacteriophage stock solutions that could only be diluted 10-fold from the concentrates we received from the manufacturer (Intralytix; Baltimore, MD).

Figure 6. Handheld spray bottles for bacteriophage manual spray application.

Ozone

A portable MPI-300 ozone generator (Fig. 7) was supplied by Del Ozone (San Luis Obispo, CA) for use with our studies at Oklahoma State University (Robert M. Kerr Food and Ag

Products Center, Stillwater, OK). In-house tap water was connected to the MPI-300 generator, and exits through an exit valve, which partly sends some of the stream through an in-line digital ozone meter while the main portion of the stream exits via the exit hose. An Analytical Technology Inc. Dissolved Ozone digital monitor (Model Q45H; Collegeville, PA) monitored the ozone concentration (ppm ozone) and temperature of the solution via a membrane-covered polarographic sensor. The MPI-300 generator also had a rheostat type dial (0-100 scale) to adjust ozone output concentration (ppm) and a flow meter to determine the output volume (gpm), both of which were affected by hose backpressure. Ozone concentration was affected by both the ozone rheostat dial and the flow meter lever as well as a change in the type of exit nozzle or spray wand which would affect flow throughput (i.e., a smaller spray nozzle opening that would slow the flow would also hold up the solution in the generator and cause a higher output of ozone ppm and visa versa).

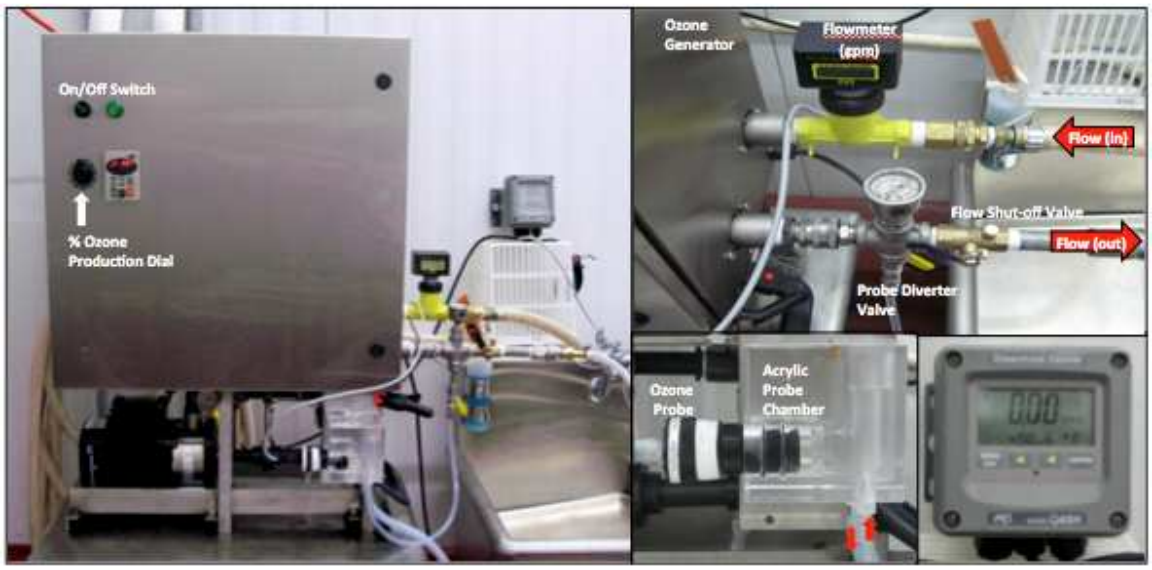

Figure 7. Portable ozone generator.

The digital ozone meter was periodically calibrated by manual ozone analysis performed with the Accuvac Ozone, High Range Hach Test Kit (calibration of the sensor was performed according to the meter manufacturer) (No. 25180-50; Loveland, CO.).

Calibration of the sensor was achieved according to directions in the ATI Q45H/64 dissolved ozone system manual (i.e., Part 7- Calibration). In brief, the ozone generator was set to produce ozone at a low concentration within the range of the manual titration kit. After analysis, the digital ozone meter 'calibration point' was then set according to the determined ozone level.

Determination of the half-life of ozone solution.

The half-life of ozone was determined using the self-contained in-line ozone meter which contains an acrylic chamber fed by a side port of the main exit line and enters the bottom of a cylindrical chamber that is connected to the horizontal ozone probe (Acrylic probe chamber, Fig. 7). Ozone half-life was determined by recording ozone measurements at 1-min intervals while flow of fresh ozone was stopped to the ozone meter.

Effect of ozone on bacterial cultures (washed and unwashed).

E. coli O157:H7 cultures (ATCC 43890, 43894, 43895, and 35150) were grown overnight, combined in equal quantities, and washed three times to remove media/protein. A 100-µl aliquot of the final washed culture was placed into five sterile glass test tubes into which ozone solution would be added. In order to obtain an ozone sample at the same level that would be used for inoculated meat testing, we chose to obtain it as it exited the spray nozzle, without losing ozone due to off-gassing, ozone was obtained from a small plastic "trough" with holes (to allow liquid to be expelled) in which the digital ozone meter was configured so that the exact level of ozone could be quantified for the ozone sample being tested and allowed the liquid solution to continuously flow out of the trough as it was flowing in (Fig. 8).

Figure 8. Plastic trough with holes at bottom to allow continuous exit of ozone as it flows into the system.

Using a sterile plastic pipette, 5 ml of solution was extracted from the trough and immediately added into one of the tubes with 100 µl of washed bacterial culture. This was repeated for triplicate replication of samples. The same procedure with water obtained in a similar manner was compared with water solution, but without the ozone generator turned on and with the ozone meter reading 0.00 ppm. Although the in-line ozone meter measures the ppm of ozone solution as it was being generated, by the time it exits through the spray nozzles it could be as much as 2 ppm lower. In this experiment ozone being released from the showerhead was approximately 4 ppm.

Tubes sat for 10 min after treatment, and were then diluted with 0.1% Buffered Peptone Water (BPW), plated on TSA containing rifamycin and gentamycin, and incubated in 30°C for 48 hours and then colonies were counted using a darkfield colony counter.

An additional assay was performed to examine the effect of ozone on washed and unwashed *E. coli* O157:H7 cells. *E. coli* strains were grown overnight and either kept in their original broth media or washed as stated in the prior description. Then 100 µl of culture (washed or unwashed) was placed in a sterile tube and then either 9 ml of water or 9 ml of ozone was

added to the tube. Tubes were allowed to sit for 10 min before being diluted using DE Neutralizing broth and plated on TSA containing rifamycin and gentamycin. Plates were then incubated in 30°C for 48 hours and then counted using a darkfield colony counter (Fig. 11, Part A).

Effect of growth media on ozone concentration.

In order to assess the effect of media directly on ozone, we added media directly to the chamber attached to the ozone machine with the probe inserted. The probe diverter to the chamber (Fig. 7) was switched off so that any remaining ozone was allowed to remain in the chamber. Either 1 ml of tryptic soy broth or 1 ml of water was added directly to the chamber and changes in ozone levels were recorded.

Effect of ozone on inoculated beef.

Pieces of beef were trimmed into round discs (20.25 cm^2, 2-inch diameter) and inoculated with 100 µl of the washed, or unwashed, inoculum culture of four *E. coli* O157:H7 strains (ATCC 43890, 43894, 43895, and 35150). Samples were placed at 5°C for 30 min to allow for bacterial attachment. Inoculated beef discs were treated with ozone by the methods characterized below. After treatment, 2 individually treated beef discs were placed in one sample bag, to which 40.5 ml of DE Neutralizing broth was applied, and then stomached using a Seward 400 Laboratory stomaching blender (Tekmar Company; Cincinnati, OH) for 2 min (60 seconds on each side of the bag). Beef-inoculated samples treated with ozone and stomached in DE Neutralizing broth were then diluted with 0.1% BPW, plated on TSA agar plates containing rifamycin and

gentamycin, and incubated at 30°C for 48 hours. After incubation, plates were enumerated using a darkfield colony counter.

Ozone treatment using the Ross Industries spray system.

Beef pieces were inoculated with washed cells and treated two at a time with either water (control) or ozone (treatment solution) through the Ross Industries blade tenderizer integrated antimicrobial spray system utilizing stainless steel nozzles. Timed spray treatment with the Ross spray system was performed by placing inoculated samples directly under the spray nozzles which were then removed manually at desired time intervals and not by using the conveyor belt. Treatment groups were divided into 15- and 30-sec applications of either water or ozone. All water (control) treatments were run in duplicate replication using paired samples. All ozone treatments were run in triplicate replication using paired samples. After treatment, samples were placed in stomacher bags and allowed to sit on ice until brought back to the lab for processing (i.e., 15 min). DE Neutralizing broth was then added to each bag and processed as stated above.

The same experiment was performed again using a set of plastic kynar nozzles. Stainless steel nozzles released a mist-like spray that may result in off-gassing of ozone solution. The plastic kynar nozzles had a larger nozzle pore size and would reduce any potential to release ozone gas.

Ozone treatment using a pressurized and non-pressurized Delta showerhead spray nozzle.

In order to evaluate different spray nozzle mechanisms and treatments, a showerhead application was used to allow a larger spray orifice for the ozone solution to flow through. Inoculated rubber discs were subjected to one of three different treatments. The treatments were divided up as: a) inoculation control (no treatment), b) spray control (water/30 sec), c) and lethality spray (ozone/30 sec). Ozone concentrations varied between 5-7 ppm through the showerhead that was attached directly to the ozone generator output hose. These trials were

performed in triplicate replication using paired samples for each replication (i.e., 6 samples total). After treatment, samples were processed as stated earlier.

Based on the results from the pressurized showerhead spray, we examined the effect of using a non-pressurized, gravity fed deluge system in combination with the Delta showerhead as a means of testing the effect of maximum ozone levels with the least loss of ozone due to off-gassing. Inoculated felt pieces were again run in duplicate replications with paired samples (water controls) or triplicate replications of paired samples (ozone treatments) using the Delta showerhead with reservoir funnel attached to allow a gravity deluge rather than a pressurized spray. Treatment groups were divided into 100 ml, 200 ml, and 300 ml applications of either water or ozone. After treatment samples were processed as stated above.

Based on the results from the previous studies, a further test was performed to investigate the effects of ozone (100 ml, 200 ml, and 300 ml) on organic materials. The experiment was then repeated using inoculated beef discs.

Electrolyzed water produced by Ultra-Lyte (Clarentis; Palm Beach Gardens, FL) generators.

Electrolyzed water treatments consisted of two different solutions: Catholyte and Anolyte (hypochlorous acid, also known as Ultra-Lyte™). Both solutions were produced with Ultra Lyte™ equipment (Clarentis LLC; Richaland, WA) and were shipped to Oklahoma State University by Johnson Diversified Products (Saint Paul, MN). Catholyte is electrochemically activated water containing 0.5% NaCl and 0.015% sodium hydroxide. It has a pH of 12.0±3 and Oxidation Reduction Potential (ORP) of -900±40mV. Anolyte is composed of 99.5% water, 0.45% sodium chloride, and 0.046% hypochlorous acid/sodium hypochlorite. Anolyte was sent to us with an original pH that ranged from 6.3-6.7.

Electrolyzed water total chlorine concentration and pH analysis.

Prior to all studies, pH and total chlorine concentration was measured for anolyte solution. The Hach Total Chlorine test kit (Cat. No. 24711-00) was used as instructed by the supplier to measure total chlorine of the solution. Steps taken for titration of anolyte were found in the kit manual; the iodometric method was used to determine the mg/L of total chlorine in the solution. This was performed by adding 5 ml of anolyte to 45 ml of deionized water and transferring the mixed solution into the provided Erlenmeyer flask. The contents of a potassium iodide pouch and a dissolved oxygen pillow were then added to the flask and stirred until all contents dissolved. A clean delivery tube was attached to a sodium thiosulfate cartridge to initiate titration of the test solution. The flask was swirled while slowly adding the contents of the cartridge until the anolyte solution turned a pale yellow color. Once this occurred, 10 drops of a starch indicator solution was added until it turned a dark blue. The tip of the titration cartridge was placed back into the flask and titrated until the solution turned clear. The digit on the counter was then recorded to calculate mg/L of total chlorine (Cl_2). A Hanna Instruments (Smithfield, RI) pHep Tester (pocket pH tester) was used to determine the pH of both the anolyte and catholyte solutions. The tester was first calibrated by using the provided Hanna pH solution packets.

Electrolyzed water effect on washed vs. unwashed Cells

The bactericidal efficacy of electrolyzed water was evaluated directly on our cultures prior to testing inoculated meat samples. Four strains of *E. coli* O157:H7 (ATCC 43890, 43894, 43895, and 35150) were grown over night at 30°C and then equal volumes of each strain were mixed and split into two portions. One portion of the mixture was washed (centrifuged, decanted, and re-suspended in sterile water). The other half of the mixture was left as is, in its own culture media broth. Sterile tubes containing 100 µl of either the washed or unwashed mixture were divided into several groups: water (5 ml), catholyte (5 ml), anolyte (5 ml), and catholyte/anolyte

(2.5 ml of each solution). We tested two replicative samples for each treatment groups using both washed and unwashed cells.

A 5 ml sample of each treatment solution was added to their specific tube, containing 100 µl of washed or unwashed cells and was gently mixed, and samples were allowed to sit for approximately 10 min. The tubes were then diluted, plated on TSA plates containing rifamycin and gentamycin, and incubated at 30°C for 48 hours before being counted on the colony counter. The anolyte solution held at room temperature (22°C) had a pH of 6.8 and 581 mg/L total chlorine prior to experiment.

Effect of electrolyzed water on inoculated beef via (non-pressurized) gravity deluge showerhead.

Four *E. coli* O157:H7 strains (229 Na1 R/25, 230 Na1 R/25, 231 Na1 R/25, ATCC 43894) were grown over night and mixed in equal portions. Pieces of beef were trimmed down into 2-in. diameter discs (20.25 cm^2) and then inoculated with 100 µl of the mixed inoculum culture. Samples were then allowed to sit at 5°C for 30 min to allow bacterial attachment.

Samples were divided into various treatments: inoculated sample (no treatment), water spray control (500 ml), a double water control (500 ml + 500 ml), catholyte (500 ml), anolyte (500 ml), catholyte plus anolyte (500 ml + 500 ml). Treatments were performed in duplicate with 2 pieces of beef for each sample replication.

Sample pieces were treated (two discs at a time) via a gravity showerhead nozzle. Once treated samples were placed in stomacher bags and allowed to sit at 5°C for 15 min. A 40.5 ml aliquot of DE Neutralizing broth was then added to each sample bag containing 2 discs and stomached for 1 minute on each side. Samples were then diluted, plated on TSA plates containing rifamycin and gentamycin, and incubated at 30°C for 48 hours before being counted using a darkfield colony counter.

Prior to the experiment, measurements were made for pH, total chlorine content, and temperature. The pH of both solutions was the same before and after being put through showerhead (anolyte: 6.0 pH; catholyte: 12.0 pH). Titration of anolyte resulted in a measurement of 776 mg/L total chlorine (same before and after). The temperature of both anolyte and catholyte were recorded at 22°C.

The same experiment was repeated to analyze the effect of using heated anolyte and catholyte solutions. For trials with heated solutions, the four *E. coli* O157:H7 strains (ATCC 43890, 43894, 43895, and 35150) were prepared as described previously. After inoculation of beef pieces, samples sat at 5°C for 30 min to allow bacterial attachment.
The catholyte, anolyte, and water solutions were heated to 105°F (41°C). Treatments were performed in duplicate replications and samples were processed as stated in previous experiment.

Prior to experiment, measurements were made of solutions for pH, total chlorine content, and temperature. The anolyte at room temperature (22°C) prior to spray treatment had a pH of 6.4 and 629 mg/L total chlorine. Post spray anolyte at room temperature (22°C) had a pH of 7.1 and 584 mg/L total chlorine. After being heated (40°C) anolyte had a pH of 6.1 and 533 mg/L total chlorine, but post spray heated solution (40°C), the pH of anolyte was 7.2 and total chlorine concentration was 492 mg/L.

Effect of heated electrolyzed water on inoculated beef via a pressurized spray system

Four strains of *E. coli* O157:H7 (ATCC 43890, 43894, 43895, and 35150) were prepared as stated previously. Pieces of beef were trimmed down into 2-inch discs (20.25 cm^2) and surface inoculated with 100 µl of the mixed inoculum culture. Inoculated samples were placed at 5°C for 30 min to allow bacterial attachment. Samples were divided into groups: inoculated sample (no treatment), water (30 sec), water (30 sec + 30 sec), catholyte (30 sec), anolyte (30 sec), and catholtye (30 sec) plus anolyte (30 sec). A 30-sec treatment from the single nozzle spray

pressurized system sprayed approximately 460 ml of solution. The catholyte, anolyte, and water solutions were heated to 147°F (64°C) in order for the solutions to hit the beef samples at approximately 112°F (44°C) after cooling off while going through the spray system. Each treatment had triplicate replications and each replication consisted of two samples of beef. Once treated, samples were stomached, diluted, and plated as stated in previous experiment. The samples were incubated at 30°C for 48 hours before being counted.

Measurements were made for pH, total chlorine content, and solution temperature. The anolyte at room temperature (22°C) prior to spraying had a pH of 5.2 and 725 mg/L total chlorine. After spraying, anolyte had a temperature of 22°C, a pH of 6.1, and 677 mg/L total chlorine. After heating anolyte had a pH of 5.7 and 714 mg/L total chlorine. Post spray system heated (44°C) pH of anolyte was 5.8 and total chlorine concentration was 653 mg/L.

A final experiment was performed with warmed beef samples (95°F) pre-treated with catholyte prior to inoculation. Attachment time for inoculum to samples was only 15 min because of the warm temperature. This experiment utilized the same *E. coli* O157:H7 cultures as stated previously. Treatments were as follows:

[Pre-inoc. treatment)+(Inoculation period)+(Post-inoc. treatment)]

1. 44°C water + Inoculated (15 min attachment)+ 44°C water plus 44°C water
2. 44°C water + Inoculated (15 min attachment)+ 44°C water plus 22°C water
3. 44°C catholyte+ Inoculated (15 min attachment)+ 44°C catholyte plus 44°C anolyte
4. 44°C catholyte+ Inoculated (15 min attachment)+ 44°C catholyte plus 22°C anolyte

Measurements of solutions were made for pH, total chlorine content, and temperature. Unheated anolyte had a temperature of 22°C , pH of 6.1, and 499 mg/L total chlorine. After spraying anolyte had a temperature of 22°C, pH of 7.1, and 584 mg/L total chlorine. After being heated to 44°C anolyte had a pH of 6.6 and 410 mg/L total chlorine. Following treatment samples were processed as stated previously.

Bacteriophage stock suspensions.

The bacteriophage concentrates, EcoShield^{Tm} (Cat # 07EP, Lot 0709K170114) and SalmoFresh^{Tm} (Cat # 02SP, Lot 0212H2001172), were received from Intralytix Inc (Baltimore, MD). The phage products were shipped to us in 500-ml clear bottles with the instructions to store in a dark place at 2-6°C and were stored in a refrigerator.

Bacteriophage spot and titer assays against test strains of *E. coli* O157:H7 or *Salmonella* serotypes.

A bacteriophage 'spot assay' was performed with bacteriophage stock concentrates, and/or dilutions, on each of our inoculum cultures to insure the sensitivity of each culture to the phage preparations. Cultures used in the phage spot assay, or phage titer assays, were utilized in a soft agar medium at a low culture inoculum level to allow some degree of growth before bacterial indicator lawns became fully grown (i.e., bacteriophage require multiple rounds of bacterial replication for them to elicit a visually-discerning lytic response). All strains were grown overnight in tryptic soy broth (TSB). Then, 100 µl of overnight culture was placed in 5ml of TSB and grown in a Lab-Line® Orbit Environ-Shaker (Lab-Line Instruments, Inc.; Melrose Park, IL) at 275 RPM (35°C) for approximately 2 hours or until the culture grew to an optical density of 0.30 measured at 590nm. Absorbance at 590 nm was measured using a Spectron 20D+ spectrophotometer (Thermo Electron Corporation; Madison, WI.). TSA plates were pre-labeled and placed in an incubator 30 min prior to experiments in order to slightly "warm" the base agar plates while soft TSA overlay agar (0.7% agar) was kept tempered at 48°C until use. Cultures inoculated in broth and shaking were harvested after reaching 0.30 O.D. 590nm and diluted 10^{-1}-10^{-3} cfu/ml using 0.1% BPW. An 'indicator lawn' of each bacteriophage test culture was made by placing 100 µl of each culture dilution into sterile screw cap tubes and adding 8 ml of 0.7% soft agar. The tubes were then shaken lightly to ensure culture and soft agar were mixed. This

mixture was then poured slowly on TSA plates to allow an even distribution. All soft agar lawns were poured in duplicate, and allowed to sit for 15 min in order to cool and solidify. Phage dilutions were made using TSB, and then 10 µl spots of every phage dilution were placed on each plate. After spotting, plates were left alone to absorb spotted solutions for 15 min, and then placed at 30°C (not inverted). Zones of confluent phage lysis, or spots containing individual plaques (if spotting dilutions of bacteriophage preparations), were observed after overnight incubation.

For phage titers, an entire plate was used to indicate the number of phage plaques (i.e., phage infections) per given amount of phage solution. Cultures were grown individually in the shaker as stated previously. Bacteriophage stock suspensions were diluted to approximately 10^4 or 10^3 pfu/ml and 100 µl of undiluted shaker-grown culture was added to 50 µl of diluted phage. The mixture was allowed to sit for approximately 2 min before being added to an 8 mls of soft TSA (0.7% agar) in sterile tubes. Tubes were then gently mixed and poured on top of base TSA plates. The plates sat for a few minutes to allow the top agar to cool and were then placed at 30°C incubator for 24 hours and then counted for pfu (plaque forming units).

***E. coli* O157:H7 and *Salmonella* susceptibility to bacteriophage mixtures in liquid medium.**

Mixed cultures were also subjected to the phage infection in liquid culture to determine the degree of infection on viability with a 15-min infection period. Strains were grown over night and mixed in equal proportions. A100 µl aliquot of either the *E. coli,* or *Salmonella,* cocktail was placed into sterile empty tubes. Tubes were divided into treatments: Water (5 ml) and Phage (EcoShield or SalmoFresh; 5 ml); each treatment had two tubes (two replications).

To each tube containing 100 µl of the *E. coli* or *Salmonella* cocktail (undiluted), 5 ml of each treatment (water or phage) was added to their specific tube, mixed gently, and samples sat for approximately 15 min (at the 7.5 minute tubes were mixed gently again). Tubes were then diluted, plated, and incubated in 30°C for 48 hours and then colonies were counted with a colony

counter to determine the remaining survivor count after the earlier phage infection.

Bacteriophage treatment of inoculated meat via an air-assisted, fine mist spray system.

Four *E. coli* O157:H7 strains (229 Na1 R/25, 230 Na1 R/25, 231 Na1 R/25, and ATCC 43894), or six *Salmonella* serovars (*S. enterica* ser. Thompson 120, *S. enterica* ser. Heidelberg F5038BG1, *S. enterica* ser. Montevideo FSIS 051, *S. enterica* ser. Hadar MF60404, *S. enterica* ser. Enteritidis H3527, *S. enterica* ser. Typhimurium H3380) were grown over night and combined in equal parts to make a mixture. All experiments were centrifuged, decanted, and re-suspended in sterile water. Pieces of beef and fat were trimmed into 2-inch diameter pieces (20.25 cm^2) and then inoculated with 100 µl of the washed culture. The meat and fat samples were then placed at 5°C for 30 min to allow bacterial attachment. Inoculated samples and water (control) treatments were performed in duplicate replication with paired samples per replication. Experimental treatments with phage were done in triplicate replication with paired samples. Each treatment (water or phage) was done with both beef and fat samples and sprayed for 15-sec using an automated digital controller and an air-assisted fine-mist spray nozzle. Each 15-sec spray period was measured to deliver approximately 20 ml of either water or phage suspension. Once treated, samples were placed in stomacher bags and allowed to sit at 5°C for 20 min. A 40.5 ml aliquot of DE neutralizing broth was then added to each sample bag (2 sample pieces) and stomached for 2 min (60-sec on each side of the bag), diluted with 0.1% BPW, plated on TSA plates containing the respective antibiotic, and incubated at 30°C for 48 hours. After incubation, plates were counted using a darkfield colony counter.

Bacteriophage treatment of inoculated meat via handheld (manual) spray bottle.

In prior treatments, use of the air-assisted sprayer required too much working stock of phage suspension on each run do to the amount of liquid required to be present in the reservoir, the manifold tubing, and for purging the system of any prior water. We made the decision to

switch to a small handheld sprayer (i.e., manual spray system), which allowed the use of smaller volumes of phage suspension and calibrated the required number of sprays to deliver the same volume.

E. coli and *Salmonella* strains were grown and prepared as stated above. Cultures were initially used at high concentrations (i/e/ 10^7 cfu/cm^2) and then switched to inoculum levels that were 3 log levels lower (10^4 cfu/cm^2), to try to improve the lytic response. Samples of beef and fat were inoculated as stated in previous experiment. Samples were then divided by treatment: inoculated sample (no treatment), water control (20 ml), or phage (20 ml of 10^9 pfu/ml). Water treatments were performed in duplicate replication and phage treatments were performed in triplicate replication. Each sample treatment consisted of two separate pieces of beef or fat (never combined). Samples were treated with the manual hand held sprayer. Once treated, samples were processed the same as in the previous experiment. The EcoShield experiment was performed on two separate occasions with the exact same treatments and number of samples.

Statistical Analysis

Trials were performed in duplicate (water controls) or triplicate (Ozone, EW, or phage treatment) replications using paired samples within each replication. Samples were serially diluted and plated in duplicate for each analysis. Experimental results were analyzed using a one-way analysis of variance (ANOVA) to determine the level of significance between the effects of each treatment. Pairwise multiple comparisons were completed using the HolmSidak method. All statistical analysis was performed using SigmaPlot (Systate Software, San Jose, CA) at a p-value of 0.05.

CHAPTER IV

RESULTS AND DISCUSSION

We examined the effectiveness of ozone, electrolyzed water, and bacteriophage that could serve as antimicrobial interventions for sanitizing meat surfaces, notably beef carcasses and mechanically tenderized beef. These studies were intended to further the understanding of the potential use of these antimicrobials as possible interventions to reduce or eliminated pathogens on beef surfaces.

Our lab had previously used inoculated beef and fat discs as convenient models to mimic contaminated meat surfaces during spray treatment with chemical antimicrobials. The effect of antimicrobial treatments can be conveniently evaluated on small beef or fat wafers/discs, than on more expensive and larger cuts of beef.

Ozone half-life studies.

Ozone equipment manufacturers often indicate the need to situate ozone generators near the point of use because of the short half-life of ozone. Therefore we evaluated the stability of ozone over short periods of time before performing subsequent experiments. After only 1 min,

ozone fell from 4.8 ppm to 3.5 ppm (73% of initial) and by the 5-minute mark ozone levels fell to less than half of initial levels (Fig. 9). Based on these results, we felt compelled to use ozone either directly from the ozone generator or as soon as possible after it was generated.

Effect of ozone on washed and unwashed bacterial cultures.

The efficacy of ozone was evaluated directly on our bacterial cultures to confirm the biocidal activity of our manufactured ozone solution before beef applications would be initiated. Ozone was used immediately in the test tube assay as soon as it was recovered from the Delta showerhead to minimize the amount of ozone depletion by natural decay. Since the showerhead pores are larger than standard spray nozzles, this helped ensure that the ozone we recovered was being used at the highest ppm possible. During discussions with the ozone equipment manufacturer they emphasized the need to use "washed" bacterial cells in antimicrobial assays, although the need to eliminate the presence of protein cannot be averted with actual beef applications. In order to accommodate these concerns we evaluated antimicrobial activity using both washed and unwashed cell inoculums. When of ozone was added to a tube containing washed *E. coli* O157:H7 cells, a complete inactivation of the pathogen occurred. There was no growth on the plates of the lowest dilution of all three samples treated with ozone, resulting in >6 log reduction when compared to the same treatment using water that plated out to 6.3-6.4 log cfu/ml (Fig. 10).

A second trial was performed to assess the effect of washed and unwashed cultures. When unwashed *E. coli* cells were placed in ozone there was no reduction compared to when unwashed cells were placed in water. However, when washed *E. coli* cells were placed in ozone there was a complete elimination of the pathogen resulting in >5 log reduction (Fig. 11, Panel A).

A third assay was performed in which (uninoculated) growth media was placed into the actual ozone chamber with the probe in it (Fig. 11, Panel B). This allowed us to see the potential drop in ozone level as the protein-rich media was added. When first adding water to the ozone chamber the ozone level dropped from 6.6 ppm to 5.7 ppm, likely due to the water diluting the ozone in the chamber. When the same procedure was performed with growth media, the ozone levels dropped from 6.6 ppm to 0.4 ppm (Fig. 11, Panel B). The protein rich-media clearly had a detrimental effect on the ozone. This demonstrated the potential lack of efficacy that can occur when ozone comes into contact with other organic media, such as unwashed cultures and possibly even beef. In order to improve our chances of demonstrating antimicrobial activity on inoculated beef, we used washed bacterial cells for our inoculum in our ozone studies.

Effect of ozone treatment through Ross Industries spray system

Trials of ozone application via the Ross Industries spray system were performed using both, stainless steel and plastic kynar nozzles. *E. coli* O157:H7 inoculated beef discs were treated with a 15- or 30-second application of water or ozone. The results indicated there were minimal differences in reduction between the two treatments, regardless of spray period (Fig. 12). The first study utilized stainless steel spray nozzles with washed culture. There was little or no difference between water and ozone applications (Fig. 12). Yoder et al. (2011) found that when using aqueous ozone to reduce pathogens on beef surfaces, 'the reduction did not differ significantly from a control tap water rinse'. Our results with ozone experiments involving beef confirm the observations made by Yoder et al. (2011). The stainless steel nozzles released a fine mist-like spray while the plastic kynar nozzles released a much more coarse stream of solution to reduce the likelihood of ozone off-gassing when spray exits from pressurized fine bore nozzles. However neither application of ozone through kynar or stainless steel nozzles showed any

practical differences in comparison to spray treatments with water (Fig. 12). These results are another example of the potential instability of ozone when it comes into contact with organic material. The overall reduction of ozone was basically the same (in some cases even slightly less effective) as the application of tap water to the inoculated beef discs.

Effect of ozone treatment through (pressurized and non-pressurized) Delta showerhead.

Additional studies with ozone were performed with the porous Delta showerhead to further minimize the potential off-gassing of ozone gas during spray application of samples. A larger nozzle pore size would seemingly allow for more potent ozone application without loss due to off-gassing. Initial experiments examined the effects of a 30-sec treatment through the showerhead directly connected to the ozone generator (pressurized). Subsequent experiments were also performed with a non-pressurized (gravity) deluge using the same Delta showerhead nozzle. These experiments with the gravity flow showerhead examined the effect of increasing amounts of ozonated solution (100 ml, 200 ml, 300 ml).

We evaluated the ability of ozone to inhibit *E. coli* when delivered by a Delta showerhead using inoculated inert discs (rubber and felt pads). Ozone treatment of inoculated rubber discs resulted in a 0.8-log reduction in comparison to similar rinse treatment with water. Additional experiments with a non-pressurized (gravity) deluge approach were performed on inoculated felt discs and the results showed a steady reduction as amount of treatment solution increased (Fig. 13). The final treatment of 300 ml of ozone resulted in a 1.2-log reduction relative to water treatment. These results showed the efficacy potential of ozone on washed *E.* coli cells on the surface of inert materials.

We applied this same approach of a gravity deluge application through the showerhead on inoculated beef discs (washed cells). In contrast to our results with inert (inoculated) discs,

results from these trials showed no significant difference between water or ozone application of the same spray volume (Fig. 14). No significant difference in cell numbers was observed when sprayed with ozone or water, regardless of volume used. These data indicate that ozone is an ineffective antimicrobial when applied to organic material such as beef.

Effect of electrolyzed water on washed vs. unwashed bacterial cells.

After experiencing the effect of growth media with unwashed bacterial cells in our ozone trials, we were interested to see if a similar phenomenon could be observed with electrolyzed water solutions. We examined the effect of electrolyzed water on washed or unwashed *E. coli* O157:H7 cells. This allowed us to understand if it was crucial for our experiments to utilize washed or unwashed cultures prior to our experiments (similar to the process we had to do for ozone).

Unlike the situation with ozone, our data showed that electrolyzed water could still have a detrimental antimicrobial effect on cultures whether they are washed or not. The protein and organic matter in unwashed cultures did not deter the electrolyzed water (i.e., hypochlorous acid) at all. No viable cells were recovered from treatments with electrolyzed water (anolyte or catholyte), resulting in almost a 7-log reduction with unwashed cells and almost a 5-log reduction with washed cells (Fig. 15). The inhibitory activity observed with unwashed cells was no doubt due to the high concentration of hypochlorous acid used in this study (500-800 ppm Cl-). The intent was to use as high a level as possible in order to obtain acceptable reduction levels of *E. coli* O157:H7 on inoculated beef samples. Once that is achieved we would then use lower levels commensurate with what is allowed on raw beef.

Effect of electrolyzed water on inoculated beef via gravity deluge showerhead (non-pressurized).

The efficacy of electrolyzed water (72°F) was tested using a Delta showerhead (gravity spray treatment) on inoculated beef discs. The application resulted in a 0.22-log reduction when discs were treated with catholyte plus anolyte (Fig. 16). The results of these trials showed that room temperature electrolyzed water was not sufficient to adequately reduce *E. coli* O157:H7 from the surface of raw meats. We therefore considered the use of heated solutions.

Electrolyzed water applied at room temperature did not have a significant antimicrobial effect and therefore, we repeated the experiment with heated solutions. Water, catholyte, and anolyte were heated to 105°F (40°C) using a water bath in the hopes that antimicrobial activity would increase with higher applied temperature. When 500 ml of heated catholyte plus 500 ml of heated anolyte was applied to beef discs, a slight, 0.24 log reduction occurred (Fig. 17). This was similar to the reduction obtained with room temperature solutions, and still not sufficient enough to show that electrolyzed water can serve as an effective antimicrobial on contaminated beef surfaces.

Effect of heated electrolyzed water (112°F) on inoculated beef via a pressurized spray system.

We tested the application of heated electrolyzed water (112°F) through a pressurized single nozzle spray system. This spray system released a constant spray onto samples in 30-sec timed increments. Since the solutions cooled rapidly as they move through the spray system, they were heated to 147°F (64°C) in order to hit the beef surface at approximately 112°F. A 30-sec spray through this system resulted in a 460 ml output of solution.

This experiment was initially performed with *E. coli* cultures used for our bacteria phage experiments (*E. coli* O157:H7 229 Na1 R/25, 230 Na1 R/25, 231 Na1 R/25, and ATCC 43894) and was repeated a second time using the *E. coli* O157:H7 strains used in the ozone experiments (ATCC 43890, 43894, 43895, and 35150). The results obtained from both experiments were averaged together (Fig. 18).

A 0.16-log reduction occurred after 30-sec of heated catholyte plus 30-sec of heated anolyte (Fig. 18). This was still not a sufficient reduction to be considered substantial in the industry.

In a final EW study, beef discs were warmed to 95°F before the pre-treatment of a 110°F application of either water or catholyte. The warming of the pieces was to resemble the warm body of a freshly killed beef carcass. The catholyte pre-treatment was to remove any excess, soluble organic matter that may occur on the meat surface prior to inoculation. A similar pre-treatment is usually performed when spraying environmental processing surfaces with an electrolyzed water treatment. An initial spray of heated catholyte or heated water was first sprayed to help "dislodge" any potential bacteria or other loosely soluble organic materials. After pre-treatment, discs were inoculated with *E. coli* O157:H7 mixed culture and then were treated a second time with the heated catholyte or heated water. The final application was either room temperature or heated anolyte solution. This was to compare their effects since previous experiments displayed that heated anolyte had a 'bleaching' effect on the appearance beef. Trindade et al. (2012) observed a discoloration due to the chlorine found in sanitizing agents on the appearance of chicken carcasses. With the most inhibitory conditions possible (exceptionally high ppm Cl-, warm beef samples, heated anolyte, and pretreatment with catholyte), we were only able to observe a 0.25 log reduction of *E. coli* O157:H7 (Fig. 19).

There was also no substantial difference in between heated anolyte vs. room temperature anolyte. In a study performed in 2008 by Kalchayanand et al. (2008), various antimicrobials were evaluated for interventions on *E.coli* inoculated bovine heads, to help examine a possible pre-evisceration carcass wash. They found that both EW and ozone had less than a 0.5 log reduction. The various experiments covered in our study show that anolyte at levels 10-15x higher than is allowable on meats, and in combination with catholyte, could not serve as an acceptable antimicrobial intervention for beef materials.

Bacteriophage spot and titer assays against test strains of *E. coli* O157:H7 or *Salmonella* serotypes.

The objective of the phage spot and phage titer assays were to find the best combination of phage dilution and *E.coli*/*Salmonella* dilutions, and to evaluate each individual strain and whether one may be more resistant to the bacteriophage than others (Fig. 21, Panels A). "Host specificity is generally found at strain level, species level, or, more rarely, at genus level" (Hagens and Loessner, 2010). In order to find the best combination of phage and bacterial pathogen to perform phage titers, we cross-examined different dilutions of bacteriophage (EcoShield or SalmoFresh) on agar lawns made with different dilutions of each individual pathogen by modifying a phage titer protocol provided by Intralytix (Fig 20). Increasing dilutions of EcoShield or SalmoFresh were "spotted" on different dilutions of pathogenic soft agar "lawns" (Fig. 21, Panels B). A sufficient combination of phage and pathogen dilutions would be the occurrence of a large "plaque" that is made up of multiple visibly smaller plaques.

After performing these bacteriophage assays, and assessing which dilutions of phage and individual strain would result in the most sufficient combination, an additional assay allowed us to plate those individual combinations and count the 'Plaque Forming Units' (i.e., pfu) (Fig. 21,

Panels C). This was determined by adding an amount of phage to an excess of bacteria that are then plated in a soft agar medium; as the phage infect the original cell to which it attaches and causes a lytic response, the released progeny phage will infect neighboring bacteria in the soft agar that are still in the process of growing. This process will continue repeatedly until the bacterial culture stops growing and with sufficient cell divisions, the initial infection can be spotted as a small zone of clearing (i.e., a 'plaque') which are enumerated as an indication of how many phage particles were in the suspension. Use of the same phage stock dilution with different cultures may yield different 'phage titers' because each strain may be either more, or less, permissive to the lytic cycle of the bacteriophage. Some bacteria have a 'restriction modification' system by which foreign DNA (i.e., bacteriophage) is recognized as 'foreign' and it is attacked by restriction endonucleases, and reduce the degree of successful lytic infections. Once a phage successfully infects a bacterial host strain, its DNA becomes modified and should be able to infect that host again at a higher efficiency.

Performing these different assay's helped us understand the potential resistance of each individual strain to its specific phage while also allowing us to recognize what dilutions of cultures and phage is best to use for the remainder of our experiments.

E. coli O157:H7 and _Salmonella_ susceptibility to bacteriophage mixtures in liquid medium.

This study was performed to assess the antimicrobial ability of the phage solution when it was applied directly to culture and plated after 15 min of uninterrupted treatment. Two separate experiments were performed when working with EcoShield. One was application of EcoShield in tubes of *E. coli* O157:H7 mixture of strains ATCC 43894, ATCC 43890, ATCC 43895, and ATCC 35150. The second application was to evaluate the effect of the EcoShield on specific *E. coli* O157:H7 strains provided by the phage manufacturer (229 Na1 R/25, 230 Na1 R/25, 231

Na1 R/25) and our own lab strain *E. coli* O157:H7 ATCC 43894. The trials performed with strains from our lab were due to the fact that in real world applications, you won't have the luxury to choose specific *E. coli* strains to contaminate your food products. The same experiment was also performed with SalmoFresh on the 6-strain *Salmonella* cocktail used in our study (our lab strains). The 15 min was to give a sufficient amount of time for phage to find and infect pathogenic *E. coli* O157:H7 or *Salmonella* cells. Tubes were gently inverted at the 7.5 minute mark in order to re-distribute phage in the phage-bacteria suspension. When the three *E. coli* O157:H7 strains provided by Intralytix (plus one from our lab) were used in liquid infection trials, we observed almost a 4-log reduction in viable *E. coli* (Fig. 22, Panel A). However, when the four-strain mix of *E. coli* O157:H7 strains from our lab was treated with EcoShield, we observed less than a 0.5 log reduction (Fig. 22, Panel A). Application of SalmoFresh to the 6-strain *Salmonella* cocktail resulted in just over a 3-log reduction (Fig. 23, Panel A). These data suggest one may experience differences in phage-based lethality due to differences in susceptibility of different strains.

When individual strains were tested for phage sensitivity by comparing phage titers obtained from the phage stock preparation, we observed a 3.3-fold difference in susceptibility from the least- to most- sensitive strain in our multi-strain *E. coli* O157:H7 cocktail (Fig. 22, Panel B).

Overall these studies showed that bacteriophage have potential to act as a decent antimicrobial. This led to our continued evaluation of bacteriophage in order to determine the product efficacy when applied to pathogen-contaminated beef or fat.

Application of SalmoFresh and EcoShield through a pressurized/automated spray system on inoculated beef and fat discs.

These experiments were performed to examine the effect of bacteriophage (SalmoFresh and EcoShield) through an automated air-assisted spray system on inoculated pieces of beef and fat. The air-assisted nozzle sprays a fine mist so that it uses a minimum amount of bacteriophage suspension. Our objective was to achieve as high a kill as possible on beef and fat discs representing contaminated carcass or beef subprimals.

The SalmoFresh phage spray application on beef and fat resulted in only a 0.48-log reduction for beef and a 0.53-log reduction for the fat treatment (Fig. 23). The EcoShield application resulted in a 1.19-log reduction for beef pieces and almost a 1.61-log reduction for fat pieces (Fig. 24). This showed that bacteriophage could produce an antimicrobial effect when applied through a pressurized spray system. Depending on the economics of commercial application, these results are nearly approaching commercial applicability. However, it should be noted that even upon demonstration of large reduction with test strains, a survey of phage sensitivity on random strains that may be found to contaminate beef may be warranted.

Bacteriophage treatment of inoculated meat and fat via manual spray system.

The phage application using our pressurized/automated spray system led to large amounts of waste of working stock phage suspensions. In order to lower the amount of solution lost during application we utilized a basic handheld manual spray bottle for treatment of phage. The objective of these experiments was to examine the effect of bacteriophage (SalmoFresh and EcoShield) through a “less waste” and “more efficient” system.

Two trials were performed for the SalmoFresh application, one with high inoculum levels

(i.e.,10^7 cfu/cm^2) and another with lower inoculum levels (10^4 cfu/cm^2). At high inoculum levels SalmoFresh resulted in a 0.51-log reduction on beef and a 0.62-log reduction on fat samples (Fig. 25). We then examined the effect of SalmoFresh using lower inoculum levels resulting in a maximum 0.54-log reduction on beef and a maximum reduction of 0.36- log for fat samples (Fig. 26). We expected better results with a lower bacterial inoculum (i.e., 3-log lower inoculum represents 1000 more phage per bacterial cell). However, it may simply represent the situation whereby there are fewer bacteria on the sample making it more difficult for the phage to find their targets and initiate a lytic attack.

Two identical trials were performed for the EcoShield application, both incorporating the reduced inoculum, presented in the previous experiment. This was to keep the protocol consistent with the second round of SalmoFresh manual application. The first EcoShield trial resulted in a 1.1-log reduction on beef and a 1-log reduction on fat samples (Fig. 27). The second trial resulted in only a 0.74-log reduction for beef and a 0.66-log reduction for fat samples (Fig. 28). These trials exhibited the potential reduction of pathogenic *E. coli* by phage utilizing a spray system that would result in less waste of product than our automated air-assisted spray system. These results were obtained using the most susceptible strains of *E. coli* O157:H7. Final considerations should be made after random strains of pathogens are also tested for susceptibility to the bacteriophage.

Our research suggests that both ozone and electrolyzed water will not serve as effective antimicrobials if directly applied to beef, whether for carcass application or non-intact beef cuts. Both may however serve as sufficient sanitizers for processing equipment, food contact surfaces, and other inert surfaces found in processing environments. Both solutions have potential to work as "green" organic antimicrobials in the food industry for products with less drastic protein contact such as vegetables and produce. Additional research should be applied in order to

understand the antimicrobial potential of these two products.

Our research also proposes that bacteriophage may be able to serve as a sufficient antimicrobial if possibly paired with other additional applications. Viazis et al. (2011), found that phage paired with essential oils helped reduce all inoculated *E. coli* cells on leafy green vegetables after 10 min. Although this particular study doesn't apply to meat and a 10 min treatment seems to be quite lengthy for the meat processing industry (during "hot box" chilling), it does show that bacteriophage have potential application if combined with another antimicrobial intervention. We did obtain a modest reduction in many of our experiments when utilizing phage alone. The issue lies in the fact that this reduction alone is not substantial enough for carcass, non-intact beef, or other industry-level applications. Therefore, future studies should examine multiple hurdles in order to improve outcomes for application of antimicrobial interventions on beef surfaces.

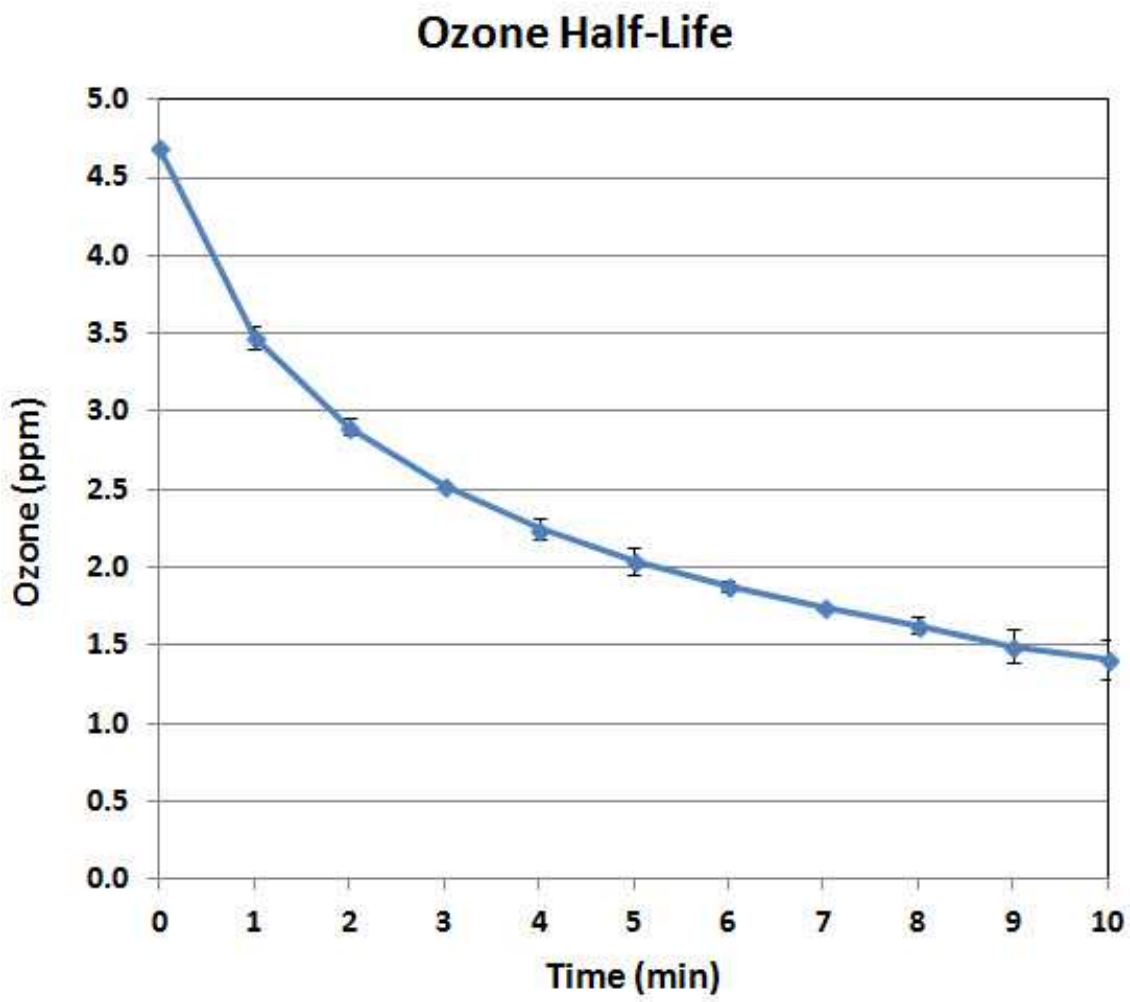

Figure 9. Ozone decay over time. Ozone decay was evaluated directly by stopping the flow into the digital ozone meter probe chamber. The data points represent the means of duplicate replications and error bars represent standard deviation from the mean.

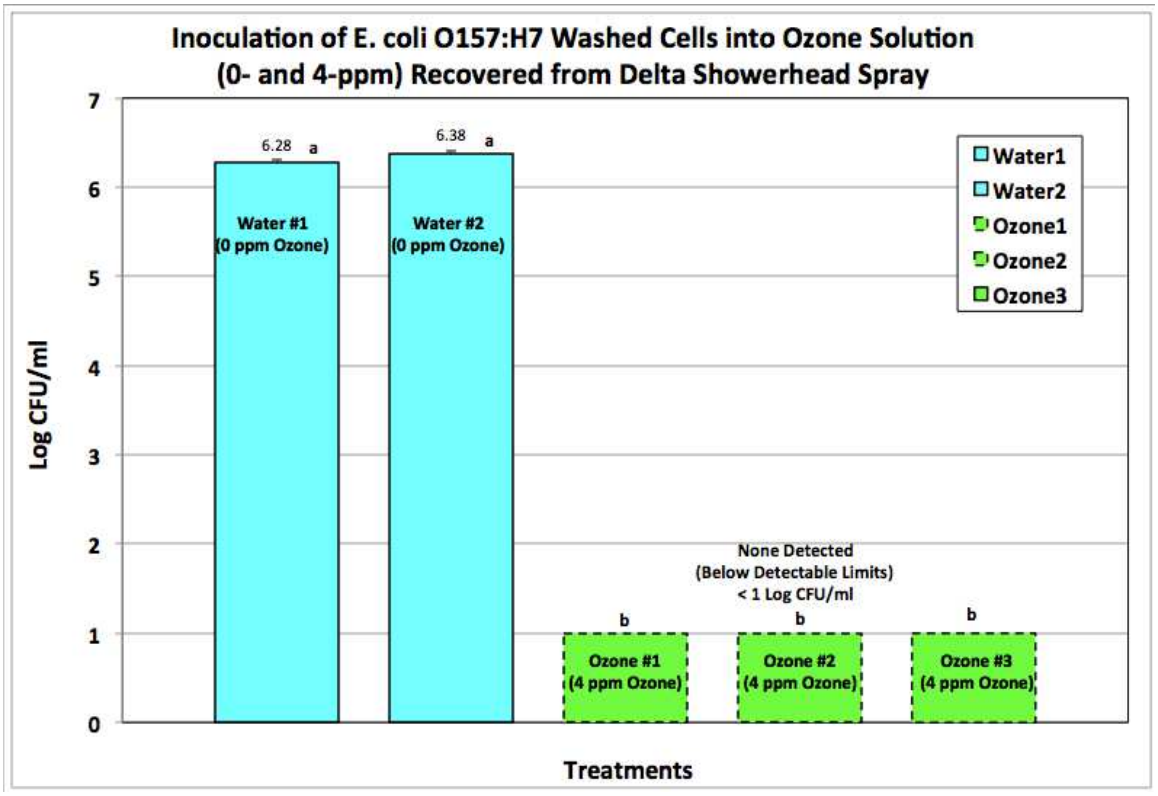

Figure 10. Effect of washed *E. coli* O157:H7 cocktail after being treated with either water or ozone (4 ppm) for 10 min. The data points represent the means of duplicate replications for water treatments, triplicate replications for ozone treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

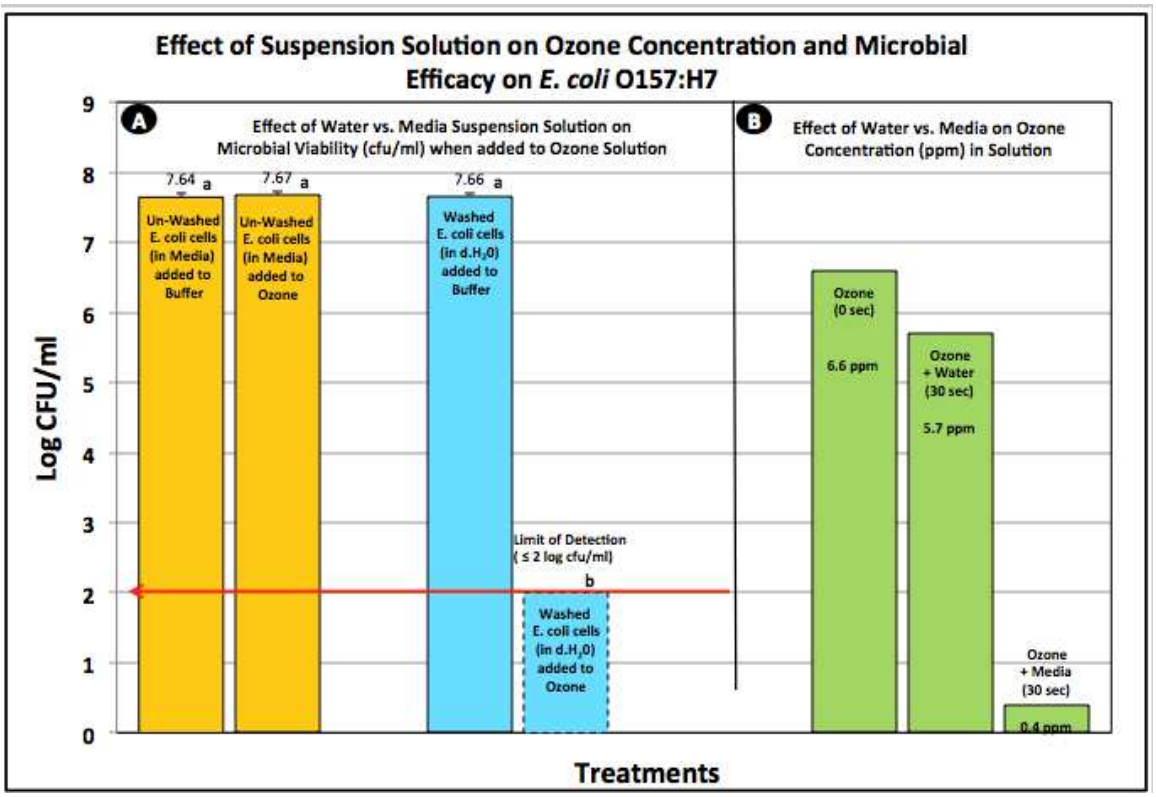

Figure 11. Panel A**.** *E. coli* O157:H7 cells at suspended in either growth media or water (washed) were placed in either buffer solution or ozone to observe the effect. Panel B**.** The effect on active ozone ppm when either media or water was added to it. The data points represent the means of duplicate replications for water treatments, triplicate replications for ozone treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different (P<0.05).

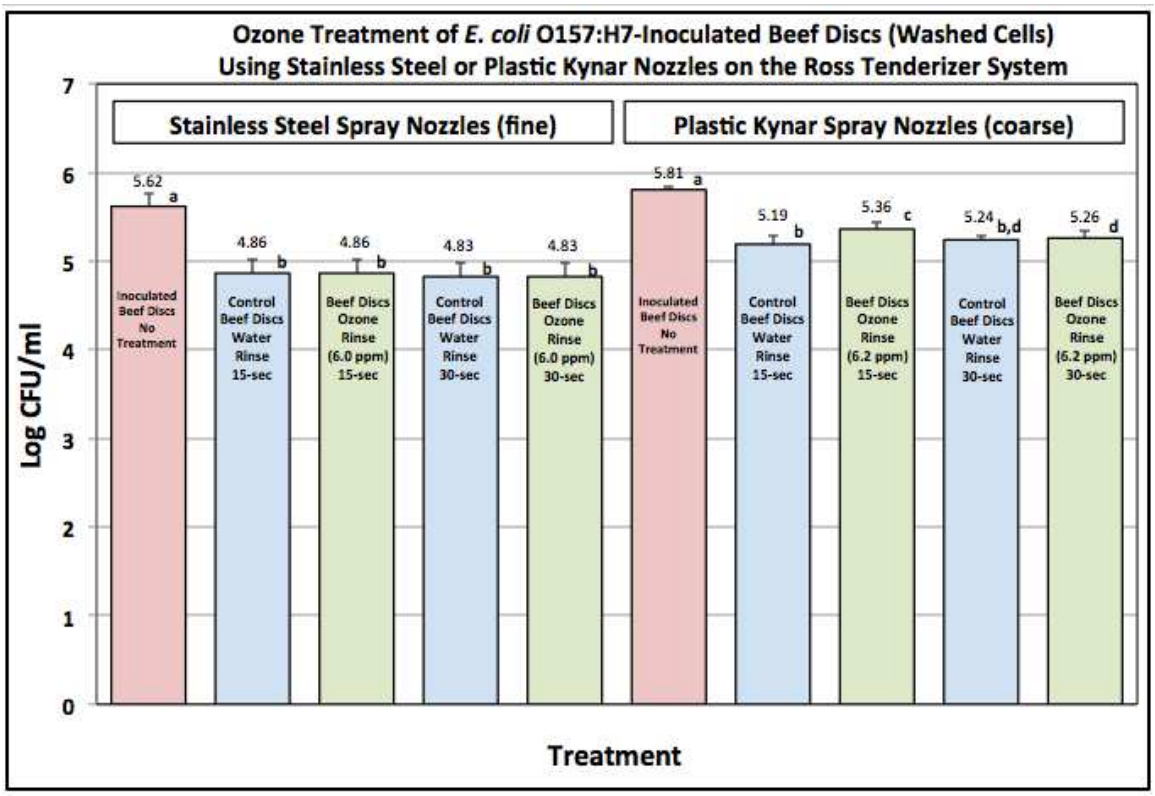

Figure 12. The effect of *E. coli* O157:H7 (washed)-inoculated beef discs treated with ozone for 15- or 30- sec through two different spray nozzles (stainless steel and plastic kynar). The data points represent the means of duplicate replications for water treatments, triplicate replications for ozone treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

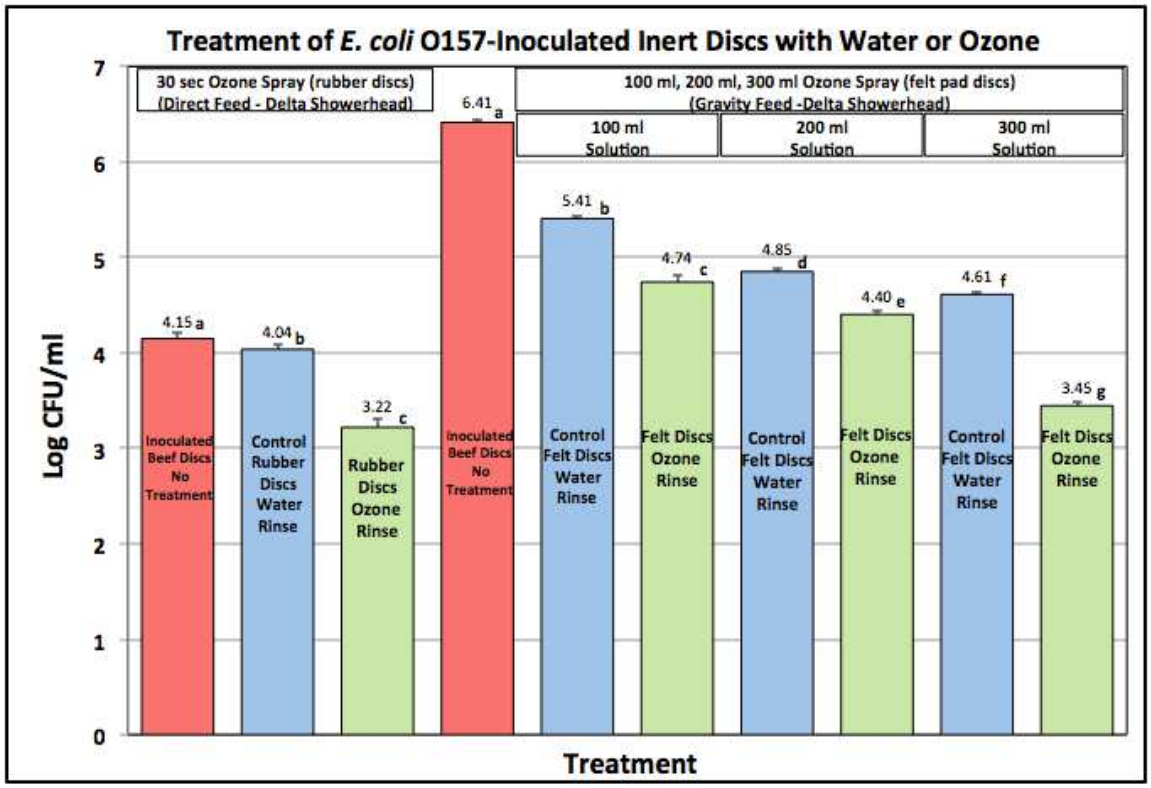

Figure 13. The effect of inert discs inoculated with *E. coli* O157:H7 (washed) and then treated with with either 30-sec of ozone (rubber discs) via pressurized Delta showerhead or of ozone through the Delta Shower head via gravity drip (felt discs). The data points represent the means of duplicate replications for water treatments, triplicate replications for ozone treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

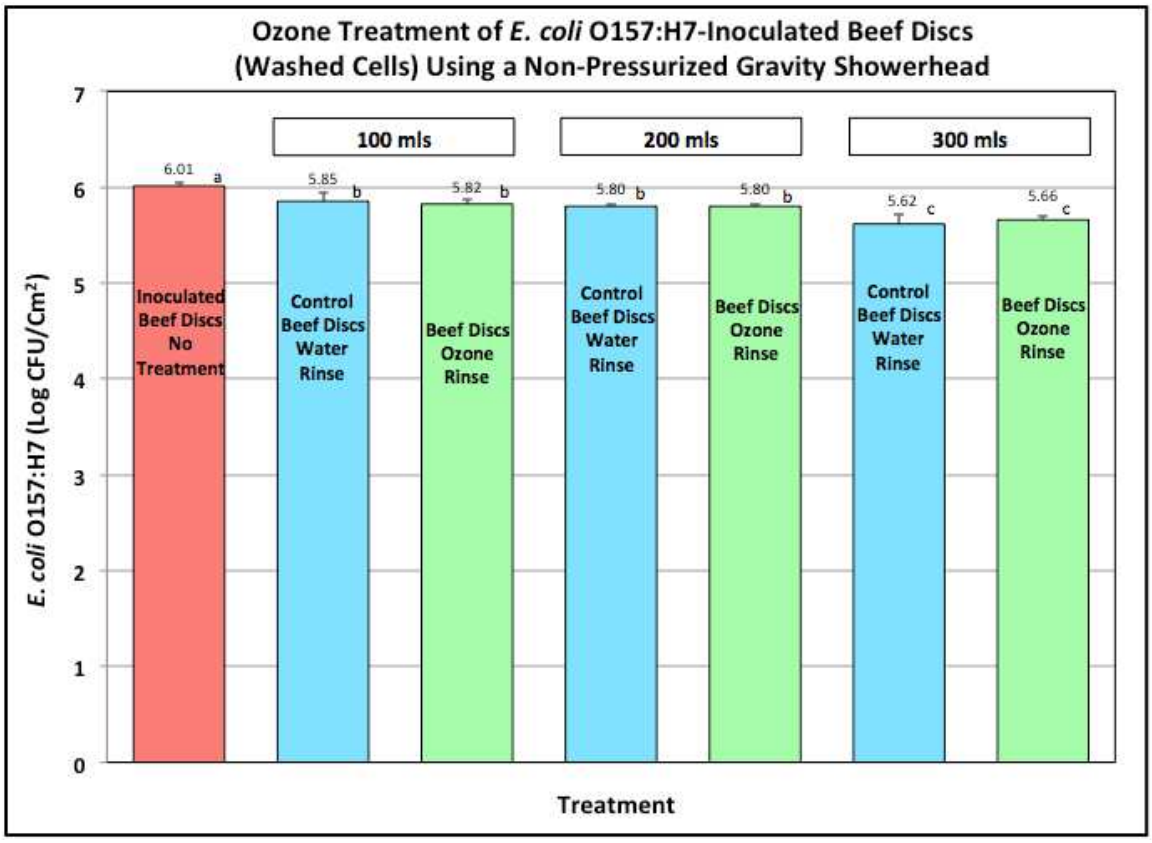

Figure 14. The effect of beef discs inoculated with *E. coli* O157:H7 (washed) and then treated with increasing amounts of ozone through the Delta showerhead via gravity drip. The data points represent the means of duplicate replications for water treatments, triplicate replications for ozone treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

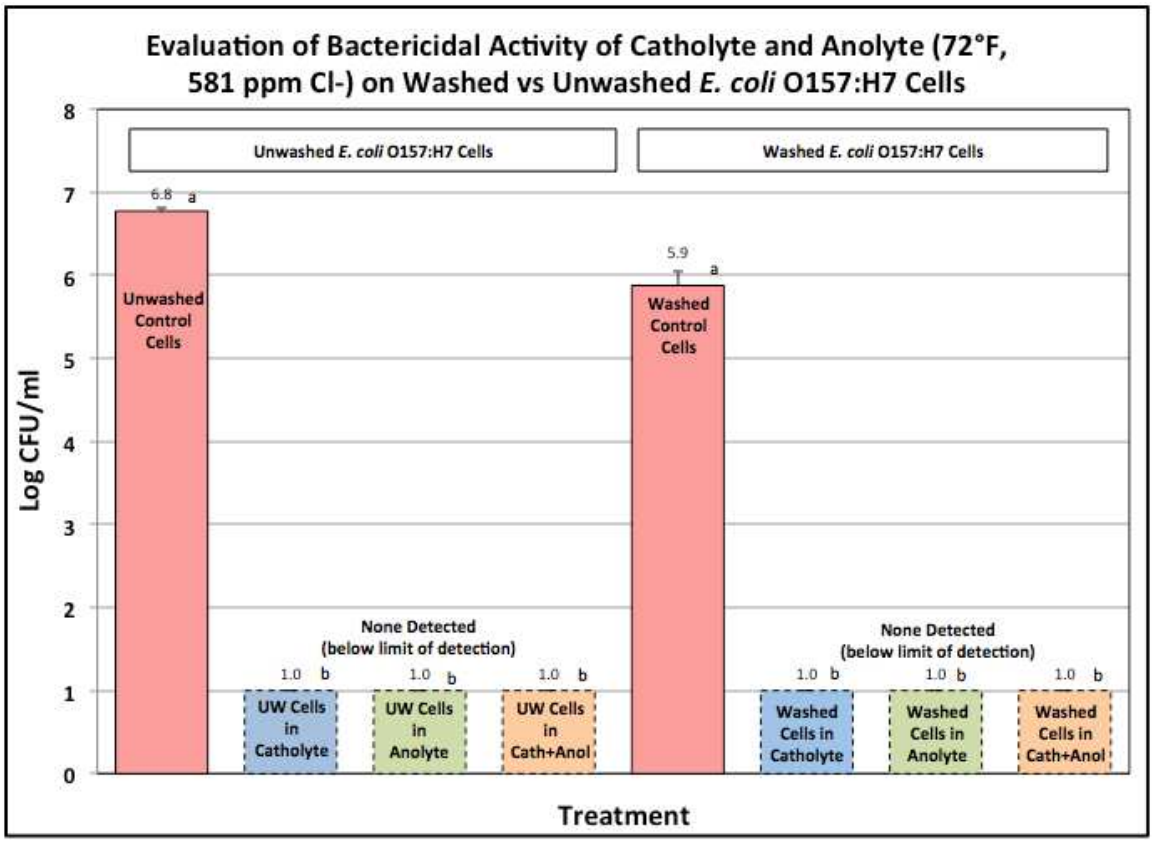

Figure 15. The effect of electrolyzed water (EW) solutions (catholyte, anolyte, or catholyte plus anolyte) on washed or unwashed *E. coli* O157:H7 cocktail. Anolyte was used at 581 ppm (as free Cl-) and pH 6.8. The data points represent the means of duplicate replications for water treatments, triplicate replications for EW treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

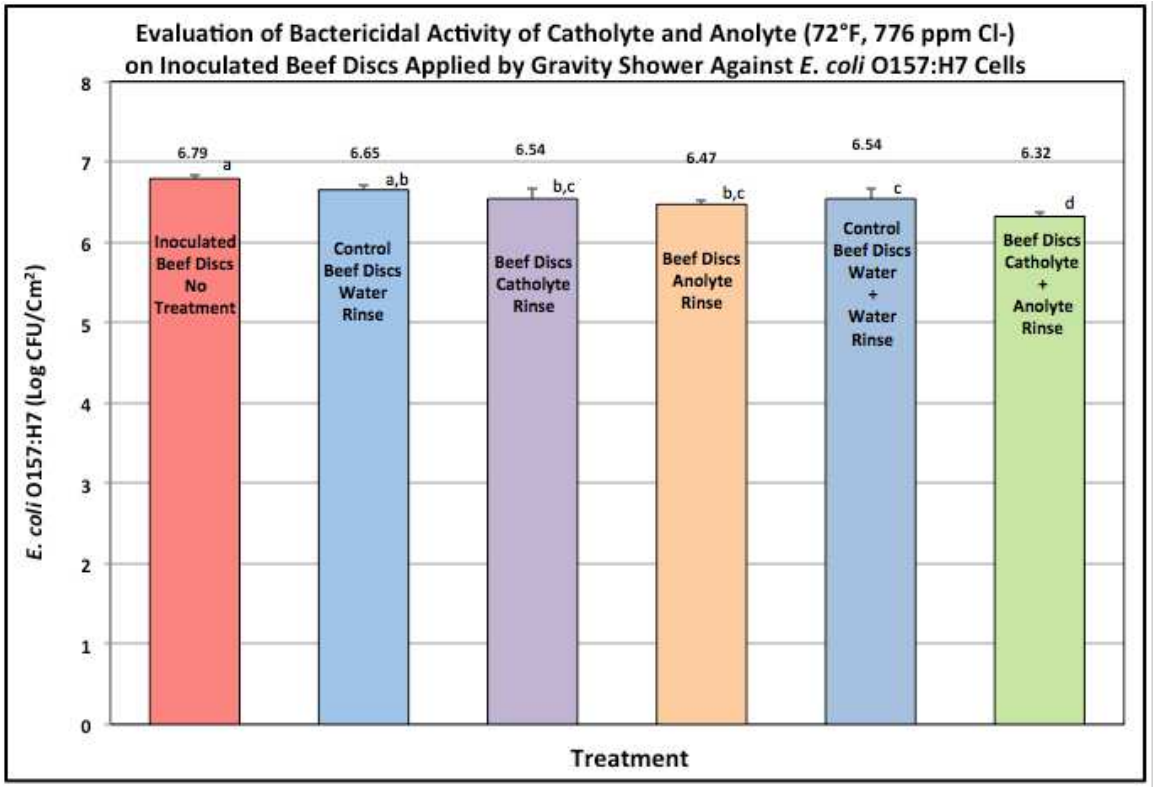

Figure 16. The effect of beef discs inoculated with *E. coli* O157:H7 and then treated via gravity shower with either water, water (twice), catholyte, anolyte, or catholyte plus anolyte solutions maintained at room temperature (72°F). Anolyte was used at 776 ppm (as free Cl-) and pH 6.0. The data points represent the means of duplicate replications for water treatments, triplicate replications for EW treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

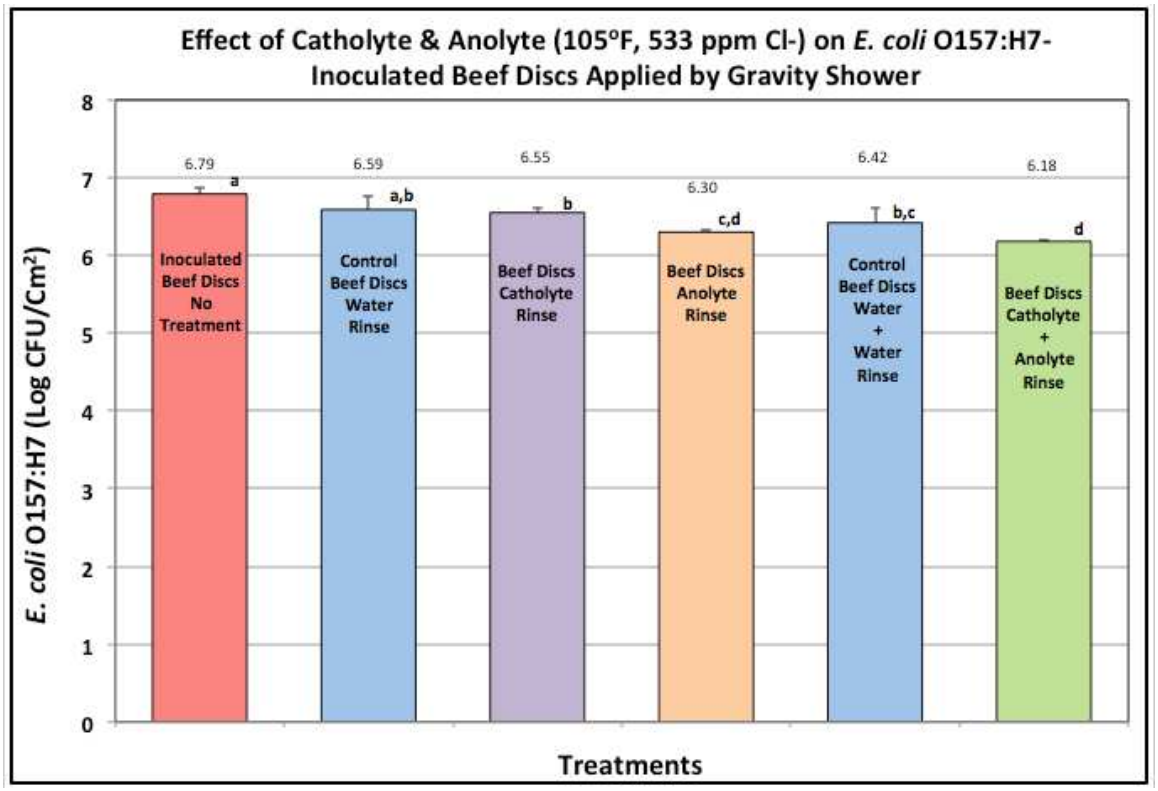

Figure 17. The effect of beef discs inoculated with *E. coli* O157:H7 and then treated via gravity showerhead with heated solutions of either water, water (twice), catholyte, anolyte, or catholyte plus anolyte. Anolyte was used at 533 ppm (as free Cl-) and pH 6.1. The data points represent the means of duplicate replications for water treatments, triplicate replications for EW treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

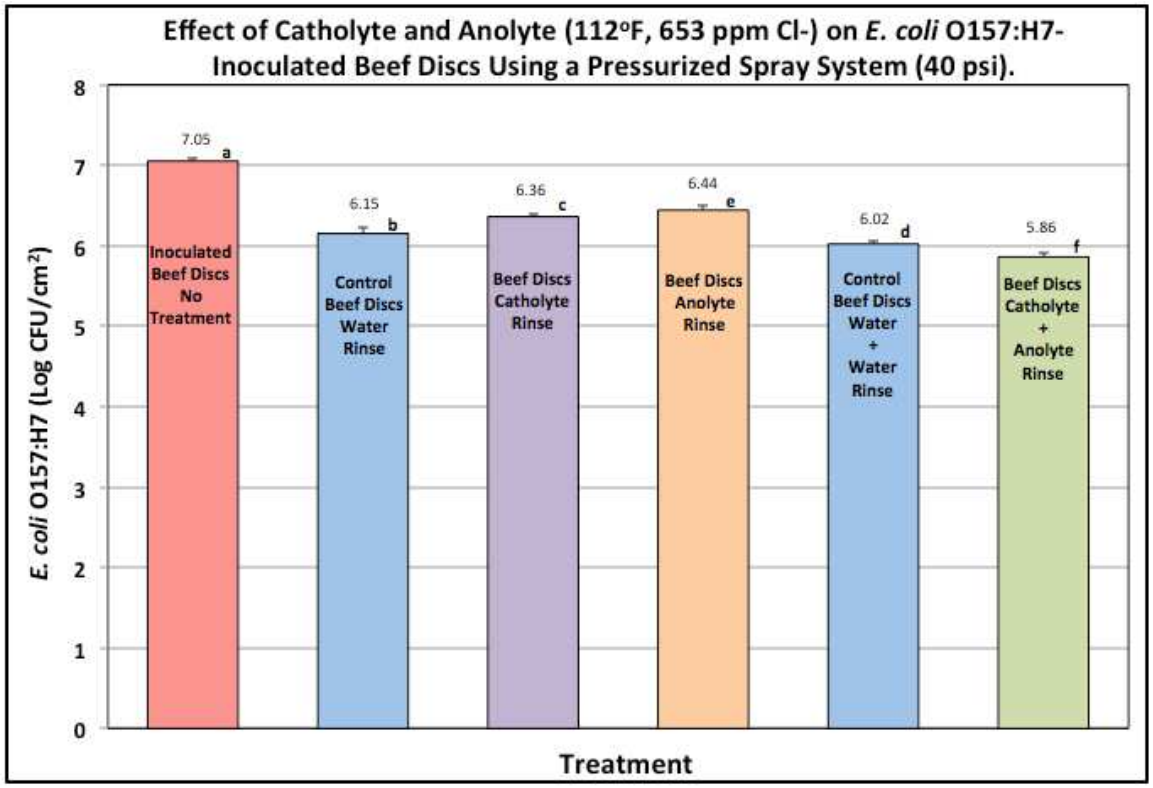

Figure 18. The effect of beef discs inoculated with *E. coli* O157:H7 and then treated via pressurized spray system with either water, water (twice), catholyte, anolyte, or catholyte plus anolyte. Anolyte was used at 653 ppm (as free Cl-) and pH 5.8. The data points represent the means of duplicate replications for water treatments, triplicate replications for EW treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

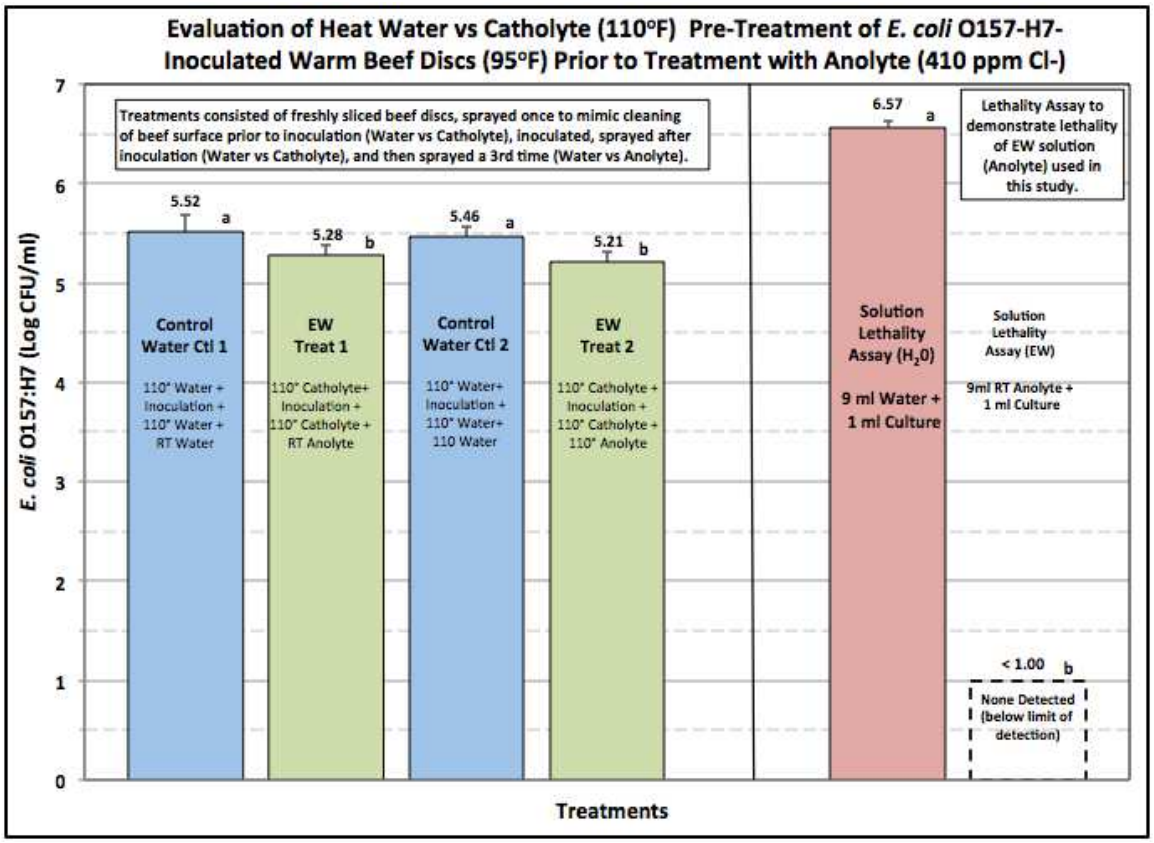

Figure 19. The effect of warmed beef discs (95°F) pre-treated with catholyte, inoculated with *E. coli* O157:H7 cocktail and then treated via pressurized spray system with various combinations of electrolyzed water (anolyte at heated or room temperature) (left). Also shown (right) a repeated lethality assessment of anolyte when manufacturers sent us an additional order of the solution to use for further experiments. Anolyte was used at 410 ppm (as free Cl-) and pH 6.6. The data points represent the means of duplicate replications for water treatments, triplicate replications for EW treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

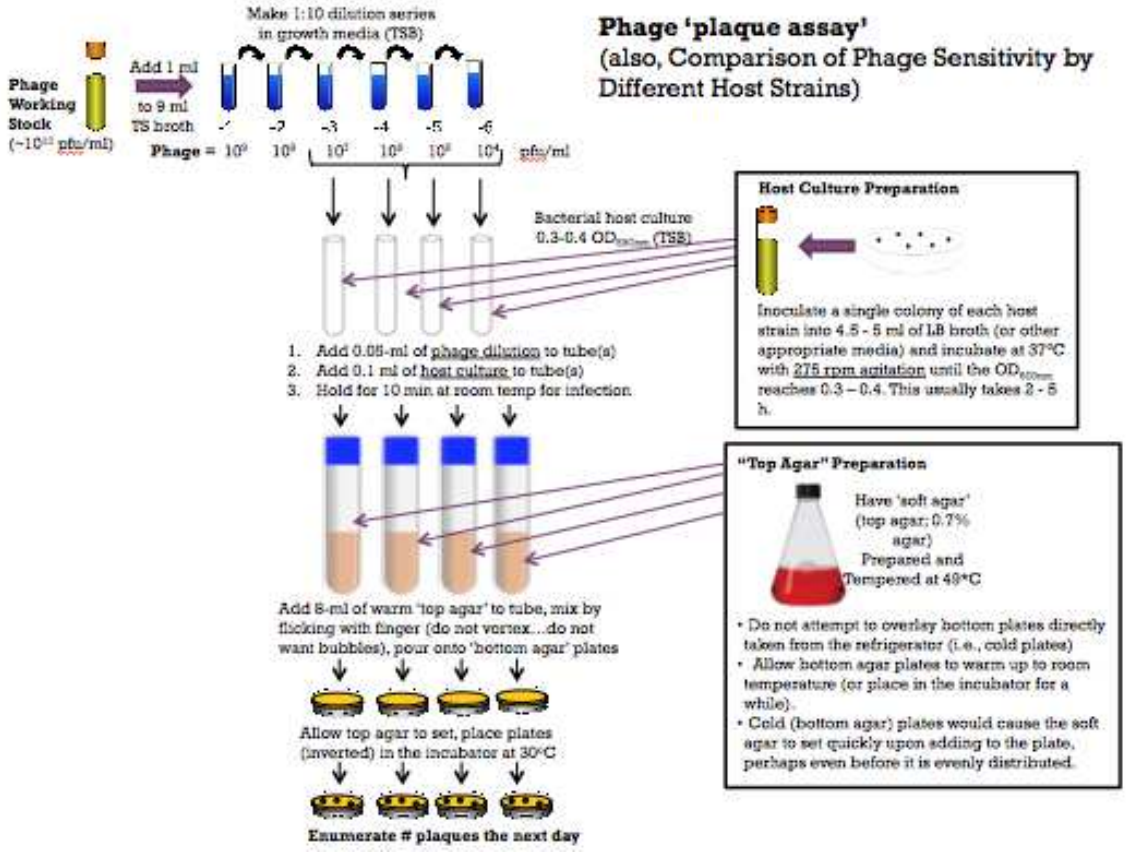

Figure 20. Bacteriophage titer assay protocol to determine phage titers on various bacterial strains. Different titers enumerated using the same phage preparation serve as a measure of the relative sensitivity of each strain to the bacteriophage.

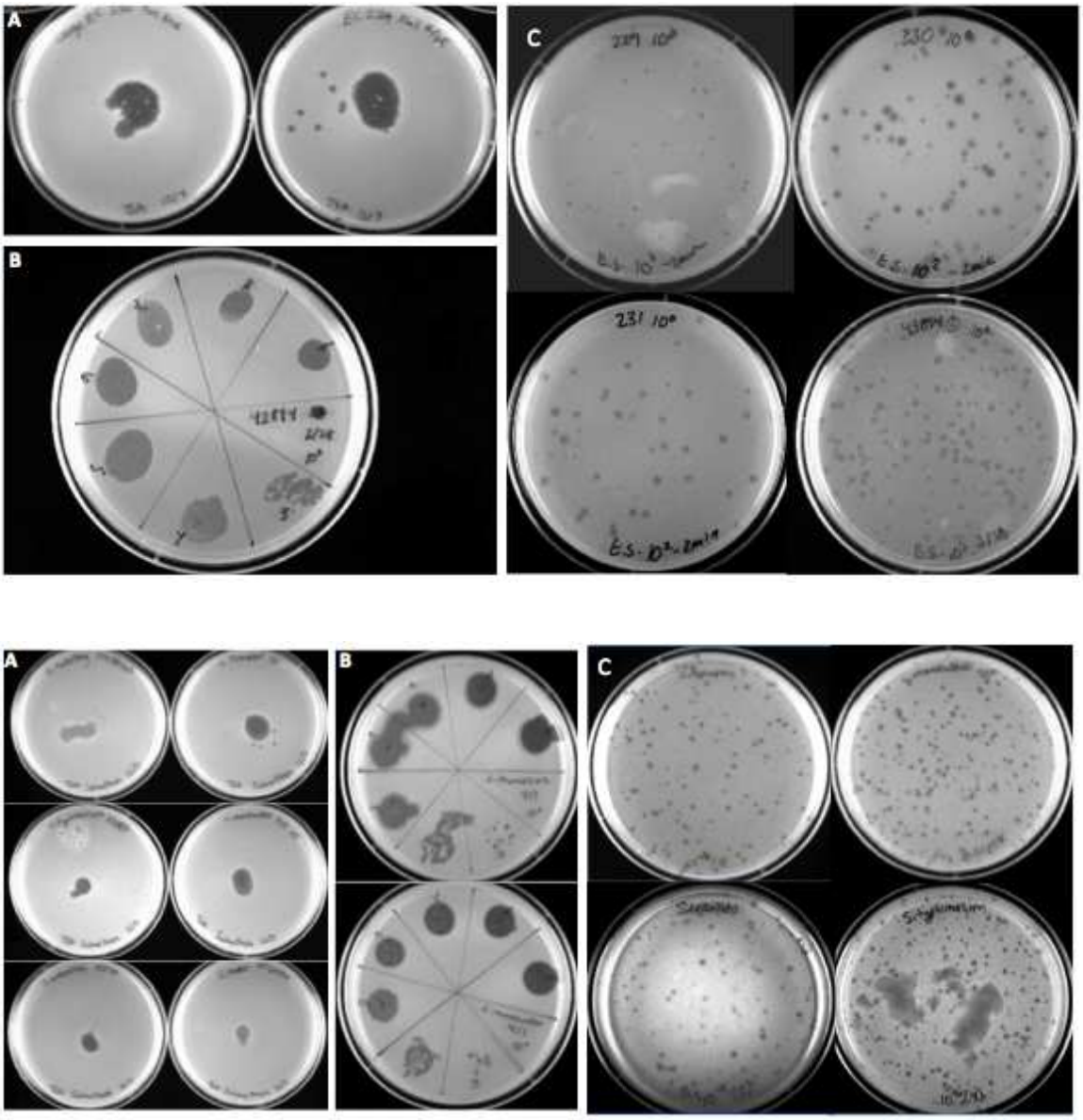

Figure 21. EcoShield phage assays, top. EcoShield spot assay (A), dilution spot assay (B), and phage titer assays (C). SalmoFresh phage assays, bottom. SalmoFresh spot assay (A), dilution spot assay (B), and phage titer assays (C).

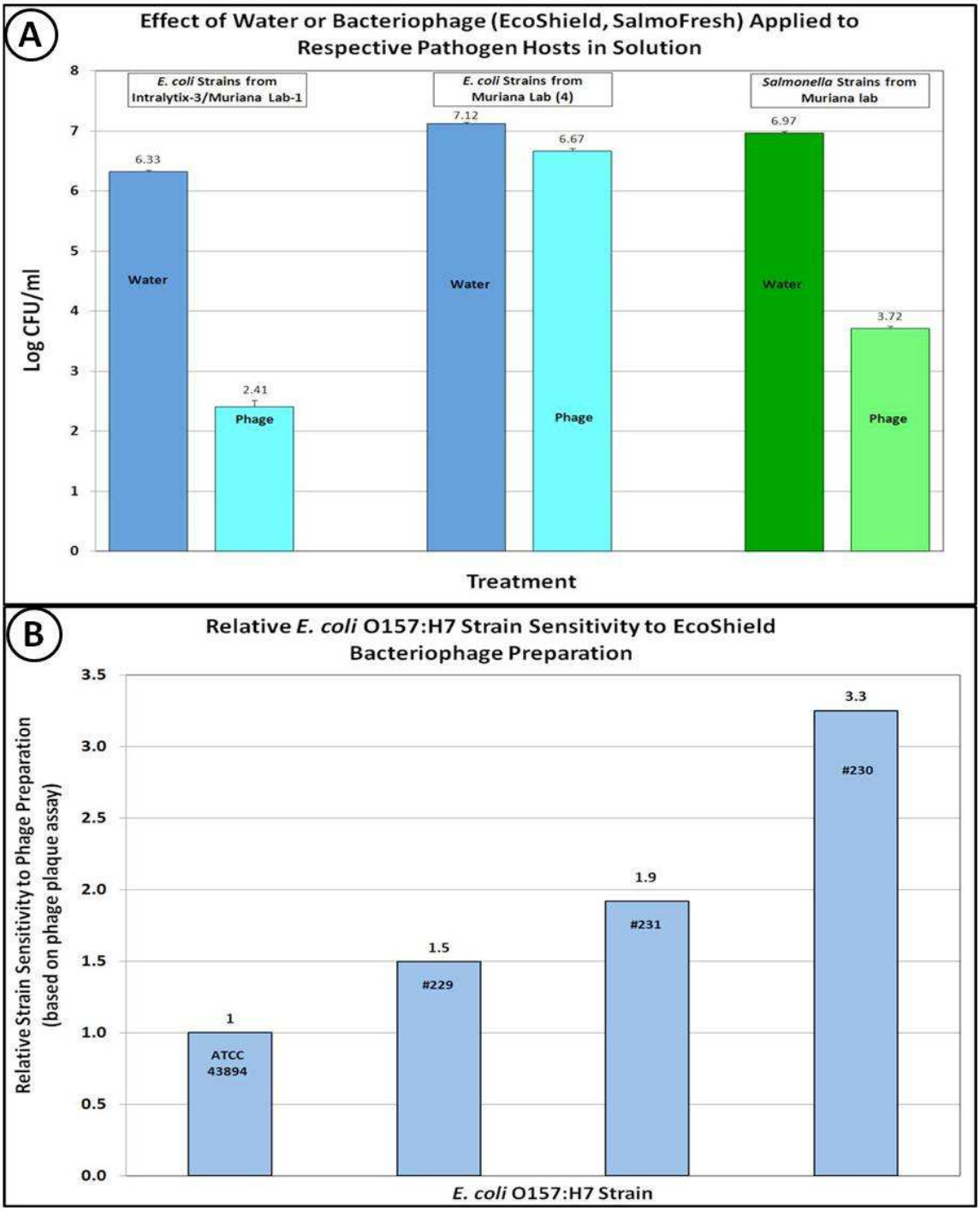

Figure 22. Lytic phage reactions in liquid media and sensitivity of select strains to phage. Panel A, treatment of *E. coli* O157:H7 multi-strain and *Salmonella* multi-strain cocktails with either water or phage. The mixtures sat for 15 min (inverted at 7.5 minute mark), then plated. The data points represent the means of triplicate replications and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$). Panel B, relative sensitivity of *E. coli* O157:H7 strains used in our applications to EcoShield phage preparation as determined from phage titers.

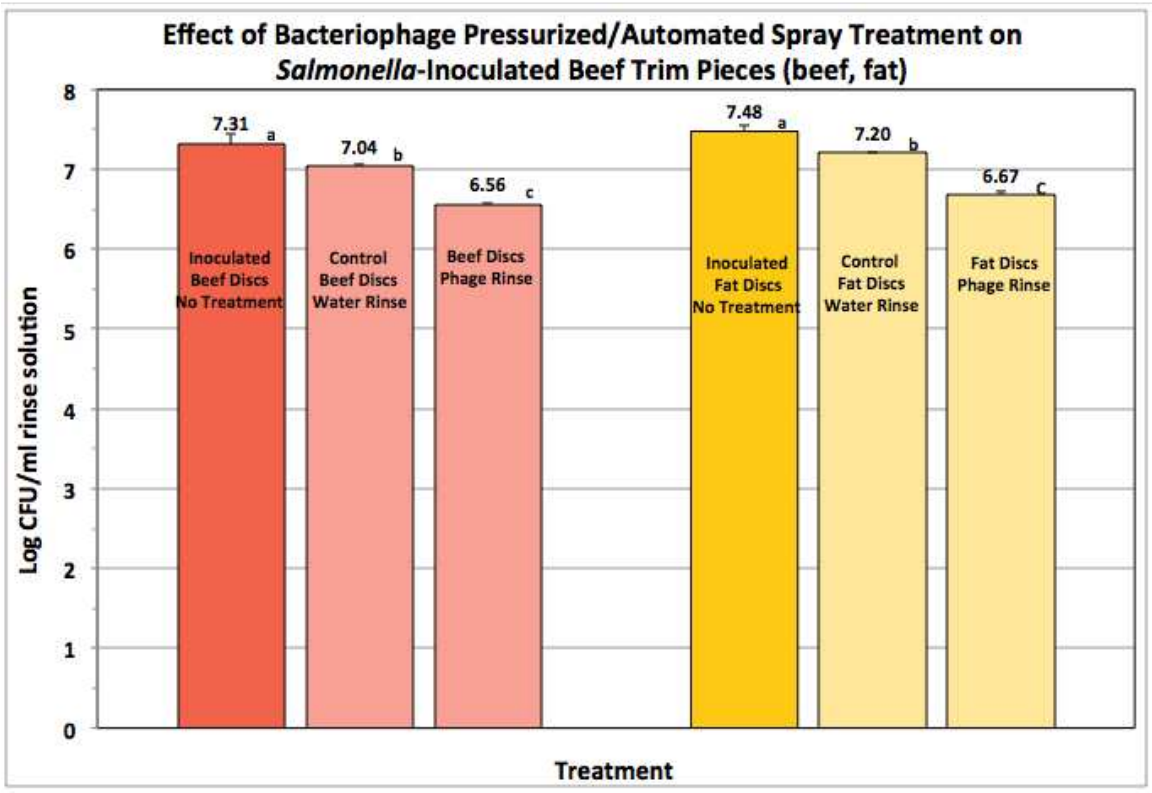

Figure 23. The effect of beef and fat trim pieces inoculated with *Salmonella* cocktail and then treated via air-assisted automated spray system with either 15-sec of water or SalmoFresh. The data points represent the means of duplicate replications for water treatments, triplicate replications for phage treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

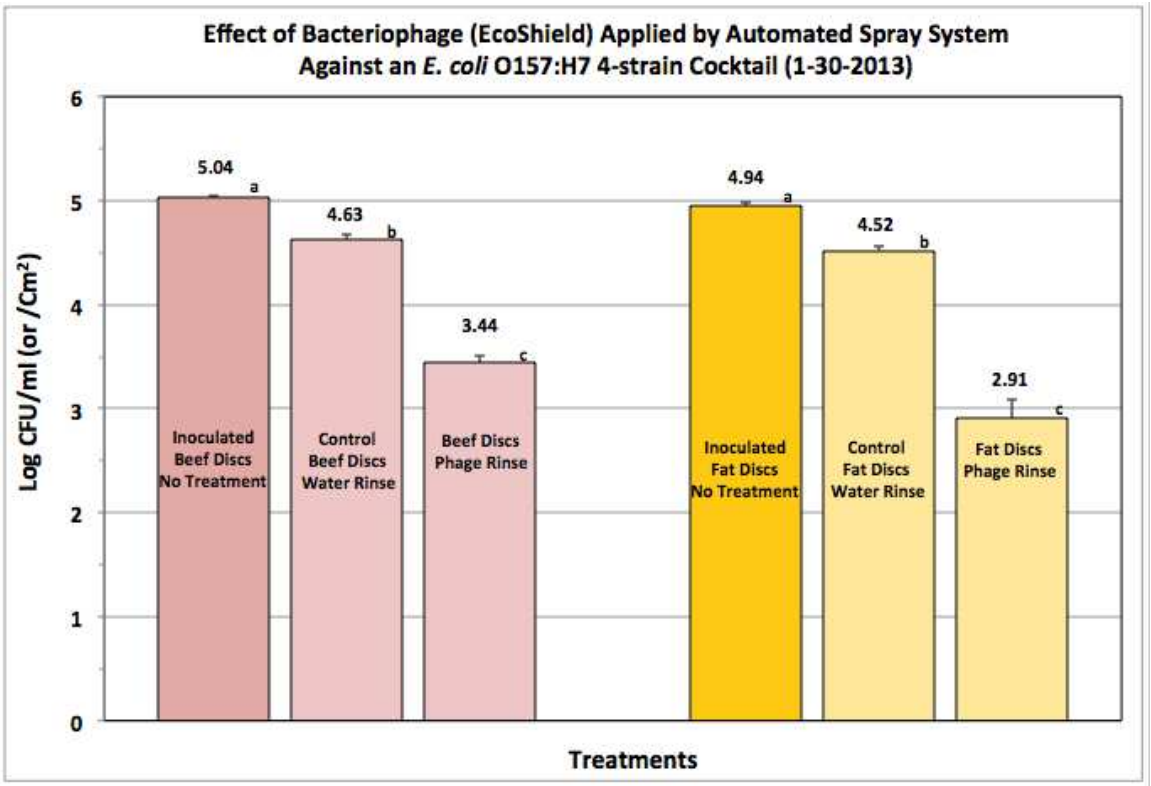

Figure 24. The effect of beef and fat trim pieces inoculated with *E. coli* O157:H7 cocktail and then treated via air-assisted automated spray system with either 15-sec of water or EcoShield. The data points represent the means of duplicate replications for water treatments, triplicate replications for phage treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

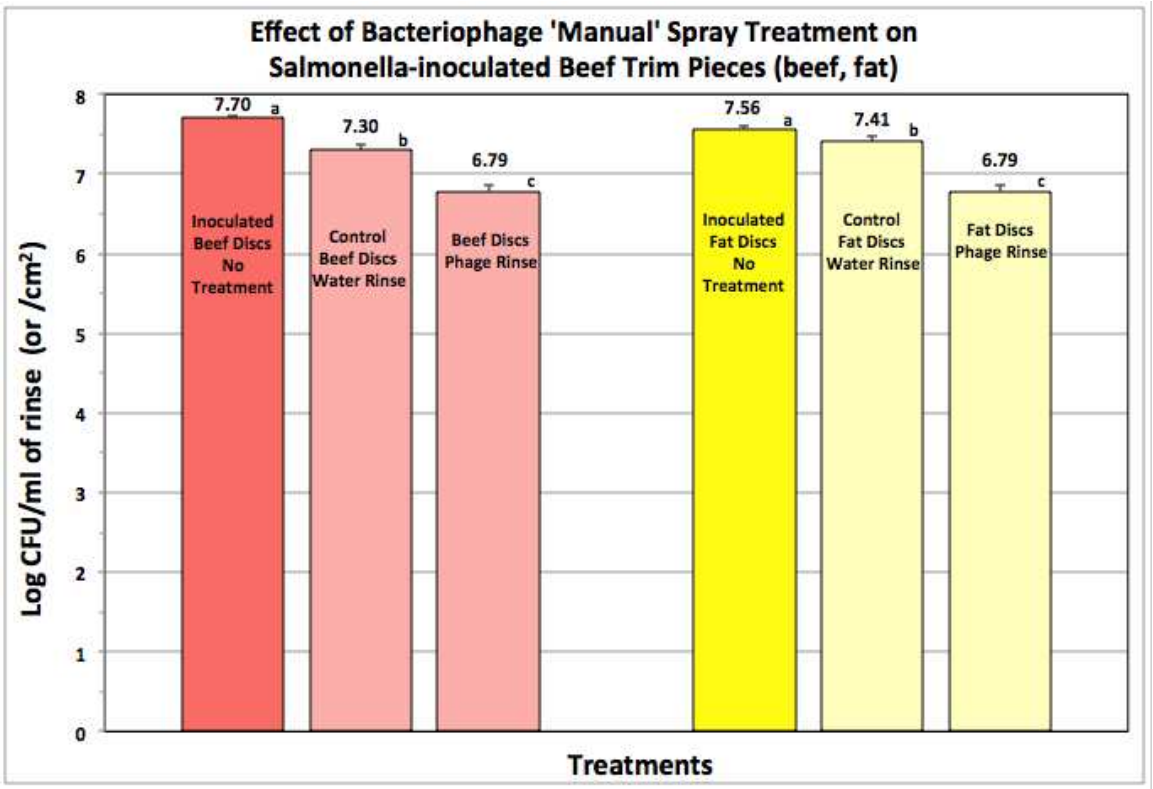

Figure 25. The effect of beef and fat trim pieces inoculated with *Salmonella* cocktail and then treated via manual spray system with either of water or SalmoFresh. The data points represent the means of duplicate replications for water treatments, triplicate replications for phage treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

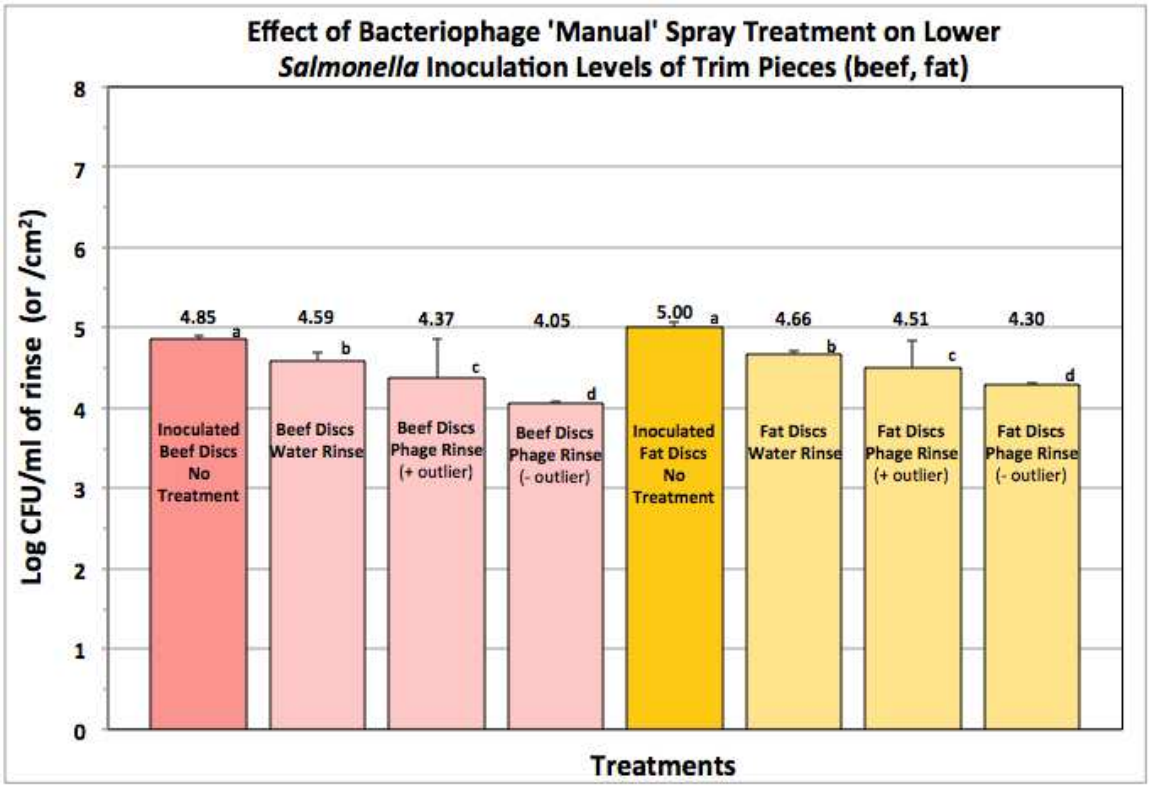

Figure 26. The effect of beef and fat trim pieces inoculated with *Salmonella* cocktail and then treated via manual spray system with either water or SalmoFresh. The data points represent the means of duplicate replications for water treatments, triplicate replications for phage treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

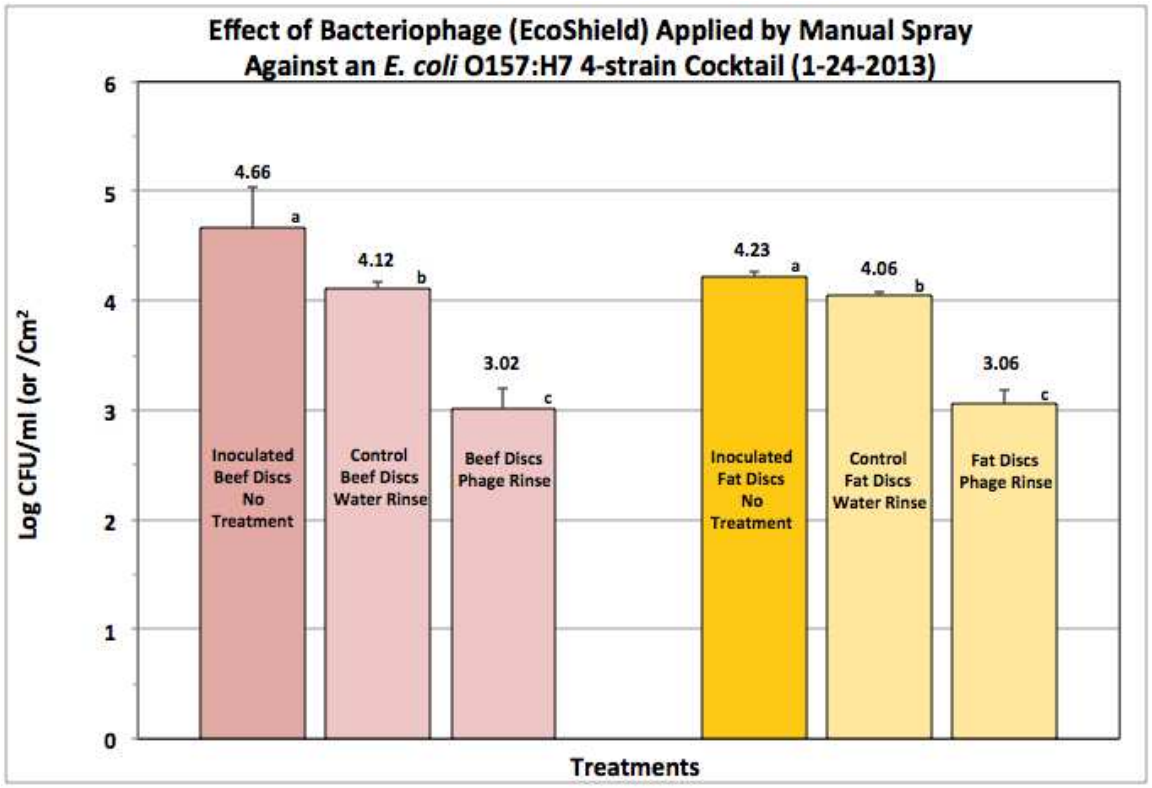

Figure 27. The effect of beef and fat trim pieces inoculated with *E. coli* O157:H7 cocktail and then treated via manual spray system with either water or EcoShield. The data points represent the means of duplicate replications for water treatments, triplicate replications for phage treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

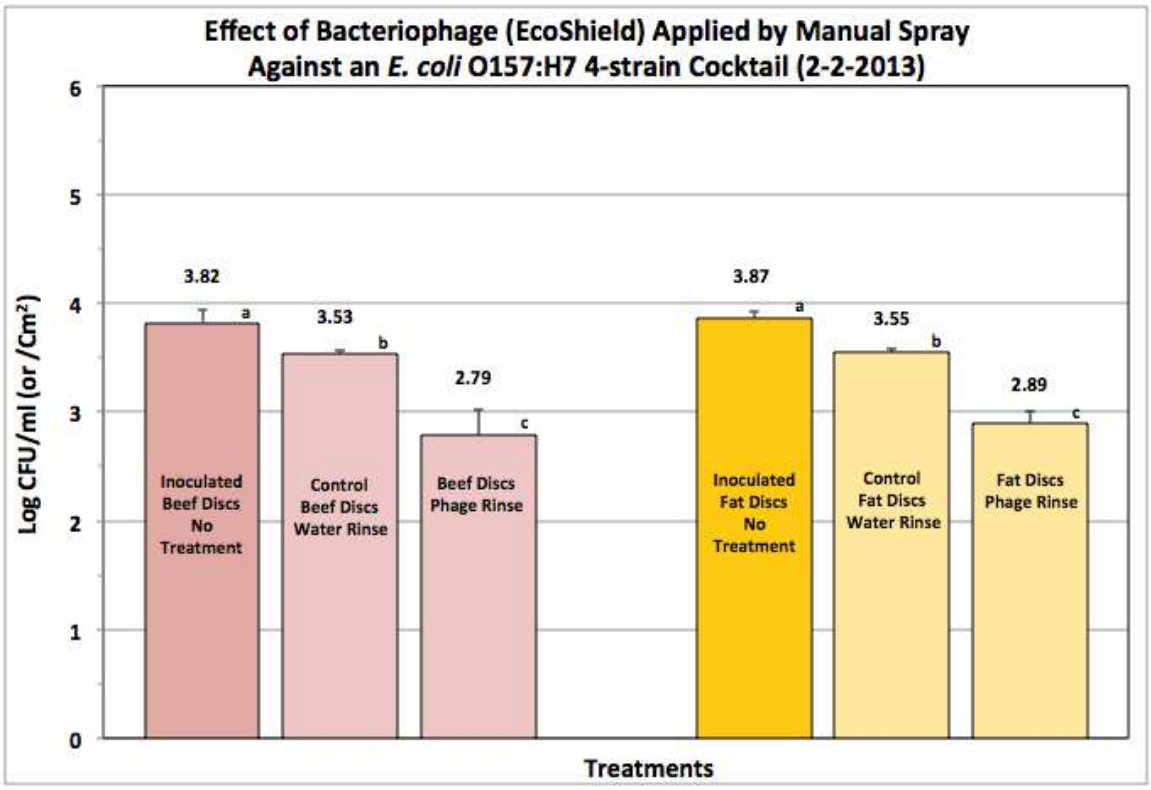

Figure 28. The effect of beef and fat trim pieces inoculated with *E. coli* O157:H7 cocktail and then treated via manual spray system with either water or EcoShield. The data points represent the means of duplicate replications for water treatments, triplicate replications for phage treatments, and error bars represent standard deviation from the mean. Treatments with different letters are significantly different ($P<0.05$).

REFERENCES

Abadias, M., J. Usall, M. Oliveira, I. Alegre, and I. Vinas. 2008. Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally processed vegetables. *Int. J. Food Microbiol*. 123: 151-158.

Abuladze, T., L. Manrong, M. Menetrez, T. Dean, A. Senecal, and A. Slakvelidze. 2008. Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 74(20): 6230-6238.

Alexandre, E.M.C., D.M. Santos-Pedro, T.R.S. Brandão, and C.L.M. Silva. 2011. Influence of aqueous ozone, blanching and combined treatments on microbial load of red bell peppers, strawberries and watercress. *J. Food Eng*. 105: 277-282.

Alonzo, G. 2012. Inactivation of *Escherichia coli* O157:H7 and spoilage yeasts in germicidal uv-c-irradiated and heat-treated clear apple juice. *Food Control*. 25: 425-432.

Belongia, E.A., K.L. MacDonald, and G. L. Parham. 1991. An outbreak of *E. coli* O157:H7 colitis associated with consumption of pre-cooked meat patties. *J. Infect. Dis*. 164: 338-343.

Bielaszewska, M., A. Mellmann, W. Zhang, R. Kock, A. Fruth, A. Bauwens, G. Peters, and H. Karch. 2011. Characterization of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *Lancet*.11: 671-676.

Bosilevac, J. M., S.D. Shackelford, D.M. Brichta, and M. Koohmaraie. 2005. Efficacy of ozonated and electrolyzed oxidative waters to decontaminate hides of cattle before slaughter. *J. Food Prot*. 68: 1393-1398.

Calder, B.L., D.I. Skonberg, K. Davis-Dentici, B.H. Hughes, and J.C. Bolton. 2011. The effectiveness of ozone and acidulant treatments in extending the refrigerated shelf life of fresh-cut potatoes. *J. Food Sci*. 76: S492-S498.

Centers for Disease Control and Prevention. 2013. Surveillance for Foodborne Disease Outbreaks-United States, 2009-2010. Morbidity and Mortality Weekly Report. January 25, 2013. 62(3): 41-47.

Chawla, A., J.W. Bell, and M.E. Janes. 2008. Optimization of ozonated water treatment of wild caught and mechanically peeled shrimp meat. *J. Aquat. Food Prod. T*. 16: 41-56.

Cheigh, C.I., M.H. Park, M.S. Chung, J.K. Shin, and Y.S. Park. 2011. Comparison of intense pulsed light and ultraviolet (UV)-induced cell damage in *Listeria monocytogenes* and *Escherichia coli* O157:H7. *Food Control*. 25: 654-659.

Code of Federal Regulations. Revised 1 April 2012. Title 21, Part 172 Food Additives permitted for Direct Addition to Food for Human Consumption., Sec. 172.785 Listeria-specific bacteriophage preparation. 3:77-78. U.S. Government Printing Office, Washington DC.

Code of Federal Regulations. Revised 1 April 2012. Title 21, Part 184 Direct Food Substances Affirmed As Generally Recognized As Safe., Sec. 184.1563 Ozone. U.S. Government Printing Office, Washington DC.

Code of Federal Regulations. Revised 1 April 2012. Title 21, Part 178 Indirect Food Additives: Adjuvants, Production Aids, and Sanitizers., Sec. 178.1010 Sanitizing Solutions. U.S. Government Printing Office, Washington DC.

Crowe, K. M., D. Skonberg, A. Bushway, and S. Baxter. 2012. Application of ozone sprays as a strategy to improve the microbial safety and quality of salmon fillets. *Food Control*. 25: 464-468.

Cui, X., Y. Shang, S. Zhengxiang, X. Hongwe, and C. Wei. 2009. Physicochemical properties and bactericidal efficiency of neutral and acidic electrolyzed water under different storage conditions. *J. Food Eng*. 91: 582-586.

Ding, T., S. M. E. Rahman, U. Purev, and D.H. Oh. 2009. Modeling of *Escherichia coli* O157:H7 growth at various storage temperatures on beef treated with electrolyzed oxidizing water. *J. Food Eng*. 97: 497-503.

Dorsa, W.J. 1997. New and established carcass decontaminating procedures commonly used in the beef processing industry. *J. Food Prot*. 60:1146-1151.

Duckworth, D.H. 1976. Who discovered bacteriophage? *Bacteriol. Rev*. 40: 793-802.

Duffy, G., C. Walsh, I.S. Blair, and D.A. McDowell. 2006. Survival of antibiotic resistant and antibiotic sensitive strains of *E. coli* O157 and *E. coli* O26 in food matrices. *Int. J. Food Microbiol*. 109:179-186.

Echeverry, A., J.C. Brooks, M.F. Miller, J.A. Collins, G.H. Loneragan, and M.M. Brashears. 2010. Validation of lactic acid bacteria, lactic acid, and acidified sodium chlorite as decontaminating interventions to control *Escherichia coli* O157:H7 and *Salmonella typhimurium* DT104 in mechanically tenderized and brine-enhanced (nonintact) beef at the purveyor. *J. Food Prot*. 73:2169-2179.

Elano, R. R., T. Kitagawa, L. Bari, S. Kawasaki, S. Kawamoto, and Y. Inats. 2010. Comparison of effectiveness of acidified sodium chlorite and sodium hypochlorite in reducing *Eschericia coli*. *Foodborne Pathog. Dis*. 7: 481-489.

Fabrizio, K. A., and C.N. Cutter. 2004. Comparison of electrolyzed oxidizing water with other antimicrobial interventions to reduce pathogens on fresh pork. *Meat Sci*. 68: 463-468.

Fabrizio, K. A., and C.N. Cutter. 2005. Application of electrolyzed oxidizing water to reduce *Listeria monocytogenes* on ready-to-eat meats. *Meat Sci*. 71: 327-333.

Fabrizio, K. A., R. R. Sharma, A. Demirici, and C. N. Cutter. 2002. Comparison of electrolyzed oxidizing water with various antimicrobial interventions to reduce *Salmonella* species on poultry. *Poultry Sci*. 81: 1598-1605.

Gamble, R., and P.M. Muriana. 2007. A microplate fluorescence assay for the measurement of the ability of strains of *Listeria monocytogenes* from meat and meat processing plants to adhere to abiotic surfaces. *Appl. Envrion. Microbiol*. 73: 5235-5244.

Gill, C.O. 2009. Effects on the microbiological condition of product of decontaminating treatments routinely applied to carcasses at beef packing plants. *J. Food Prot*. 72: 1790-1801.

Gill, C.O., and C. Landers. 2003. Microbiological effects of carcass decontaminating treatments at four beef packing plants. *Meat Sci*. 65: 1005-1011.

Gill, C.O., L.F. Moza, and S. Barbut. 2009. Survival of bacteria in less than thorough cooked, brine-injected steaks. *Food Control*. 20: 501-507.

Gomez-Lopez, V.M., P. Ragaert, J. Ryckeboer, V. Jeyachchandran, J. Debevere, and F. Devlieghere. 2007. Shelf-life of minimally processed cabbage treated with neutral electrolyzed oxidizing water and stored under equilibrium modified atmosphere. *Int. J. Food Microbiol*. 117: 91-98.

Granier, S. A., C. Moubareck, C. Colaneri, A. Lemire, S. Roussel, T.T. Dao, P. Courvalin, and A. Brisabois. 2011. Antimicrobial resistance of *Listeria monocytogenes* isolates from food and the environment in France over a 10-year period. *Appl. Environ. Microbiol*. 77: 2788-2790.

Greer, G.G. 2005. Bacteriophage control of foodborne bacteria. *J. Food Protect*. 68: 1102-1111.

Gross, M. 2011. Revived interest in bacteriophages. *Curr. Biol*. 21:267-270.

Guenther, S., O. Herzig, L. Fieseler, J. Klumpp, and M. Loessner. 2012. Biocontrol of *Salmonella typhimurium* in RTE foods with the virulent bacteriophage fo1-e2. *Int. J. Food Microbiol*. 154: 66-72.

Guentzel, J.L., K.L. Lam, M.A. Callan, S.A. Emmons, and V.I. Dunham. 2008. Reduction of bacteria on spinach, lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. *Food Microbiol*. 25: 36-41.

Gyles, C.L., 2006. Shiga toxin producing *Escherichia coli*: An overview. *J. Anim. Sci*. 85: E45-E62.

Hagens, S., and M. Loessner. 2010. Bacteriophage for biocontrol of foodborne pathogens: Calculations and considerations. *Curr. Pharm. Biotechno*. 2010: 56-68.

Hagens, S., and M. Loessne. 2007. Application of bacteriophages for detection and control of foodborne pathogens. *Appl. Microbiol. Biot*. 76: 513-519.

Hajmeer, M.N., E. Ceylan, J.L. Marsden, and R.K. Phebus. 2000. Translocation of natural micro flora from muscle surface to interior by blade tenderization. Available at http://www.ksre.ksu.edu/library/lvstk2/srp850.pdf Accessed 4 April 2012.

Harakeh, S., I. Saleh, O. Zouhairi, E. Baydoun, E. Barbour, and N. Alwan. 2009. Antiicrobial resistance of *Listeria monocytogenes* isolated from dairy-based food products. *Sc. Total Environ*. 407: 4022-4027.

Harris, K., M.F. Miller, G.H. Loneragan, and M.M. Brashears. 2006. Validation of the use of organic acids and acidified sodium chlorite to reduce *Escherichia coli* O157 and *Salmonella typhimurium* in beef trim and ground beef in a simulated processing environment. *J. Food Prot*. 69: 1802-1807.

Harris, D., M.M. Brashears, A.J. Garmyn, J.C. Brooks, and M.F. Miller. 2012. Microbiological and oranoleptic characteristics of beef trim and ground beef treated with acetic acid, lactic acid, acidified sodium chlorite, or sterile water in a simulated commercial processing environment to reduce *Escherichia coli* O157:H7 and *Salmonella*. *Meat Sci*. 90: 783-788.

Hauser, E., A. Mellmann, T. Semmler, H. Stoeber, L.H. Wieler, H. Karch, N. Kuebler, A. Fruth, D. Harmsen, T. Weniger, E. Tietze, H. Schmidt. 2013. Phylogenetic and Molecular Analysis of Food-Borne Shiga Toxin-Producing *Escherichia coli*. *Appl. Environ. Microbiol*. 79: 2731-2740.

Huffman, R.D. 2002. Current and future technologies for the decontamination of carcasses and fresh meat. *Meat Sci*. 62: 285-294.

Jalali, M., and D. Abedi. 2008. Prevalence of *Listeria* species in food products in Isfahan, Iran. *Int. J. Food Microbiol*. 122: 336-340.

James, W. O., R. L. Brewer, J. C. Prucha, W. O. Williams, and D.R. Parham. 1992. Effects of chlorination of chiller water on the bacteriologic profile of raw chicken carcasses and giblets. *J. Am. Vet. Med. Assoc*. 200: 60-63.

Kalchayanand, N., T. Arthur, J. Bosilevac, D. Brichta-Harhay, M. Guerini, T. Wheeler, and M. Koohmaraie. 2008. Evaluation of various antimicrobial interventions for the reduction of *Escherichia coli* O157:H7 on bovine heads during processing. *J. Food Prot*. 71: 621-624.

Kannan, P., H. Yao Yong, L. Reiman, C. Cleaver, P. Patel, and A. Bhagwat. 2010. Bacteriophage-based rapid and sensitive detection of *Escherichia coli* O157:H7 isolates from ground beef. *Foodborne Pathog*. Dis. 7: 1551-1558.

Khadre, M.A., A. E. Yousef, and J.-G. Kim. 2001. Microbiological Aspects of Ozone Applications in Food: A Review. *J. Food Sci*. 66: 1242-1252.

Kim, J-G., A. E. Yousef, and S. Dave. 1999. Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J. Food Prot*. 62:1071-1087.

King, D.A., L.M. Lucia, A. Castillo, G.R. Acuff, K.B. Harris, and J.W. Savell. 2005. Evaluation of peroxyacetic acid as a post-chilling intervention for control of *Escherichia coli* O157:H7 and *Salmonella typhimurium*. *Food Prot. Trends*. 25: 14-22.

Koohmaraie, M., T.M. Arthur, J.M. Bosilevac, M. Guerini, S.D. Shackelford, and T.L. Wheeler. 2005. Post-harvest interventions to reduce/eliminate pathogens in beef. *Meat Sci*. 71: 79-91.

Laster, B.A., K.B. Harris, L.M. Lucia, A. Castillo, and J.W. Savel. 2012. Efficacy of trimming chilled beef during fabrication to control *Escherichia coli* O157:H7 surrogates on subsequent subprimals. *Meat Sci*. 90: 420-425.

Leverentz, B., W.S. Conway, Z. Alavidse, W.J. Janisiewicx, Y. Fuchs, M.J. Camp, E. Chighladze, and A. Sulakvelidze. 2001. Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: A model study. *J. Food Prot*. 64: 1116-121.

Liangji, X. Use of Ozone to improve the safety of fresh fruits and vegetables. *J. Food Tech*. 53:58–61.

Liao, L.B., W.M. Chen, and X.M. Xia. 2007. The generation and inactivation mechanism of oxidation-reduction potential of electrolyzed oxidizing water. *J. Food Eng*. 78: 1326-1332.

Mahmoud, B. S. M. (2007). Electrolyzed water: A new technology for food decontamination-a review. *Deut. Lebensm-Rundsch*. 103: 212-221.

Martin, A., and L. Beutin. 2011. Characteristics of Shiga toxin-producing *Escherichia coli* from meat and milk products of different origins and association with food producing animals as main contamination sources. *Int. J. Food Microbiol*. 146: 99-104.

Mataragas, M., M. H. Zwietering, P.N. Skandamis, and E.H. Drosinos. 2010. Quantitative microbiological risk assessment as a tool to obtain useful information for risk managers- Specific application to *Listeria monocytogenes* and read-to-eat meat products. *Int. J. Food Microbiol*. 141: S170-S179.

Matthews KR. Microbiology of Fresh Produce-Emerging Issues on Food and Safety. Washington, DC: ASM Press, 2006. [MHLW] Ministry of health, labor and welfare. About the results of a surveillance of Foodborne Pathogens in Foods in 2008 FY. Japan: Ministry of health, labor and welfare, 2009.

Mead, P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis*. 5: 607-625.

New Zealand Food Safety Authority (NZFSA), 2008. Microbial pathogen data sheets-*Listeria monocytogenes*. http://www.nzfsa.govt.nz/science/daa-sheets/index.htm. Accessed 17 March 2013.

O'Flynn, G., R. P. Ross, G.F. Fitzgerald, and A. Coffey. 2004. Evaluation of a cocktail of three bacteriophages for biocontrol for *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 70:3417-3424.

Pimentel, D., S. McNAir, J. Janecka, J. Wightman, C. Simmonds, C. O'Connell, E. Wong, L. Russel, J. Zern, T. Aquino, and T. Tsomondo. 2001. Economic and Environmental threats of alien plant, animal, and microbe invasions. *Agr. Ecosyst. Environ*. 84: 1020.

Pittman, C.I., I. Geornaras, D.R. Woerner, K.K. Nightingale, J.N. Sofos, L. Goodridge, and K.E. Belk. 2012. Evaluation of Lactic Acid as an Initial and Secondary Subprimal Intervention for *Escherichia coli* O157:H7, Non-O157 Shiga Toxin-Producing *E. coli*, and a Nonpathogenic *E. coli* Surrogate for *E. coli* O157:H7. *J. Food Prot*. 75: 1701-108.

Pryor, A., and R. G. Rice. 1999. Introduction to the use of ozone in food processing application, p. 26-36. In 14th Ozone World Congress. Dearborn, MI.

Rahman, S., J. Park, J. Wang, and D. Oh. 2012. Stability of low concentration electrolyzed water and its sanitization potential against foodborne pathogens. *J. Food Eng*. 113: 548-553.

Rajkowski, K.T. 2012. Thermal inactivation of *Escherichia coli* O157:H7 and *Salmonella* on catfish and tilapia. *Food Microbiol*. 30: 427-431.

Scanga, J.A., A.D. Grona, K.E. Belk, J.N. Sofos, G.R. Bellinger, and G.C. Smith. 2000. Microbiological contamination of raw beef trimmings and ground beef. *Meat Sci*. 56: 145-152.

Schlech, W. F., P.M. Lavigne, P.M. Borotolussi, A.C. Allen, E.V. Haldane, A. J. Wort, A.W. Hightower, S.E. Johnson, S.H. King, E.S. Nicholls, and C.V. Broome. 1983. Epidemic listeriosis-evidence for transmission by food. *New Engl. J. Med*. 308: 203-206.

Sizer, C., and V. Balasubramaniam. 1999. New intervention process for minimally processed juices. *Food Technol*. 53: 64-67.

Smith, B.A., A. Fazil, and A.M. Lammerding. 2013. A risk assessment model for *Escherichia coli* O157:H7 in ground beef and beef cuts in Canada: Evaluating the effects of interventions. *Food Control*. 29: 364-381.

Sofos, J. N., I. Geomaras, K. E. Belk, and G. C. Smith. 2008. Nonintact whole muscle food safety: the problem and research needs, p. 1-7. Proceedings of the 61st American Meat Science Association Reciprocal Meat Conference, Muscle Biology/Molecular Biology Section, Gainesville, FL.

Stivarius, M.R., F.W. Pohlman, K.S. McElyea, and J.K. Apple. 2002. The effects of acetic acid, gluconic cid and trisodium citrate treatment of beef trimmings on microbial, color and odor characteristics of ground beef through simulated retail display. *Meat Sci*. 60: 245-252.

Sulakvelidze, A. 2005. Phage therapy: an attractive option for dealing with antibiotic-resistant bacterial infections. *Drug Discov Today*. 10: 807-809.

Sulakvelidze, A, Z. Alavidze, and J.G. Morris. 2001. Bacteriophage therapy. *Antimicrob. Agents Ch*. 45:649-659

Trindade, M. A., M.M. Kushida, N.D. Montes Villanueva, D. Uenaka Dos Santos Pereira, and C.E. Lins De Oliveira. 2012. Comparison of ozone and chlorine in low concentrations as sanitizing agents of chicken carcasses in the water immersion chiller. *J. Food Prot. 75*: 1139-1143.

Unluturk, S. M. Atilgan, A. Handan Bysal, and C. Tari. 2008. Use of UV-C radiation as a non-thermal process for liquid egg products (LEP). *J. Food Eng*. 85: 561-568.

U.S. Department of Agriculture, Food Safety and Inspection Service. 1999. 9 CFR Chapter III [Docket No. 97-068N] Beef Products Contaminated with *Escherichia coli* O157:H7. U.S. Department of Agriculture, Washington, D.C.

U.S. Department of Agriculture, Food Safety and Inspection Service. 2013. [Docket No. FSIS-2008-0017] Mandatory Labeling for Mechanically Tenderized Beef Products. U.S. Department of Agriculture, Washington, D.C.

U.S. Department of Agriculture, Food Safety and Inspection Service. (2004a). Listeria Guidelines for Industry. U.S. Department of Agriculture, Washington, D.C.

Viazis, S., M. Akhtar, J. Feirtag, and F. Diez-Gonzalez. 2011. Reduction of *Escherichia coli* O157:H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and *trans*-cinnamaldehyde. *Food Microbiol*. 28: 149-157.

Wang, X., X. Lu, L. Yin, H. Liu, W. Zhang, W. Si, S. Yu, and M. Shao. 2013. Occurrence and antimicrobial susceptibility of *Listeria monocytogenes* isolates from retail raw foods. *Food Control*. 32: 153-158.

Yoder, S. F., W.R. Henning, E.W. Mills, S. Doores, N. Ostiguy, and C. Cutter. 2012. Investigation of chemical rinses suitable for very small meat plants to reduce pathogens on beef surfaces. *J. Food Prot*. 75: 14-21.

Yoon, Y., A. Mukherjee, K.E. Belk, J.A. Scanga, G.C. Sith, J.N. Sofos. 2009. Effect of tenderizers combined with organic acids on *Escherichia coli* O157:H7 thermal resistance in non-intact beef. *Int. J. Food Microbiol*. 133: 78-85.

Yoon, Y., A. Mukherjee, I. Geornaras, K.E. Belk, J.A. Scanga, G.C. Smith, and J.N. Sofos. 2011. Inactivation of *Escherichia coli* O157:H7 during cooking of non-intact beef treated with tenderization/marinating and flavoring ingredients. *Food Control*. 22: 1859-1864.

Youssef, M.K., X. Yang, M. Badoni, and C.O. Gill. 2012. Effects of spray volume, type of surface tissue and inoculum level on the survival of *Escherichia coli* on beef sprayed with 5% lactic acid. *Food Control*. 25: 717-722.

Zhao, S., K. Blickenstaff, S. Bodeis-Jones, S.A. Gaines, E. Tong, and P.F. McDermott. 2012. Comparison of the prevalence and antimicrobial resistances of *Escherichia coli* isolates from different retail meats in the United States, 2002 to 2008. *Appl. Environ. Microb*. 78: 1701-1707.

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