EVALUATION OF THE MICROBIAL ASPECTS OF AMMONIUM HYDROXIDE WHEN USED IN BRINE SOLUTIONS

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WHEN USED IN BRINE SOLUTIONS

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Enhanced or value-added meat and poultry products are raw products that contain brine solutions added through marination or needle injection. The injection of brine solutions into meat products is a common practice to decrease variability in tenderness and juiciness, and reduce the loss of water (purge or drip loss) while meat is in retail display (*22*). Brine is a strong solution of water and salt (*24*). Aside from salt (NaCl) brines often contain phosphates. The addition of phosphate has the greatest effect on tenderness due the improvement of water holding capacity by raising meat pH and solubilizing myofibrillar proteins (*2*). In addition, phosphates have been reported to act as antimicrobial agents (*1, 11, 20*). Unfortunately, phosphates are contributing as much sodium to fresh meat products as the salt incorporated in the brine solution. Phosphates are being added in commercial injection brines at about 3-5% (phosphates are permitted by USDA law up to 0.5% of final product weight; *14*). A non-sodium alternative to improve water holding capacity, tenderness and juiciness might be ammonium hydroxide (AH). Ammonium hydroxide is considered a safe and suitable ingredient as a pH control agent in brine solutions for meat products (*25*). It can be used to increase a brine solution to pH 11.6 (*25*). Currently, limited research has been published on the formulation brines using AH. The available studies focused on palatability parameters (*4, 7, 10*), optimum pumping level (*9*), and consumer acceptability (*15*). We previously studied the effect of

injecting brine solutions containing AH into beef loins, which were subsequently cut into steaks, packaged under a high-oxygen modified atmosphere packing (80% O_2/20% CO_2,MAP), and placed into retail storage for 14 days after an initial storage period of 5 days in the dark to mimic transportation conditions. Quality factors such as tenderness, juiciness, color, cook yield, and sensory acceptability as well as aerobic (APC) and anaerobic total counts (AnPC) were evaluated (*3*). Quality parameters of loins injected with the brine solution containing ammonium hydroxide (0.1% AH, brine pH 10) were not as good as the control (phosphate steaks); however, the aerobic (APC) and anaerobic plate counts (AnPC) were lower. Therefore, a subsequent study was conducted using a higher level of ammonium hydroxide (1% AH; brine pH ~11, *16, 17*). In the study by Parsons et al. (*16, 17*), quality parameters were comparable and final meat pH of AH-brine and control-brine (phosphate brine) were 5.96 and 5.86, respectively. However, APC and AnPC were not significantly different until day 14 of the study (*16, 17*). These results look promising for the industry since a phosphate-free and reduced-sodium brine can be produced. However, the impact of AH on microbial populations has not been fully addressed.

It is known that the use of invasive technologies such as blade tenderization, brine injection or mechanical tenderization serve as a vehicle for bacteria to be internalized by the needles from a contaminated surface into the sterile deep beef tissue (*7, 12, 13, 18, 19*). One of the most common bacteria that has been associated with the consumption of undercooked beef is the causative agent of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans known as *Escherichia coli* O157:H7 (*6*). The Food Safety and Inspection Service (FSIS) has classified *E. coli* O157:H7 as an adulterant and

set a zero tolerance policy for non-intact meats (*13*). Therefore, new applicable technologies are needed to reduce the prevalence of pathogens such as *E. coli* O157:H7 in meat and meat products. Again, a practical alternative may be the use of AH in the formulation of brines. It has been suggested that AH possesses an antimicrobial effect against Gram-negative pathogens. For example, U.S. patent Nº 7,022,361 describes a method to inject gas or aqueous solution containing ammonia-based-compounds into the interior of a meat product to raise the pH sufficiently to inactivate meat pathogens (*21*). Stopforth et al. (*23*) inoculated boneless beef samples (5 x 2.5 x 1 cm) with *E. coli* O157:H7 and *S.* Typhimurium and then treated the inoculated product by dipping into a 0.1% AH solution (pH 10.89, *23*). It was observed that treatment with 0.1% ammonium hydroxide did not reduce the pathogen population. Therefore, the authors concluded that the solution did not sufficiently increase final meat pH (pH 7.6) to have the expected bactericidal effect (*23*). Gupta et al. (*8*), however, evaluated the effect of AH on goat ground-meat. Different concentrations (from 0.5% to 2.6% w/w) were prepared by adding from 1 to 5 ml of AH to 11 g of ground meat sample, and incubated at 37ºC, 4ºC, and -20ºC. At 37ºC, spoilage was evident after 2 days (*8*). However, when concentrations of AH were ≥ 1.6% and meat was pH 9.5, no-increase in aerobic bacteria was observed in samples maintained at 4ºC for up to 11 days; while in samples stored at -20ºC a reduction of > 1 $\log_{10}$ was described (*8*). The antibacterial effect observed was attributed to the toxicity of AH rather than a change in pH (*8*).

The objective of this thesis project was to evaluate the microbial aspects of AH when used in brine solutions. The first study was conducted with the aim to determine if a brine containing AH impacts meat microbial flora differently than a conventional brine

containing phosphates. The following studies were focused on the pathogen *Escherichia coli* O157:H7. A multi-nozzle spray system was used to spray water, 1%, 2%, and 3% AH solutions onto inoculated meat-disk-samples with *E. coli* O157:H7. Finally, the objective of the third study was to determine if ammonium hydroxide possesses an immediate, and/or a long term antimicrobial effect against *E. coli* O157:H7 when used as an alkaline aid in the formulation of brines applied through needle injection to striploins.

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CHAPTER II

REVIEW OF LITERATURE

1. Meat Industry Overview

According to the American Meat Institute (AMI), the U. S. meat industry processed 32.4 million cattle in 2008 (*7*). Americans have been eating a yearly average of 29 kg of beef (*107*). The U.S. meat and poultry industries are one of the largest segments of the U.S. agricultural economy (*7*). The U.S. meat industry contributed $8 billion to the $23 billion surplus in the agricultural sector in 2009. Total beef, pork and poultry production in 2009 exceeded 40 billion kg (*6*). However, beef consumption has declined from 28% in 1996 to slightly more than 24% in 2006 (*93*). Declining demand for beef has been attributed to competitive pricing, safety concerns, changing consumer lifestyles, quality issues, and convenience issues (*93*). Demographics and health concerns are important demand drivers along with prices, competing foods, and information (*31*). Additionally, time-pressed consumers purchase more on convenience, while looking for quality, variety, and value (*31*). The consumer demand for food is shifting toward products that are easy to prepare while also promising safe eating, improved nutrition, and greater consistency (*31*).

According to Hendrickson et al. (*46*), during the 20th century the American agriculture industry experienced dramatic changes due to interactions between social/political, economic, environmental, and technological factors. They concluded that the highly specialized systems such as supply chain livestock production are vulnerable to future changes. They also pointed out that sustainable agricultural systems will need balance among various domains to be able to adapt and survive. Therefore, they recommend approaching dynamic, integrated agricultural practices to increase flexibility in agricultural systems as a key factor in adaptation and survival (*46*). Additionally, according to Ferrara and Ward (*31*), the beef industry needs to put forth considerable effort to provide variety within the product category, which is one way to potentially influence demand.

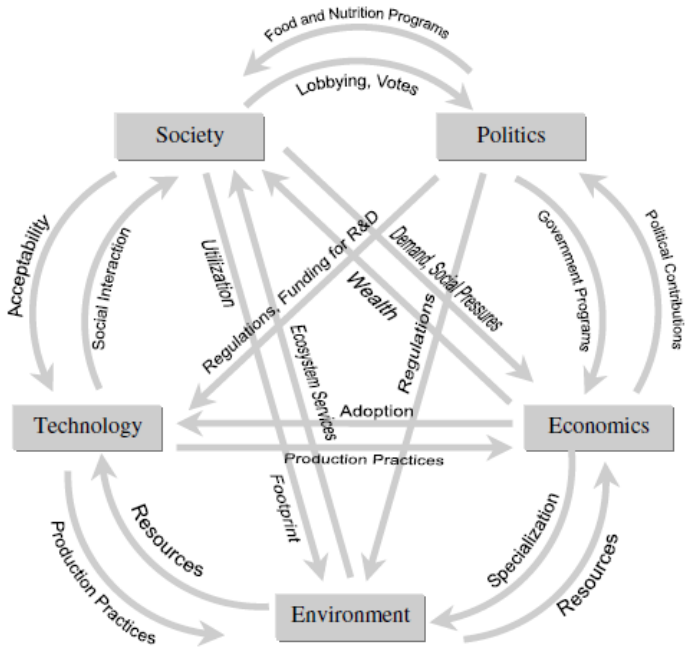

FIGURE 2.1. Interactions between social, political, environmental, economical and technical drivers of agricultural production systems. From Hendrickson et al. (*46*).

2. Use of Brines by the Meat Industry

Enhanced or value-added meat and poultry products are raw products that contain brine solutions added through marinating, needle injecting, or soaking. The production of value-added products throughout the injection of brine solutions into meat products is a common practice to decrease variability in tenderness and juiciness (*96*). An enhancement solution injected at 6-10% may also help to decrease the lipid oxidation process (*94*). The marination technology through injection has made considerable advances in the pork and beef industry in recent years (*114*). Currently in the US, the large meat companies have increased the production of water-added pork, beef, and poultry that are prepackaged for case-ready products, which is gradually replacing the traditional non-treated meats at retail stores (*114*). The primary purpose of adding brine solutions is to increase the meat pH, which leads to an increase the water holding capacity (WHC) and thus improves juiciness and tenderness. The ability of meat to retain water (swelling) is known as water holding capacity. This characteristic is essential for meat palatability in terms of juiciness and tenderness.

Water holding capacity in meats is greatly affected by pH and the meat proteins' ion environment (*13*). During the conversion of muscle to meat, lactic acid builds in tissue causing a reduction of meat pH from neutrality to 5.4 - 5.5 (*49*). Once the meat pH is between 5.0 - 5.5, the majority of the meat proteins have reached the isoelectric point (pI), especially myosin with a pI of 5.4 (*49*). At the isoelectric point, the net charge of the proteins is zero, meaning the number of positive and negative charges on the protein is equal (*49*). Within the protein, these positive and negative groups are attracted to each

other resulting in a reduction of the amount of water that can be attracted and held by the protein (*49*) consequently meat swelling or WHC is minimal as seen in Fig 2.2. Because like charges repel, as the net charge of the meat proteins reaches zero, repulsion of structures within the myofibril is reduced allowing those structures to pack more closely together, causing a reduction of space within the myofibril (*49*). Additionally, during the postmortem period, a rise in the ionic strength is caused in part by the inability of ATP-dependent calcium, sodium and potassium pumps to function.

The figure 2.2 depicts the relationship between meat pH and purge or water loss:

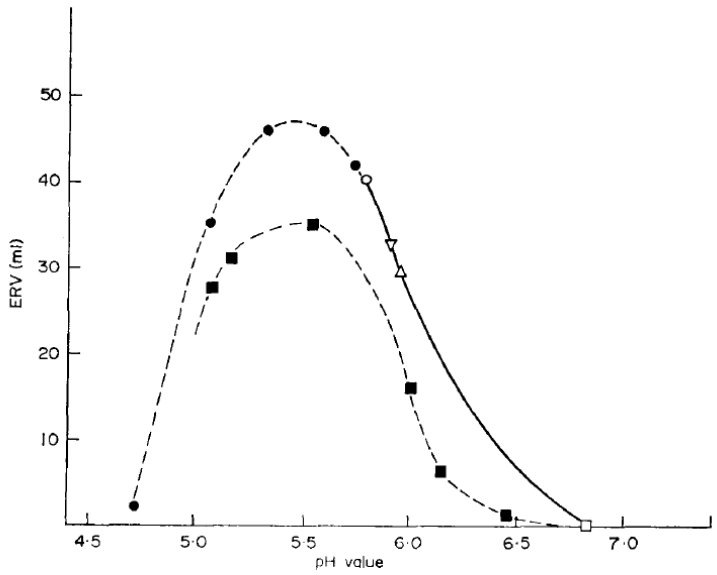

FIGURE 2.2. Relationship between extract-release volume (ERV, or purge) and pH value of ground beef held from freshness to spoilage. Dots represent purge values of fresh meat. Squares represent purge values of meat after 7 days. From Shelef (*97*).

Besides improving WHC and impacting sensorial quality of meat, water is also important in enhanced meats to serve as a carrier of ingredients, and to replace moisture

lost during thermal processing (*1*). Additionally, moisture is often associated with WHC and may be partially responsible for other physical and structural properties, including color, texture, firmness, and tenderness (*1*).

3. Ingredients used in the Formulation of Brining Solutions

Typically, enhanced beef is injected with a water solution including salt, phosphate, sodium lactate, seasonings, and flavorings. The addition of rosemary extract in enhancement solutions contributes to longer shelf-life (*71*).

3.1. Role of Sodium Chloride in Brining Solutions

Salt or sodium chloride (NaCl) is a common ingredient used in the formulation of brines. Salt has been used as anti-spoilage agent in foods, it is also an important flavoring agent, and contributes to technological and functional aspects of product development (*100*). The inclusion of salt in the brine improves yield and palatability characteristics, and also impacts meat color and shelf life of injected meat (*12*). Boles and Swan (*15*) reported that a sodium chloride (NaCl) solution increases cooking yields, decreases post mortem pH decline, and increases water-binding. In emulsion-type food products, for example, the addition of salt not only improves favor, it is also known to solubilize salt-soluble myofibrilar proteins. These proteins form a matrix, which upon heat coagulation binds fats, water, lean meat and other ingredients, which increases the yield and overall quality (*100*). In general, salt alters the meat and moisture binding ability of processed foods (*105*). Xiong et al. (*114*) conducted a study to determine alterations in the ultrastructure of myofibrils (swelling) of brine-incorporated meat. It

was concluded that no structural changes of the muscle myofibrils occurred at concentrations of 0.5 M NaCl or less. However, a significant transverse enlargement (or swelling) of the myofibrils was observed at 0.6 M NaCl and continued up to 0.8 M NaCl, where a maximum swelling was reached (*114*). They attributed this swelling to electrostatic repulsion between myofilaments as well as depolymerization of myosin filaments, which lead to expanded filamental spaces for water entrapment (*114*).

3.2. Antibacterial Aspects of Sodium Chloride

Sodium compounds are known to contribute to product preservation and extending shelf life by inhibiting microbial growth (*100*). In earlier times, the major method of preserving foods was by adding high amounts of salt to produce shelf stable dried and smoked meats. In this case, salt serves as a direct (primary or sole) antimicrobial preservative (*100*). In other food products, however, the antimicrobial action of salt is known to be indirect and it serves as a synergetic antimicrobial in combination with other factors such as pH and water activity (a_w; *100*). In fermented vegetable products, for example, lower amounts of salt are used to favor the growth of lactic acid bacteria, which produce lactic acid and inhibit the growth of pathogenic microorganisms (*100*).

Among food ingredients containing sodium, NaCl is the most effective in preventing the growth of foodborne pathogens and spoilage bacteria (*105*). The amount of NaCl permitting microbial growth varies with groups of microorganisms. However, most foodborne pathogenic bacteria do not grow at concentrations of 10% NaCl (*100*). It has been generally accepted that salt inhibits microbial growth in foods mainly by

lowering the a_w of the food products (*100*). Salt lowers a_w by causing a hyperosmotic shock on cells. The hyperosmotic shock causes shrinkage of the cytoplasmic volume called plasmolysis (*105*). However, there are some bacteria that can tolerate salt, examples of these foodborne bacteria are: *Listeria monocytogenes*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus* (*105*). Fungi are more resistant than bacteria to salt and thrive and survive in low a_w foods, and some yeast can even survive in concentrations of salt up to 11% (*105*). Other factors involved in the antimicrobial effect of salt are: direct toxicity of Cl^-, removal of oxygen from the medium, sensitization of the organisms to CO_2, and interference with rapid action of proteolytic enzymes (*105*).

3.3. Health Effects Associated with Sodium Consumption

Currently, the food industry is under pressure from consumers and government to deliver products with lower amounts of sodium due to its relationship with hypertension. Frieden and Briss (*34*) reported that excess sodium consumption was the principal cause of increasing blood pressure in the United States. They reported that most Americans consume more salt than is healthy. Sodium intake has increased over the past 30 years to more than double the recommended amount, which increased the risk for heart attack and stroke (*34*). And, according to the American Heart Association (AHA), high blood pressure (hypertension) killed 56,561 people in the United States in 2006 (*5*). Worldwide cardiovascular disease is the leading cause of death in elderly people (60 years or older), and second among people between 15 and 49 years (*34*). One third of the adults in the United States have hypertension and another 28% have levels above the desirable range (*34*). Additionally, Cappuccio and Capewell (*19*) affirm that currently approximately

62% of all strokes and 49% of coronary heart disease are attributable to high blood pressure. Each 20-mm Hg increase in systolic blood pressure above 115 mmHg doubles the risk of heart attack and stroke (*34*). However, it should be noted that sensitivity to sodium can vary among individuals (*105*). Consumer groups and Federal agencies have indicated the need for reducing the amount of sodium in the human diet (*100*). In most developed countries, 80% of the salt is added before food is sold (*19*). Hence, in 2008, Congress asked the Institute of Medicine (IOM) to recommend strategies to reduce the sodium intake of Americans (*51*). Therefore, the IOM has published the report "Strategies to Reduce Sodium Intake in the United States" in April of 2010. According to this report, more than 100,000 deaths annually can be prevented by reducing the amount of sodium intake (*34, 51*). Even though the recommended amount of sodium per day should be no more than 2,300 mg, it was established that Americans consumed more than 3,400 mg of sodium per day (*51*). The report identifies the consumption of high levels of salt in processed and restaurants foods as a reason for this high sodium intake. Therefore, the IOM has recommended the FDA set mandatory national standards for the sodium content in foods (*51*). However, they also recommended the reduction of sodium contents in foods gradually, since it was proven that consumer's taste preferences can be changed over time (*51*).

3.4. Role of Phosphates in Brining Solutions

The ability to withstand rapid meat pH changes when acid or alkali is added is referred as buffering capacity. Buffering capacity of a weak acid or base is a function of pH and it changes depending on how its pK_a value is related to the pH value of meat (*60*).

Thus, phosphate compounds with pK_a values between 6.1 and 7.1 are known to be one of the most influential compounds in meat systems with a pH range of 5.5 to 7.0 (*60, 86*). Currently, sodium phosphates are widely used as ingredient in commercial brine solutions. The use of sodium phosphates is also regulated by the FSIS; meat products can not contain more than 0.5% in the final product (*70*). According to Alvarado and McKee (*4*), sodium tripolyphosphate accounts for approximately 80% of the phosphates used in further-processed meat products.

The mechanism of action of phosphates in meat has been previously reported. Sodium tripolyphosphate has an alkaline pH and its addition to meats systems increases the final pH. Raising the final pH improves water holding capacity (WHC) of the meat by moving the protein isoelectric point (~5.5) to a more neutral pH due to an increase of negative charges (*70*). The net negative charges increase the electrostatic repulsion between muscle-fibers; consequently, there are more sites available for water binding (*83*).

The utilization of phosphates might have a positive or negative impact on other characteristics of the product such as: yield, meat color, juiciness, tenderness, and lipid oxidation. For example, yield, juiciness, and tenderness are improved due to increased water holding capacity. The addition of phosphates retards lipid oxidation (*70*). However, color is altered negatively since there is less water to reflect light and the meat appears darker in color. In terms of flavor, it has been shown that phosphates at high levels result in “soapy” or bitter tastes (*4*).

3.5. Antibacterial Aspects of Phosphates

It has been demonstrated that phosphates also possess an antibacterial effect. Pohlman et al. (*85*) has reported that trisodium phosphate (TSP) reduces *Escherichia coli*, *Salmonella*, coliforms, and aerobic bacteria in ground beef when applied to beef trimmings before grinding. In addition, an effective treatment to be used for microbial decontamination of beef and poultry carcasses is the use of TSP in washing operations (*85*). The TSP forms a complex linkage with bivalent metals essentials to the microorganism cell, consequently the cell wall stability is affected (*85*). Not all the phosphates, however, have the same antibacterial effect against bacteria. Additionally, it was found that sodium tripolyphosphate (STP) increases its antimicrobial effect when formulated with 1.25% NaCl (*2*). The STP and TSP have an antimicrobial effect at both 1 and 2% concentration levels in minced beef (*2*). In another study, TSP was used to remove attached *E. coli* O157:H7 and *S.* Typhimurium from beef surfaces (57). In this case, beef surfaces were inoculated with 10^9 CFU/ml of *E. coli* O157:H7 and *S.* Typhimurium. Surfaces were then rinsed with 10% TSP solution for 15 sec. The *E. coli* O157:H7 was reduced by 1.35 log CFU/ml and *S.* Typhimurium by 0.92 logs (57).

3.6. Health Effects Associated with Consumption of Phosphates

A large survey of nutrient consumption in the U.S. found that the average phosphorus intake was 1,495 mg/day in men and 1,024 mg/day in women (*47*). The average phosphorus intake by an average American has increased 10% to 15% over the past 20 years (*47*). This increment might be attributed to the addition of phosphoric acid in soft drinks and the use of phosphate additives in processed foods (*47*). One of the

problems related with high phosphate levels in the blood is the fact that it causes a reduction in the formation of the active form of vitamin D (calcitriol) in the kidneys, which reduces blood calcium, and leads to increased parathyroid hormone (PTH) release by the parathyroid glands (*47*).

The prevalence of chronic kidney disease (CKD) is increasing worldwide. The CKD is associated with cardiovascular disease, chronic volume overload, and abnormal calcium-phosphate metabolism (*106*). Poor controlled metabolic bone disease contributes to excess cardiovascular risk of CKD through putative effects on arteriosclerosis (*106*). In addition, serum phosphates are associated with death and myocardial infarction in patients with 3-4 CKD (*106*). Additionally, there is an increased prevalence of coronary artery calcification in people with stage 3-4 CKD (*106*). There are also data demonstrating a strong association between serum phosphate and all-cause mortality, cardiovascular mortality, and fracture rates (*45*). Elevated serum phosphate directly influences the development of hyperparathyroidism (*45*).

In addition, high consumption of phosphates can cause allergies, diarrhea, hardening of soft tissues or organs, and interferes with adsorption of iron, calcium, magnesium, and zinc (*32, 95, 113*).

3.7. Role of Ammonium Hydroxide in Brining Solutions

Ammonium hydroxide (AH) is Generally Recognized as Safe (GRAS) by FDA when used in accordance with Good Manufacturing Practices (GMP), and contains no residues of heavy metals or other contaminants in excess of FDA tolerances (*78*).

According to the FSIS Directive 7120.1, Revision 2, AH is part of the "Table of Safe and Suitable Ingredients" list and it is recognized as pH control agent in brine solutions for meat products. Ammonium hydroxide can be used in amounts sufficient for purposes of achieving a brine solution with a pH of 11.6 and it has no-labeling requirements under the accepted conditions of use (*108*). There are some studies that reported on the injection of brine solutions using AH and salt into beef cuts and meat products. Among these, the consumer acceptability of 5 different muscles injected with a solution containing AH, water and salt was studied (*74*). Later, a study conducted by Hamling and Calkins (*42*) focused on finding the optimum pumping level to improve palatability characteristics of chuck and loin muscles injected with a solution containing AH, water and salt (*42*). In another study, the effects of pH enhancement on aging were evaluated. This study was conducted to determine whether the benefits of enhancement (tenderness, juiciness, and flavor) of beef chuck and round muscles with AH and salt were reduced by aging (*43*). Moreover, with the aim to evaluate visual appearance and juiciness, several meat products (grilled chicken breast, grilled pork loin, deli-style roast beef, beef prime rib, beef pot roast, barbeque beef brisket and hams) were also injected with a brine solution containing AH (*29, 30*). In addition, Cerruto et al. (*20*) injected striploins with a 0.1% AH, brine pH 10. Quality parameters in the striploins injected with AH brine were not rated as highly as those of controls (phosphate-based brine). Therefore, it was concluded that a higher concentration of AH needed to increase in order to raise final meat pH in the product sufficiently for it to be more competitive in terms of color stability, water holding ability, and tenderness (*20*). Later in 2011, Parsons et al (*81, 82*) reported the use of a 1% AH to replace phosphate-based ingredients in the formulation of brines. In this case,

quality parameters were comparable to the control and meat pH was higher for AH injected steaks than the control (5.96 vs. 5.86). The aerobic and anaerobic plate counts were not different until day 14.

3.8. Antibacterial Aspects of Ammonium Hydroxide

Although several studies have focused on the use of AH and ammonium compounds as antimicrobial agents in foodstuffs; the role of AH in meat products is not fully understood and conflicting results have been reported. Certain authors reported no-antimicrobial effect (*44, 55*), while others stated that it is effective only when meat pH is higher than 9 (*103*). Still, other investigators affirm that AH effectively reduces microbial growth (*41, 76***).**

For example, samples of boneless beef plates inoculated with *E. coli* O157:H7 and *S.* Typhimurium were dipped into a 0.1% AH solution (pH 10.89) for 30 s at 23ºC (*103*). In this case, the treatment raised the meat pH from ~5.7 to 7.6. At pH 7.6, pathogen populations were not affected by AH solution. Hence, authors concluded, for AH solution to have an effective antimicrobial effect, meat pH must be 9 or higher (*103*). Later in 2008, Hamling et al. (*44*) reported the injection of *triceps brancii* beef steaks with a solution containing AH (pH 11.4). Injected beef steaks were low-oxygen MAP packaged, kept in dark storage at 4ºC for 1, 2, or 3 weeks, and then placed under retail display at 4ºC for an additional period of 7 days. The APCs were performed every week for 3 weeks, and APC plates were incubated at 32ºC under aerobic and anaerobic conditions (*44*). They have reported higher APC counts in AH injected steaks than in controls (un-injected steaks). Counts started at 2.58 $\log_{10}$, for treated steaks, and after 4

weeks (3 weeks in dark, plus one week under retail display) counts reached 7.36 $\log_{10}$ (*44*).

In contrast to these findings, texturized meat was exposed to 0, 250, 500, and 1000 ppm AH for 15 minutes. An increased meat pH (from 6.48 to 9.41) was observed, but, no significant reductions on APC counts were obtained (*55*). In another study, boneless lean beef inoculated (at levels of 6 $\log_{10}$ CFU/g) with *Salmonella, Listeria monocytogenes,* and *E. coli* O157:H7 were treated with ammonia gas. The meat pH was raised to 9.6. Bacterial populations were reduced by approximately 4, 3, and 1 $\log_{10}$ cycles respectively (Niebuhr and Dickson, 2003). Also Gupta et al. (*41*) evaluated the effect of AH on goat ground-meat. Different concentrations (from 0.134 to 0.67 M) were prepared by adding from 1 to 5 ml of AH to the ground meat sample, and incubated at 37ºC, 4ºC, and -20ºC. At 37ºC, spoilage was evident after 2 days (*41*). However, when concentrations of AH were ≥ 0.4 M and meat was pH 9.5, no-growth of aerobic bacteria was observed in samples maintained at 4ºC for up to 11 days; while for samples stored at -20ºC a reduction of > 1 $\log_{10}$ was described **(***41*). The antibacterial effect observed was attributed to the toxicity of AH rather than a change in pH (*41***)**. According to Silipo and others (2002), lipid A constitutes the endotoxic principle of lipopolysaccharides, which are founded in the external membrane of almost all Gram-negative bacteria. Applying AH in a 3:1 diluted solution for 16 hours at room temperature causes hydrolysis of the Lipid A (*98*).

4. Meat pH Changes Associated with Bacterial Spoilage

Spoilage in meats is directly correlated with the initial microbial quality and storage conditions (*64*). The final predominant bacterial flora depends on the packaging system used. Thus, *Pseudomonas* spp. are the most predominant bacteria that cause spoilage in meat and meat products under aerobic conditions (*37, 54, 59, 64*), Enterobacteriaceae are predominant in temperature-abused meats, lactic acid bacteria and Micrococcaceae in meats packaged with preservatives and *Brochothrix thermosphacta* in vacuum- and modified-packaged meat products (*64*).

Immediately after slaughter meat muscle is soft, limp and dry. However after a few hours, rigor mortis occurs (*13*). At this point, anaerobic conditions start to develop, and energy-rich compounds such as ATP and ADP are degraded. The glycolysis process forms lactic acid, which remains in the muscle decreasing the meat pH (*13*). Although changes in pH are dependent on the type of muscle, in general, meat pH goes from about 6.5-7.1 (1 hour post morten) to about 5.4-5.8 (24 hours post morten; *13, 16*). Bowdell et al. (*16*) observed a reduction of beef pH from 6.99 to 5.74 after 24 hours post mortem. They reported that 46.5 µM of lactic acid per gram of muscle were produced for every unit decrease in pH. In addition, glycogen in muscle was found to reduce from 56.7 to 10.1, while amount of glucose increase from 7.9 at slaughter to 18.1 after 24 hours (*16*). It was concluded that glycogen in beef muscle is degraded to glucose and lactic acid during the post-mortem glycolysis process (*16*).

Although the concentration of carbohydrates (glucose and glycogen) is low in comparison to proteins, the initial proliferation of microbial populations in meats is

supported by the depletion of these carbohydrates (*64*). Initially, under aerobic conditions, and due to a high growth rate, *Pseudomonas* spp. can easily dominate the meat environment of high oxygen MAP atmospheres when temperatures are between 2 and 15°C (*37, 38, 59*). Other bacteria that may be present in beef meats under aerobic and chilled conditions are *Acinetobacter*, *Moraxella*, *Enterobacter* spp. and *Microbacterium thermophactum* (*38*). It was reported that *Pseudomonas* spp. have preference for glucose as a substrate (*38*). *Pseudomonas* spp. and other microorganisms present in meat surface start attacking free amino acids for energy only when glucose is completely deleted (*38*, 41, *64*) and amino acids are consumed before lactate (*38*). During amino acid metabolism many bacteria (including *Pseudomonas* spp.) produce volatile byproducts including ammonia, which causes an increase in meat pH (*41, 64*). Gill and Newton (*38*) attribute the short shelf life of dark firm and dry (DFD) meat to the utilization of amino acids by *Pseudomonas* spp. from the start since there an absence of glucose in DFD meats (*38*).

The meat environment of high oxygen MAP atmospheres eventually changes. An uptake of O_2 and increase of CO_2 caused by the tissue respiration and growth of microorganism present in meats occurs (*66*). It has been demonstrated that films relatively impermeable to O_2 and CO_2 allow the accumulation of CO_2 (*104*). These enlarged amounts of CO_2 eventually inhibit microbial growth of Gram-negative aerobic spoilage microorganisms like *Pseudomonas* spp. allowing the growth of facultative or anaerobic gram positive bacteria (*66*). Thus, when availability of oxygen is reduced, lactobacillus and *Brochothrix thermosphacta* are predominant (*59, 64*). The *Br. thermosphacta* population increases only after meat pH is equal or higher than 6 (*52*)

since this bacterium is unable to grow in pH below 5.8 under anaerobic conditions (*52*). The anaerobic metabolism of *Br. thermosphacta* produces lactic acid as the major end product and small amounts of diacetyl, acetoin, isovaleric, isobutyric and acetic acids (*59*) which reduces the pH.

The following figure (Fig. 2.3) depicts the hypothetical changes in meat pH associated with spoilage bacterial growth under MAP-packaging conditions:

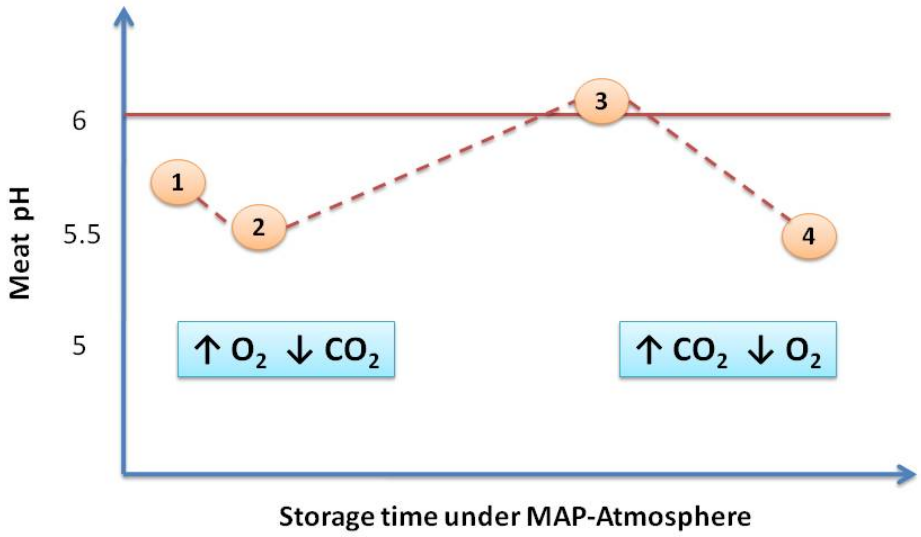

FIGURE 2.3. Hypothetical meat pH changes caused by spoilage bacteria under MAP atmospheres. Where: 1, initial meat pH. 2, meat pH at initial microbial proliferation, glucose is the main bacterial substrate. 3, increase in meat pH caused by the proteolytic activity. 4, a change in the bacterial population, lactobacillus and *Brochothrix thermosphacta* are predominant in the environment producing acidic metabolites, which reduces the meat pH.

5. Effect of High pH on Microbial Growth

It is well known that because of differences in the chemistry of the bacterial cell, Gram-negative bacteria are more susceptible to high-pH solutions than gram-positive bacteria (*11, 68, 76*). A study conducted by Vasseur et al. (*109*) found that 30 min exposure of a pH 10.5 solution (using NaOH) caused a reduction of 4-logs to an early stationary culture of *Pseudomonas* spp. (*109*). Interestingly, a mixture of ground meat and 0.4 M AH (pH 9.5) incubated at 4ºC had ~0.5 $\log_{10}$ lower Gram-negative populations than extracted ground meat adjusted at the same pH using sodium hydroxide after 11 days of study (*41*). Humphrey et al. (*50*) affirm that at pH 9.0 the death rate of *S.* Typhimurium is increased (*D*52ºC reduced from 34.5 to 1.25), and APCs and coliforms counts are also effectively reduced. In addition, Dickson (*24*) observed that washing lean and fat beef tissues with a high pH solution (using concentrated sodium or potassium hydroxide) populations of *S.* Typhimurium and *L. monocytogenes* were reduced by 2-3 log10 and 1-2 log10 cycles, respectively. According to Mendoca et al (*68*) the very thin peptidoglycan layer of Gram-negative bacteria lacks the capability of preventing the cytoplasmic membrane from bursting once it is weakened by a high pH solution. Solubilization of proteins as well as saponification of the lipids are suggested to weaken the bacterial membrane (*68*). Interestingly, some Gram-negative bacteria can survive the high pH treatments and do not exhibit injury. Therefore, Mendoca et al. (*68*) proposed an all-or-nothing event caused by high pH.

6. Use of Ammonia-Based Compounds as Antimicrobials

Ammonia-compounds are known to be high in pH. Several studies have reported the use of ammonia-compounds to reduce pathogens. For example, Himathongkham et al. (2001) showed a reduction of 2-3 logs in alfalfa seeds and 3-5 logs in mung beans experimentally inoculated with *E. coli* O157:H7 and *S.* Typhimurium (10^8 to 10^9 CFU/g) and then treated with ammonia gas (*48*). Later, Park and Diez-Gonzales (2003) reported that the level of ammonia gas, at which reductions of *E. coli* O157:H7 and *S.* Typhimurium started to be observed was ~ 5mM for inoculated cattle manure (*79*). Other studies focused on meat products, like Niebuhr and Dickson (2002) who reported the reduction of *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* by 4.5, 3.0 and 0.5 $\log_{10}$ cycles, respectively, in boneless lean beef trimmings when ammonia gas was applied and pH was raised to ~9.6 (*76*). Moreover, several patents were awarded to Freezing Machines Inc. for treating meat products with ammonia-based compounds; which are used as pH modifying agents to inhibit microbial activity, mainly *E. coli*, coliforms, and *Staphylococcus aureus* (*88, 89, 90, 91, 92*). These patents involve the injection of ammonia-based compound (ammonia gas, ammonium hydroxide or a mixture of both) to cause a rapid increase of pH, followed by the injection of a pH decreasing material (carbon dioxide gas, in most cases), and a final step where the applied gases are removed, while the meat products are in frozen state. It is claimed that this process can be used in ground beef, steaks, roast, or larger cuts of meats (*88, 89, 90, 91, 92*).

7. Mechanisms to Reduce Bacterial Growth in Fresh Beef

Although several treatments (pre and post harvest) are applied to prevent pathogenic food contamination (*65*), each year in the US food borne pathogens still cause 2,718 deaths, of which 1,809 are attributable to foodborne transmission with bacteria responsible for 72% of these deaths (*67*). The contamination of sterile meat muscle is a result of slaughtering and dressing of animal carcasses (*64*). A large variety of pathogenic and spoilage bacteria can be found in meats. Many of these bacteria can be pathogenic, and therefore, become a risk for the consumers. Thus, in order to ensure meat safety in the meat industry during the whole process of production, several efforts have focused on retarding bacterial growth or killing bacteria. Several treatments are applied to the carcasses to prevent growth of pathogens or spoilage microorganisms, including physical, chemical or biological treatments (*64*). In addition, on July 25, 1996 the FSIS issued the landmark rule: Pathogen Reduction "Hazard Analysis and Critical Control Points" (HACCP) Systems (*73*). The HACCP system is the most effective means for systematically developing food safety protocols that can reduce the risk of foodborne diseases (*17*). The system includes 7 principles that effectively minimize physical, chemical, and biological hazards rather than rely on finished product inspection to detect hazards after the fact. Thus, often a step lethal to the pathogen is included in the process (*17*). This lethality treatment is meant to eliminate pathogens and prevent subsequent cross contamination (*17*).

Additionally, there are several strategies to prevent microbial contamination before harvesting, during harvesting and after harvesting. A partial list of these interventions follows.

7.1. During Pre-harvest

The use of vaccines is not a new process. Vaccination involves exposing animals to an attenuated pathogen or antigen of a virulent microorganism (*17*). Unfortunately not all pathogens can be controlled using vaccines. For example, the vaccination against *E. coli* O157:H7 has not been successful. It was found that cattle exposed to *E. coli* O157:H7 were not protected from re-infection (*17*).

Recently, the use of lactic acid bacteria to control pathogens has been approved by the FDA. The patent US 7,291,326 B2 (2007), has been awarded for a method to reduce pathogens using a mix of lactic acid-producing bacteria (LAB mixture). The LAB mixture can be administrated to a live animal, a carcass, meat, and meat products. It is claimed that the oral microbial supplementation using different strains of *Lactobacillus* reduces the prevalence of *E. coli* O157:H7 in cattle and also increases the feed efficiency, live weight gain, and stimulates immunity against pathogenic bacteria (*18*).

The use of antibiotics in animal feeds is another intervention commonly used in US farming. According to Ritterman (*87*) more than 70% of the antibiotics used in the United States are for non-therapeutic purposes in animal feed (beef cattle, swine, and poultry). Antimicrobial agents have been used in agriculture, including livestock and poultry, since the early 1950s to treat infections and improve growth and feed efficiency

(*10*). A substantial amount of antimicrobial agents is given to food animals in subtherapeutic doses for promotion of growth in the absence of diseases (*10*). However, the practice of using antimicrobials in the animal feeds is coming under scrutiny, since antibiotic resistance in bacteria that cause disease in humans is an issue of major concern. According to Angulo et al. (*10*), the World Health Organization, following consultations in 1997 and 1999, has recommended discontinuing use of antimicrobial growth promoters that belong to an antimicrobial class used in humans, and in the United States, the Institute of Medicine (IOM) made this same recommendation in 2003.

7.2. During Harvesting

Several decontamination approaches have been studied to reduce microbial contamination and enhance food safety at the processing plants. There are several critical steps during the whole process, for example, operator skills are needed to avoid spilling fecal matter onto skinned carcasses (*64*). Therefore, several treatments are commonly applied to carcasses to eliminate bacterial growth. These treatments include: application of organic acids, hot water, steam pasteurization, and steam carcass vacuuming (*64*).

Often decontamination is achieved by soaking or spraying a meat surface with organic acids (lactic and acetic acid). Lactic acid is commonly used as it is a neutral meat compound produced during the postmortem glycolysis. According to Pipek et al. (*84*), the lactate anion retards the growth of surviving microorganisms during storage. Thus, lactic acid solutions (1 to 2%) are effective reducing the coliform counts, *Salmonella tiphymurium*, *Campylobacter jejuni*, and *E. coli* O157:H7 without increasing any health hazard (*84*). High pH values (pH 10-11) can also increase the thermal destruction of *E.*

coli serotype O157:H7 (*17*). During processing, carcass washes with organic acids such as acetic acid, citric acid, lactic acid do not reduce *E. coli* O157:H7 populations at low concentrations (up to 1.5%, *80*). But the number of *E. coli* serotype O157:H7 is effectively reduced about 2-log CFU/cm^2 at concentrations of 5% (*80*). Disinfectants appear to be more effective than organic acids; Chlorhexidine is effective in decontaminating fat, while hydrogen peroxide is more effective for connective tissue (*80*).

The use of heat, in the form of hot water and steam, for decontamination of meat is a common practice in United States, Canada, and Australia (*65*). Often after slaughter and chilling, a steam pasteurization process is useful for reducing pathogen counts as steam may be effective in sanitizing meat contaminated with pathogens (*65*). Vacuum-hot water cleaning (either water or steam at temperature greater than 82ºC), pasteurizing treatments (105ºC for 6.5 s) and subsequently spray-cooling can reduce log mean numbers of coliforms and *E. coli* by > 2 and total numbers of total aerobic bacteria by > 1 (*84*). The use of hot water has been demonstrated to reduce bacterial counts by 2 logs (applied at 80°C for 2 min). However, hot water washes have not been adopted by the industry since large volumes of water would be needed to uniformly wash carcasses. Thus, economical treatments will require the use of re-circulated water, which could affect the sanitary aspects (*64*). Thus, a practical alternative is the utilization of steam pasteurization; the advantage of using steam over the use of liquids is that steam is able to enter into the small pores of the rough surface of the carcass, as surface tension prevents liquids from entering into these pores and killing bacteria (*84*). Steam pasteurization

facilitates the growth of gram positive bacteria and reduces thermally susceptible gram-negative bacteria (*64*).

7.3. During Post-harvest

Another alternative to reducing pathogens in foods is the utilization of gamma irradiation rays. The FDA doses approved for poultry products is 1.5 and 3.0 kGy (1 kGy = 100 kilorads) as minimum and maximum, respectively. In ground beef products the use of irradiation is also FDA approved at the same doses. However, this technique lacks acceptance by consumers (*80*).

The addition of preservatives is also a common practice to reduce bacterial populations in foods. For example monolaurin (an emulsifier, 100-250 ppm) interacts with eugenol (a natural spice extract, 500 – 1000 ppm) to inhibit spoilage bacteria and *E. coli* O157:H7 in meat containing products (*79*). The FDA has determined that LAB mixture is GRAS approved when used to control the growth of pathogenic bacteria in fresh, chopped/ground, whole muscle cuts, and carcasses of meat and poultry at levels used between 10^6 to 10^8 CFU of lactobacilli per gram of product (*19, 99*).

Additionally cooking meats thoroughly and following cooking instructions is an effective way to control food pathogens. For example, it was determined that an internal temperature of 68.3°C for 15 sec will kill *E. coli* O157:H7 in ground beef (*69*). Therefore the recommended internal temperature to avoid bacterial outbreaks is 160°F or 71.2°C (*69*).

8. Microbial Analyses as Indicators of Sanitary Conditions in Fresh Beef Products

8.1. Aerobic Plate Counts (APC)

The aerobic plate count, also called aerobic colony count, or total plate count is used as indicator of bacterial populations on the meat sample (*72*). This test is based on the assumption that each cell will form a visible colony when is poured into a media containing the appropriate nutrients (*72*). Although APC does not differentiate types of bacteria present on the product, it is a good indicator of sanitary conditions as well as spoilage detection (*53, 72*). The recommended incubation temperature and time for fresh meat products is 29ºC to 31ºC for 48 h in PCA media (*53*). Aerobic bacterial counts on surfaces of 3.0 $\log_{10}/cm^{-2}$ are indicative of good hygiene and efficient commercial operations (*77*). In terms of spoilage, a limit of 10^6/g for raw food meats was established (*54*). It is suggested that raw meats with APCs of $< 10^4$ to 10^5 are free of spoilage (*54, 59*). When counts reach 10^7 to 10^8 CFU/g, it is an indication that spoilage has taken place; at a level of $\geq 10^9$, changes in texture and odor became evident (*54*, *59*). Mesophiles are those bacteria that grow well between 20ºC and 45ºC, and have an optimal growth temperature between 30ºC and 40ºC (*54*). The term psychrotrophic is used for bacteria that are able to grow well at or below 5ºC although their optimal growth temperature is 25ºC to 30ºC (*59*). Since meat is commonly maintained at refrigeration temperatures, psychrotrophic bacteria are the principal cause of spoilage in meat products and reduce shelf life (*77*)**.**

8.2. Coliform Counts

Coliforms, by definition, are “aerobic and anaerobic, Gram-negative, non-spore-forming rods that ferment lactose, forming acid and gas within 48 hr at 35ºC” (*58*). Coliform tests are common to determine possible fecal contamination (Gonzales 2003). The presence of coliforms in foods is used as indicator to determine possible presence of pathogens and overall food quality (*58*). For this reason, testing for coliform is widely practiced by the food industry (*39, 58*). Frequently coliform levels are correlated with the presence of *E. coli*; however, other genera such as *Klebsiella*, *Enterobacter*, and *Citrobacter* species are also considered coliforms. Probably the most common media to determine coliforms is violet red bile agar (VRBA); which allows coliform detection and enumeration in foods in 24 h (*39*). The limit indicated by the Food Risk Evaluation Committee (*33*) is 1,000 or 10^3 CFU/g for coliforms in fresh ground meat and meat trimmings.

8.3. Gram-negative Bacteria

Because Gram-negative pathogens cause the most cases of food borne illness, their control in meat products is a primary challenge for the meat industry (*68*). Among Gram-negative meat-borne pathogens, *Escherichia coli* O157:H7 and *Salmonella enterica* serotype Typhimurium are the greatest concern due to an elevated number of outbreaks caused by their incidence on meat and meat products (*25, 36, 40*).

8.4. Lactic Acid Bacteria

The Lactic Acid Bacteria (LAB) are known to play an important role in refrigerated meats under anaerobic conditions (*27, 35, 40*). Studies report that LAB dominate MAP packages because of elevated CO_2 and low oxygen (anaerobic conditions), which inhibits the growth of Gram-negative spoilage bacteria (*27*). It is also known that among lactic acid bacteria, *Lactobacillus sakei* is the predominant species found in meats (*8, 9, 28*). In a study conducted by Ercolini et al. (*28*) beef samples were stored at 5ºC under different MAP atmospheres to monitor the microbial spoilage using molecular techniques. *Pseudomonas* spp. and *Lactobacillus sakei* were found to be dominant under higher oxygen and lower carbon dioxide atmospheres. Therefore, the authors concluded that among LAB (under MAP conditions), mainly *L. sakei* plays an important role in the development of microbial spoilage of refrigerated raw meat (*28*).

9. *Escherichia coli* Serotype O157:H7 as a Main Pathogen Related with Fresh Beef

Contamination of meat is unavoidable during slaughter and processing. This recognition is important to protect consumers from food poisoning (*65*). A study conducted by Mead et al. (*67*) concluded that each year in the US, food borne pathogens cause 2,718 deaths, of which 1,809 are attributable to foodborne transmission. Bacteria cause 72% of these deaths; *E. coli* O157:H7 accounts for 3% of the total bacteria-caused deaths (*67*). Therefore, the Food Safety and Inspection Service (FSIS) has established a zero tolerance policy for *E. coli* O157:H7 (*33*).

Over the history of public health, *E. coli* O157:H7 has evolved as a major problem for primary-care practitioners, pediatric nephrologists, infectious-disease physicians, public health authorities, child-care setting, and the food industry (*21*). The consumption of undercooked meat is thought to be the primary cause of infection with *E. coli* O157:H7. Also, the cross contamination that commonly occurs when *E. coli* O157:H7 in raw meat or its juices are spread to other food products or utensils also accounts for illness. The Food Safety and Inspection Service (FSIS) has classified *E. coli* O157:H7 as an adulterant in raw ground beef, hence banning the sale of any ground beef contaminated with the bacteria (*22*). In addition, the National Beef Cattleman Association (NCBA) affirms that the occurrence of *E. coli* O157:H7 within the food supply is a source of great concern for both public health and the beef industry (*75*). Although the primary vehicle for transmission of *E. coli* O157:H7 is ground beef, recently at least four outbreaks of *E. coli* O157:H7 associated with the consumption of nonintact, mechanically and/or chemically tenderized steaks (sometimes also referred to as injected-tenderized steaks) have occurred (*62*). Commonly, intact (whole) and comminuted meats are needle injected or mechanically tenderized using solid or hollow needle injectors or blades (*62*). Consequently, the normally sterile internal tissues may become contaminated with microbes from the nonsterile external surface of the meats, some of which may include foodborne pathogens such as *E. coli* O157:H7 (*62*).

Among foodborne pathogens, *E. coli* O157:H7 has been identified as a major risk for the population. A number of factors contribute to its severity. First, while *E. coli* O157:H7 can cause harmful consequences of infection that affect all age groups, children and elderly people appear to be at greatest risk to get infected and consequently suffer

serious complications (*73*). Second, the infective dose has been estimated to be as low as < 100 cells (*63*). Third, it was shown that *E. coli* O157:H7 posses an unusual tolerance to acids, which can increase its occurrence in acidic food products (*80*). Consequently, the organism may survive gastric acidity and cause infection. Finally, it has a special association with ruminants as well as fresh produce that are used in several types of foods (*17*). The virulence of *E. coli* O157:H7 is attributable mainly to the production of one or more Shiga toxins and cytotoxic enzymes (*80*).

9.1. History of *E. coli* serotype O157:H7as Human Pathogen

After two hemorrhagic colitis outbreaks occurred in 1982, *Escherichia coli* serotype O157:H7 was recognized as a human pathogen. Strains of *E. coli* were first isolated in 1885 from children's feces by the German bacteriologist Theodor Escherich (*26*). Until 1982, three major strains that cause enteritis were described: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enterotoxigenic *E. coli* (ETEC). However, in 1982 *E. coli* serotype O157:H7 was linked with two outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) which involved hamburgers from fast food chains in Oregon and Michigan. The new serotype of *E. coli* was classified as verocytotoxigenic *E. coli* (VTEC; *26*). The name verocytotoxigenic was adopted in 1977, when Konowalchuck found that certain strains of *E. coli* produce a cytotoxin that can kill Vero cells, hence the name verotoxin (*80*). However, it is common that authors use the term "enterohemorrhagic *E. coli*" (EHEC) to refer *E. coli* O157:H7 since in 1972 Keusch reported that Shiga toxins contribute to the bloody diarrhea (*80*).

9.2. Life Cycle and Geographic Distribution of *E. coli* O157:H7

Although *E. coli* O157:H7 has been found in birds, sheep, dogs, deer, and humans, cattle are recognized as the most important reservoir of enterohemorrhagic *E. coli* O157:H7 strains (*23*). The majority of foodborne outbreaks involving *E. coli* O157:H7 have been associated with beef. There are several studies that have documented the presence of *E. coli* O157 and non-O157 in feces of healthy cattle. The National Beef Cattleman Association (*75*) affirms that the organism is widely distributed among feedlot cattle and their environment, as *E. coli* is carried by cattle in their intestines into the feedlot. It also is found on hides within beef processing facilities. The initial infection with *E. coli* occurs early in life and does not induce protective immunity against later colonization (*61*). The prevalence among fecal samples collected in summer from feedlot cattle can be as high as 28 percent (*115*).

Escherichia coli O157:H7 has been shown to have long-term survival in both manure and water (*110, 111*). Thus, crops may become contaminated through the use of manure as a fertilizer (*56*), irrigation of the plants with untreated water or sewage (*14, 102*), surface water runoff from nearby cattle pastures (*3*), even though bacteria entering the plant via the root system (*56, 101, 112*).

9.3. Prevalence of *E. coli* O157:H7 in Beef Products

Several products have been associated with infections caused by *E. coli* O157:H7, however, raw or undercooked bovine meat is the most common vehicle for the infection. Some factors such as temperature, pH, amount of sodium chloride and dehydration, and

fate in water can determine the prevalence of *E. coli* O157:H7 in foods. Unlike most Gram-negative bacteria, *Escherichia coli* serotype O157:H7 appears to be heat sensitive. Previous studies have determined thermal D values of *E. coli* O157:H7 in 90% lean ground beef. The D values at 55°C, 57.5°C, 60°C, 62.5°C, and 65°C were 21.13 min, 4.95 min, 3.17 min, 0.93 min, and 0.39 min, respectively (*80*). In addition, it is also reported that increasing the fat content also increases prevalence of *E. coli* O157:H7. In contrast with its heat vulnerability, the serotype can stay alive in frozen ground beef at -80ºC and then stored at -20ºC up to 9 months with a very little change in bacterial numbers (*80*). Another interesting aspect of *E. coli* O157:H7 is its extraordinary acid tolerance. Low pH products associated with outbreaks were homemade jerky, apple cider, and yogurt, among others. It was established that *E. coli* O157:H7 can resist pH as low as 2.5. However, the acid resistance is dependent on growth phase. Thus, the maximal resistance is exhibited at stationary phase and not at log phase (*80*). In addition, the survival in acidic foods is extended greatly when stored at refrigeration temperatures. For example, the serotype survive only 2 to 3 days at 25ºC in apple cider, while at 8ºC it can survive up to 31 days (*17*). The resistance to acidic conditions is a main pathogenic characteristic of the serotype. The organism after being ingested can survive and pass through gastric conditions (pH 2.0) reaching the intestinal tract of humans, and consequently cause illness. A different characteristic of *E. coli* O157:H7 is the lack of salt tolerance. The serotype is unable to growth in trypticase soy broth (TSB) containing 6.5% of NaCl. However, *E. coli* O157:H7 has an unusual high tolerance to dryness. Thus, in 1994 dry-cured salami (pH 4.63 and water activity of 0.99) stored at 5ºC for 32 days was a vehicle for *E. coli* O157:H7 and caused a new outbreak (*80*). In recent years,

it was recognized that water can be a source of contamination of *E. coli* O157:H7. Drinking water and irrigation water can be contaminated with *E. coli* O157:H7 by cattle manure slurry. It was documented that the organism can survive at 5ºC for 63 to 70 days with a moisture content of 74% in feces (*80*).

10. Literature Review Conclusion

Although the use of injection brines by the meat industry is useful to increase tenderness and reduce variability in meat products, there are several concerns associated with this practice. One of the major concerns is the fact that contamination of meat surfaces might occur during slaughtering and processing. Hence, if pathogenic bacteria such as *E. coli* O157:H7 are present, they can be translocated into the deeper sterile tissues by needle injection, or by other invasive technologies. Inside the meat tissue, bacteria can grow and reach dangerous levels which can lead to an outbreak. Another concern with using brines is the fact that conventional brines used by the industry have in their formulation phosphate ingredients, which are mainly used in the sodium form. Consumers are demanding low-sodium and sodium-free, and low-phosphate and phosphate-free foods. An alternative for the meat industry could be AH. Ammonium hydroxide is an alkaline agent which can be used to replace conventional phosphate-based ingredients to reduce the amount of sodium in the brine formulation while maintaining quality parameters. Even though the replacement of phosphate-ingredients with AH looks promising, there are few published studies that have evaluated the injection of brine solutions containing AH in meats and its effect on the microbial flora. It has been hypothesized that since AH is an alkaline solution, it can cause disruption of

the cell membrane, which has a greater impact on gram-negative bacteria. However, there are other published studies that suggest AH lacks antibacterial effect. At this point, the mechanism of action of AH as an antimicrobial agent when used in brines remains unclear.

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CHAPTER III

ANTIMICROBIAL EFFECT OF AMMONIUM HYDROXIDE WHEN USED AS AN ALKALINE AGENT IN THE FORMULATION OF INJECTION BRINE SOLUTION

ABSTRACT

Paired USDA Select striploins were injected with either a conventional brine (4.5% of a potassium and sodium polyphosphates blend [Brifisol®750], 3.6% NaCl, 1% Herbalox seasoning HT-S, and 90.9% of ice water) or the ammonium hydroxide (AH) brine (1% AH, 3.6% NaCl, 1% Herbalox seasoning HT-S, and 94.4% ice water). Steaks were sliced, high-oxygen MAP-packaged, placed at 5ºC in dark storage for 5 days, and then transferred to a retail display at 5ºC for another 14 days. Steaks injected with AH brine appeared to have lower counts of psychrotrophic, mesophilic, and Gram-negative counts. Immediately after injection, there was ~ 1 $\log_{10}$ cfu/g difference between treatments in Gram-negative counts. No differences in coliforms and lactic acid bacteria counts were found.

INTRODUCTION

Ammonium hydroxide (AH) is considered a safe and suitable ingredient as a pH control agent in brines for meat products up to final brine pH of 11.6 (*16*). We previously studied the replacement of phosphates by AH in the formulation of meat brines. When 1% AH was used, in terms of quality parameters such as tenderness,

juiciness, color stability, and lipid oxidation, results were equivalent to those obtained from phosphate-injected steaks (*2, 11*). These results look promising for the industry since this would allow the meat industry to produce phosphate-free and lower sodium content meat products. However, the impact on the microbial characteristics of products injected with a brine containing ammonium hydroxide instead of phosphate was not fully addressed by the previous studies. Previous researches suggest that AH impacts bacterial growth in meat systems. For example, Gupta et al. (*4*) evaluated the effect of AH on goat ground-meat. He was concluded that AH retards meat spoilage, improves shelf life and has no-negative effects on meat color (*4*). Hand et al. (*6*) reported that injected steaks with a brine solution containing AH had lower counts of *Escherichia coli* O157 than non-injected steaks (*6*). Hamling et al. (*5*) indicated that injected steaks with AH-containing brine had numerically higher Aerobic Plate Counts (APC) and Anaerobic Plate Counts (AnP) than non-injected steaks (*5*). Additionally, the U.S. patent Nº 7,022,361 describes a method to inject gas or aqueous solution containing ammonia-based-compounds into the interior of a meat product to raise the pH sufficiently to inactivate meat pathogens (*13*). However, there are also studies that imply that AH lacks these antibacterial effects. For instance, Stopforth et al. (*15*) inoculated boneless beef samples with *E. coli* O157:H7 and *Salmonella* Typhimurium and then treated 0.1% AH solution (pH 10.89). In this case, 0.1% AH did not reduce the pathogen population. Later, textured meat was exposed to 0.025%, 0.05%, and 0.1% AH for 15 minutes. An increased meat pH (from 6.48 to 9.41) was observed, but, no significant reductions on APC counts were obtained (*7*). To this point, the research on the antimicrobial action of AH is limited, incomplete, at times contradictory, and lacks of practical and commercial applicability. Hence, this

study was conducted with the aim to determine if a brine containing AH impacts meat microbial flora differently than a conventional brine containing sodium phosphates. Thus, in order to achieve the objectives of this study, the following microbial tests were selected: APC (aerobic psychrotrophic and aerobic mesophilic counts) as indicators of sanitary conditions as well as spoilage detection. Coliform counts to determine possible fecal contamination; and also as indicator to determine possible presence of pathogens. Gram-negative bacterial counts, since this group includes pathogens that cause the most cases of food borne illness. The LAB bacteria are known to play an important role in spoilage of refrigerated raw meats. Thus, LAB bacteria were also included in this study.

MATERIALS AND METHODS

Injection, packaging and storage of samples. Ten paired USDA Select striploins were collected and labeled at a beef fabrication facility. Striploins were vacuum-packaged, placed into coolers containing ice and transported to the Robert M. Kerr Food and Agricultural Product Center (FAPC) at Oklahoma State University where they were stored overnight at 4ºC. The next morning, striploins were trimmed and the initial weight of each striploin was recorded. Each pair of striploins (left and right side) were separated and assigned to either conventional (CON) or AH group following a complete randomization. Then, using a stitch pump enhancer (Fomaco Reiser, FoodMachine Co., MA) calibrated to inject at 110% of the recorded initial weight, striploins were injected with either the AH- or the CON-brine. The AH brine consisted of 1% w/w food grade AH [≥ 25% as ammonia (NH_3), 35.04 M, Fisher Scientific, Fair Lawn, NJ], 3.6% w/w sodium chloride, 1% w/w Herbalox seasoning type HT-S (Kalsec,

Kalamazoo, MI), and 94.4% w/w ice water. The CON-brine was prepared using 4.5% w/w agglomerated blend of potassium and sodium polyphosphates (Brifisol®750; BK Giulini Corporation, Simi Valley, CA), 3.6% w/w sodium chloride, 1% w/w Herbalox seasoning type HT-S, and 90.9% w/w ice water. Five stiploins from the left side and 5 striploins from the right side were injected with the CON brine and the remaining loins (opposite sides from same animals, the other 5 right and 5 left) were injected with the AH-brine. The weight of the striploins after injection was also recorded. After 30 min of injection; striploins were cut into 2.54 cm steaks using a standard 13 inch manual slicer (Model 3600P, Globe Food Equipment Co., OH). From each striploin, 10 steaks were sliced and numbered as they were cut from 1 to 10. Steaks were placed into 5.08 cm deep pre-padded (absorbent pads: Dri-Loc® Ac-50, Duncan, SC) trays (Cryovac 17:CS977 Duncan, SC) and packaged under a high-oxygen (79.2% O_2/15.8% CO_2 /6% N) modified atmosphere packing (MAP) using a MAP machine (G. Mondini CV/VG-S Brescia, Italy). Trays were sealed with Cryovac LID 1050 film (Duncan, SC) with an oxygen transmission rate of less than 20 cc (24 h, m^2 at 4.44ºC and 100% Relative Humidity). Packaged steaks were labeled, placed into boxes, and then moved to a dark storage room at 4.45ºC (40ºF) where they were held for the next 4 days. The 4 days in dark storage are meant to simulate maximum time for transportation. Thus, day 5 will be equivalent to day 0 at the retail market. On day 5, packaged steaks were transferred to a retail display at 5ºC (41ºF), under continuous lightening (40 watt Rapid Start T12 Fluorescent Platinum lights; Promolux, B.C., Canada). Steaks were stored under retail display for 2 weeks or until they were analyzed.

Sampling Procedure. Steaks were collected for microbial evaluation on the day they were cut (day 0) and again the day they were placed under retail lights (day 5). Steaks were also analyzed after they had been under retail light one week (day 12) and two weeks (day 19). On each day of microbial evaluation one steak from each loin (n = 20) was randomly selected and transported to the laboratory on ice. At the laboratory, packages were opened aseptically and steaks were transferred from the original package onto sterile cutting boards. Each steak was cut into two pieces with the aid of a sterile knife and a fork, one piece was placed into a pre-labeled Whirl-Pak® bag (Nasco Whirl-Pak® bag, Fort Atkinson, WI) for further pH analysis. The remaining half of the steak was cut into small pieces (smaller than 0.5 cm) and transferred aseptically to a pre-labeled sterile Whirl-Pak® bag. Sample was mixed thoroughly by massaging the bag. A 50 g sample was weighed into a sterile stomacher bag (Nasco Whirl-Pak® filter bag, model B01318, Fort Atkinson, WI) and 450 ml of 0.1% peptone water solution was added. Sample was homogenized for 2 min at high speed using a stomacher (Laboratory Blender Stomacher 400; Tekmar Company, Cincinnati, OH).

Microbial Analysis. From the stomacher slurry, appropriate additional serial dilutions were prepared using a buffered peptone solution (0.1%). Duplicate samples were pour-plated on the appropriate media to enumerate bacteria present. Plate count agar (PCA; BD Difco^{TM}, Sparks, MD) was used to enumerate total aerobic plate count (APC). Prior to pour-plating PCA plates, 1 ml of 0.5% aqueous solution of 2,3,5-Triphenyl tetrazolium chloride (TTC; BioChemika, Sigma-Aldrich, St. Louis, MO) was added to each 100 ml of molten media. Two sets of plates were poured for APC, one set of plates was incubated at 25ºC and the other set at 32ºC, for psychrotrophic and

mesophilic bacteria, respectively. Violet red bile agar (VRBA; Difco™, Sparks, MD) was used to count coliforms. Plates were overlaid and incubated at 37ºC for 24 h. Crystal violet tetrazolium (CVT) was used to determine the numbers of Gram-negative bacteria. Plates were incubated aerobically at 25ºC for 48 h. Lactobacillus selection (LBS) agar was used to enumerate lactobacillus bacteria. The LBS was prepared from individual ingredients according to the manufacturer's formulation (Baltimore Biological Laboratories, Cockeysville, MD). Plates were also prepared with the pour-plate method with an overlay, then incubated anaerobically by placing them in an anaerobic chamber containing GasPak Plus with Palladium Catalyst (BD BBL™, Sparks, MD) and incubated for 48 h at 35ºC. To verify anaerobic conditions, Dry Anaerobic Indicator Strips (BD BBL™, Sparks, MD) were placed inside anaerobic chambers. In all cases, typical colonies were enumerated using an electronic colony counter (eCount™ Colony Counter; Heathrow Scientific, IL). Means were calculated and bacterial numbers were expressed as $\log_{10}$ colony forming units per gram (CFU/g) of meat sample.

Analysis of pH. Direct pH measurements of steak halves were recorded using a Model IQ150 pH meter (Scientific Instruments Inc., Carlsbad, CA). Three readings were taken from each beefsteak and then averaged for further statistical analysis. Readings of pH were obtained prior meat injection and then every day of microbial analysis, days 0, 5, 12, and 19.

Statistical Analysis. Means and standard deviation were calculated on the assumption of a normal distribution. The experiment was arranged as a 2 x 4 factorial and set out in a randomized complete block design (RCBD) with repeated measures and 10 reps or blocks per treatment. Data were analyzed using SAS, version 9.2 (SAS

Institute, Cary, NC). Fixed variables were day (n=4) and treatment (n=2). Random block variable was the animal ID. In addition, correlation between pH and microbial growth was analyzed. The least significance difference (LSD) type approach was used to determine significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

As intended, the percentage of brine solution injected into subprimals was not different ($P = 0.92$) across brines. The initial weight was increased by 10.55 ± 1.1% and 10.58 ± 1.25% for the subprimals injected with the conventional brine and with the AH brine, respectively. No significantly differences were found in meat pH before injection ($P = 0.58$); 5.64 ± 0.06 vs. 5.66 ± 0.05) for AH and the CON-brine, respectively. However, after injection (day 0) and also for days 5, 12, and 19, meat pH of AH-injected steaks were statistically higher than the CON-injected steaks ($P = 0.003$ and $P < 0.05$, respectively). In a practical scenario, however, those pH values would be considered equivalent since the highest pH difference among treatments was a numerical value of 0.07 (on both, day 12 and day 19) and the lowest difference was 0.03. The pH was significantly different across days ($P < 0.05$, Fig. 3.1); the highest pH was observed after 30 min injection, the mean values were 5.82 ± 0.07 and 5.77 ± 0.04 for AH and for CON-brine injected steaks, respectively. By day 5, those values were slightly reduced to 5.74 ± 0.04 for AH- injected steaks and 5.71 ± 0.08 for CON-brine injected steaks. Changes in pH over time were likely due to absorbance of CO_2 since steaks were packaged with MAP. Lower bacterial counts were observed on steaks injected with a brine containing AH when compared with a conventional phosphate-based brine (*2*). However, it was not

elucidated whether those reduced counts were caused by a direct effect of AH (toxic effect) or if it was caused by the pH being lower than the conventional brine injected steaks. Subsequent research using higher levels of AH in the brine (*11*), which resulted in nearly equivalent meat pH between the phosphate and AH treated meat, indicated that differences in microbial counts did not occur until towards the end of meat shelf-life. In this case, AH treated meats were higher in APC and AnPC than phosphate treated meats. Because a difference was observed, it suggested that perhaps specific types of microbial flora were being impacted by the treatments. Hence, one of the challenges of this study was to determine what kinds of microbial flora were being impacted by the use of AH as compared to phosphates.

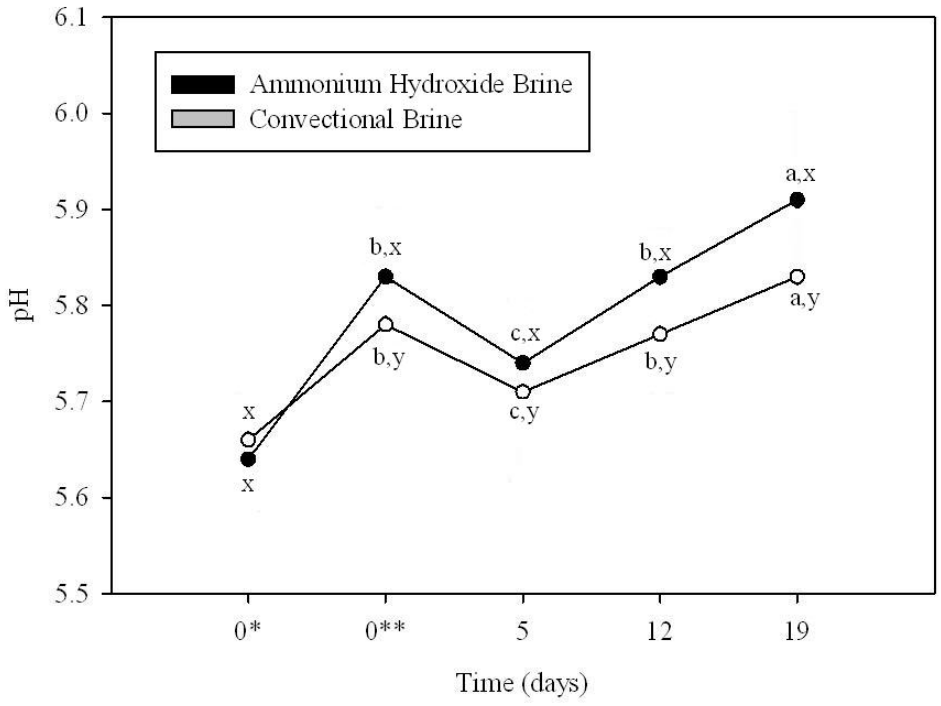

FIGURE 3.1. The pH values of striploin steaks after injection to 110% initial weight with an alkaline brine solution containing ammonium hydroxide or a conventional sodium

phosphate-based brine. Data points represent the means from ten repetitions. 0* represents meat pH prior to injection, these data were not compared with after injection data. 0** represents meat pH after 30 min injection. [a-d] Means with differing letters within brine (ammonium hydroxide or conventional brine) are significantly different ($P < 0.05$). [x-y] Means with differing letters within day (0**, 5, 12, or 19) are significantly different ($P < 0.05$).

Additionally, in order to determine if initial pH (pH before injection) had any further effect on final meat pH, analysis of covariance (ANCOVA) was performed. Results of ANCOVA were not significant, therefore excluded from the statistical model.

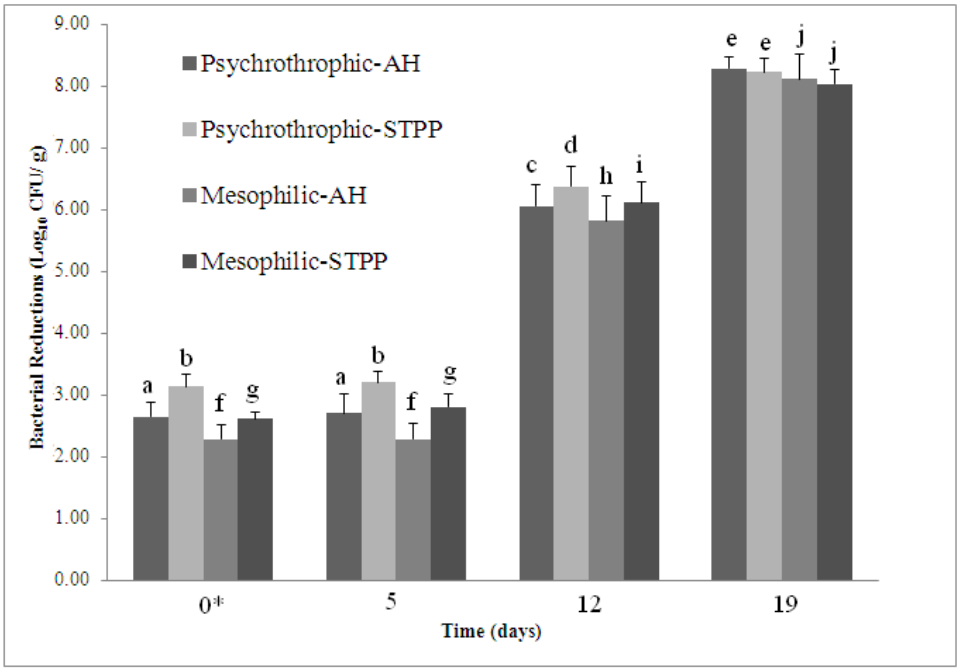

FIGURE 3.2. Psychrotrophic and mesophilic counts (log_{10} cfu /g) of striploin steaks after injection to 110% initial weight with an alkaline brine solution containing ammonium hydroxide or a conventional sodium phosphate-based brine. Bars represent

the standard deviation of the mean. [a-e] Means with differing letters within psychrotrophic analysis indicate significant differences ($P < 0.05$). [f-j] Means with differing letters within mesophilic analysis indicate significant differences ($P < 0.05$). 0* represents analysis after injection.

Regardless of treatment, there were more colonies enumerated under psychrotrophic conditions than mesophilic (Fig. 3.2). From day 0 to day 5 there was no significant change in microbial populations for either treatment. However, by day 12 there was a > 2 $\log_{10}$ CFU/g increase in psychrotrophic counts for both treatments (brines) with populations increasing to > 8 $\log_{10}$ CFU/g by day 19. Mesophilic growth patterns were almost identical to psychrotrophic (Fig. 3.2). There were no significant differences on day 19 ($P < 0.05$) for both, psychrotrophic and mesophilic counts. Our observations are in agreement with Steinbruegge and Maxy (*14*), who evaluated the growth of bacteria from ground beef at 25°Cand 32°C. They have concluded that one third of the bacteria that grow at 25°C are unable to grow at 32ºC after 48 h incubation period (*14*). The pattern of bacterial growth we report in this study was also observed by Hamling et al. (*5*). Their initial (2.58 vs. 2.30 $\log_{10}$ cfu/g, respectively) and final plate counts (7.36 vs. 8.13 $\log_{10}$ cfu/g, respectively) were similar to this study. They reported that the spoilage state (7.22 $\log_{10}$ cfu/cm^2) in product (high oxygen MAP) was reached in AH-injected steaks after 3 weeks under dark storage and 4 days in retail display. Authors concluded that in higher meat pH samples, as the storage time increased, bacterial growth appeared to be faster. In a study conducted by Gupta et al. (*4*) the preservative effect of different concentrations (from 0.5% to 2.6% w/w or from 0.134 to 0.67 M) of AH and ground goat solutions was studied. Meat homogenates were prepared by adding from 1

to 5 ml of AH to 11 g of ground meat sample, and incubated at 37ºC, 4ºC, and -20ºC. At 37ºC, spoilage was evident after 2 days (*4*). When concentrations of AH were ≥ 1.6% and meat was pH 9.5 no-increase in aerobic bacteria was observed in samples maintained at 4ºC for up to 11 days; while in samples stored at -20ºC a reduction of > 1 $\log_{10}$ was described (*4*). The antibacterial effect observed was attributed to the toxicity of AH rather than a change in pH (*4*). Hence, results from Hamling et al. (*5*) and Gupta et al. (*4*) along with our results, suggest that AH slightly reduces psychrotrophic and mesophilic bacterial growth when used in the formulation of injection brine solutions.

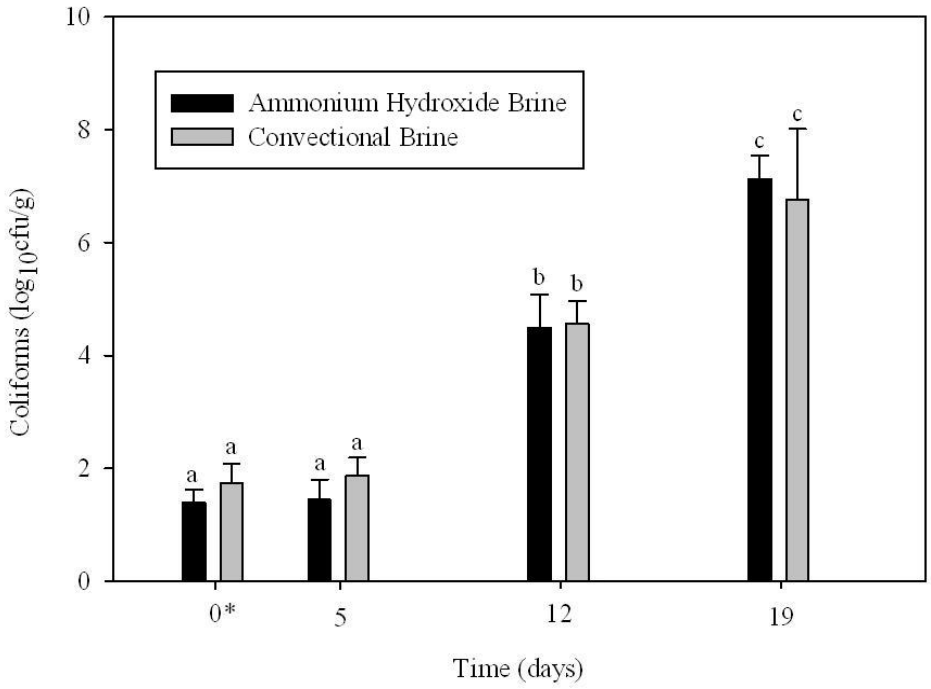

FIGURE 3.3. Coliforms counts ($\log_{10}$ cfu/g) of striploin steaks after injection to 110% initial weight with an alkaline brine solution containing ammonium hydroxide or a conventional sodium phosphate-based brine. Bars represent the standard deviation of the mean. Letters above bars indicate significant differences ($P < 0.05$). Data points represent the means from ten repetitions. 0* represents analysis after injection.

Coliform counts did not differ between brines ($P = 0.37$); however, a significant day effect was observed ($P < 0.001$). The interaction between brines and day was not significant ($P = 0.2602$; Fig. 3.3). For both brines, coliform counts started at around 1.5 $\log_{10}$ CFU/g and a change was not noted until day 19. The mean coliform count was ~4.5 $\log_{10}$ CFU/g and increased an additional 2 $\log_{10}$ CFU/g by day 19. The pattern of microbial growth observed for coliforms was very similar to that seen for psychrotrophic and mesophilic microorganisms.

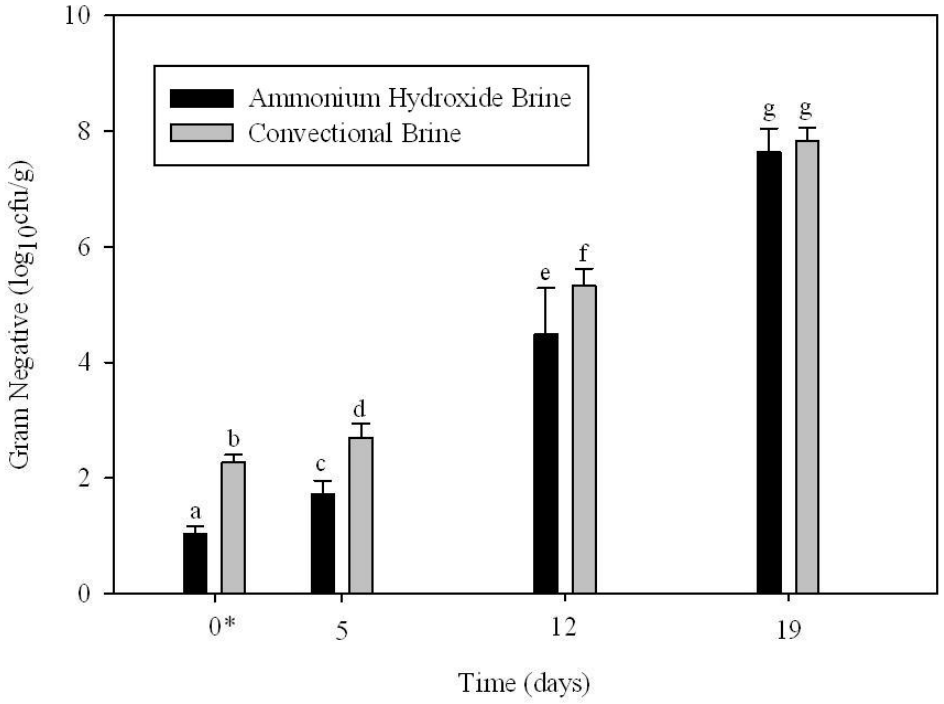

FIGURE 3.4. Gram negative counts ($\log_{10}$ cfu /g) of striploin steaks after injection to 110% initial weight with an alkaline brine solution containing ammonium hydroxide or a conventional sodium phosphate-based brine. Bars represent the standard deviation of the mean. Letters above bars indicate significant differences ($P < 0.05$). Data points represent the means from ten repetitions. 0* represents analysis after injection.

The analysis of Gram-negative bacteria indicated that bacterial counts were significantly affected by treatment, day, and their interaction ($P < 0.001$; Fig. 3.4). The AH treated steaks had lower counts than the CON-brine steaks until day 19. There does appear to be an immediate impact on Gram-negative counts 30 min after beef steaks were injected with AH. The difference between brines was over one full $\log_{10}$ CFU/g. The one log difference between brines continued on day 5 and 12 (Fig. 3.4). However, by day 19 differences between brines were no longer discernable ($P = 0.21$). The reduction observed in Gram-negatives is likely explained by the reported susceptibility of Gram-negative bacteria to high-pH solutions, which causes cell wall disruption (*8*). The proposed mechanisms of action by which alkaline solutions harm Gram-negative bacteria, are: disruption of the cytoplasmic membrane and leakage of the internal contents (solubilization of membrane proteins and/or saponification of membrane lipids), and separation of cytoplasmic contents (precipitation of proteins, causing separation from DNA, *8*). This suggests that perhaps more than just alkalinity is a factor in bacterial reduction when AH is used. Aside from the reduction of Gram-negative bacteria by alkaline pH, ammonia itself is well known for its cytotoxic effects (*9*)**.** In moderate concentrations of ammonium in the media, the diffusion of NH_3 across the cytoplasm is sufficient to actually promote growth. However, at high concentrations (> 750 mM), it causes osmotic and ionic stress to the cell membrane (*9*). The Initial level of NH_3 in the brine used in the current study was ~192 mM.

Lastly, in this study, after 19 days of storage, LAB counts were $< 10^1$ CFU/g. Lactic acid bacteria are known to play an important spoilage role in refrigerated meats (*3*). In a study conducted by Ercolini et al. (*3*) beef samples were stored at 5ºC under

different MAP atmospheres (0% and 60% O_2) to monitor the microbial spoilage using molecular techniques. *Pseudomonas* spp. and *Lactobacillus sakei* were found to be dominant under higher oxygen and lower carbon dioxide atmospheres (*3*). This suggests that lactic acid bacteria should have been detected in this study. The absence of LAB growth, however, might be explained by the use of LBS media, which is more acidic (pH 5.5 vs. 6.2 to 6.5) and has higher salt content than de Man-Rogosa-Sharpe (MRS) media. However, it seems that some strains of *L. sakei* are inhibited by high concentrations of salt (*1*) and acidic conditions (*12*).

In conclusion, when AH was used as an alkaline agent in the formulation brines intended for meat injection (1%, pH 10.66), it effectively reduced select microbial populations when compared with a conventional phosphate-based brine solution. The highest inhibition occurred with Gram-negative bacteria, followed by APCs. However, further research is needed to identify more precisely the bacterial taxa that are being inhibited by AH and also the specific levels at which bacteria are harmed.

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CHAPTER IV

COMPARISON OF 1%, 2% AND 3% AMMONIUM HYDROXIDE SOLUTIONS TO CONTROL *ESCHERICHIA COLI* O157:H7 ON BEEF LEAN SURFACES

ABSTRACT

Ammonium hydroxide (AH) is a processing aid which can be used in brines injected into meats. This study was conducted to determine whether 1%, 2% and 3% AH solutions could reduce *Escherichia coli* O157:H7. These levels were selected because they were found to be the most effective in improving meat functionality. Meat beef disks (20.5 cm^2) were fabricated from beef top butts and inoculated at ~10^6 log cfu/cm^2 of an antibiotic-resistant 4-strain cocktail of *E. coli* O157:H7. A multi-nozzle spray system developed from Ross Industries Inc. (Model TC700M, Midland, VA) was utilized to spray 0%, 1%, 2%, and 3% AH onto inoculated meat disk samples that were conveyed through the system at 5.5 ft/min with a spray dwell time of 18 s and an application spray rate of 1.5 gpm. Meat samples were analyzed on days 0, 1, 7, and 14 (those not tested immediately were vacuum packaged and kept at 4ºC until analysis). The results showed that 1%, 2%, and 3% AH solutions were effective in controlling *E. coli* O157:H7 compared to the control ($P < 0.05$) for each day of analysis. However, no significant differences were observed between the 1%, 2% and 3% AH treatments against *E. coli* O157:H7. The reduction of *E. coli* O157:H7 by AH increased from 0.23 – 0.25 $\log_{10}$

CFU/cm^2 on day 0 to 1.34 – 1.69 $\log_{10}$ CFU/cm^2 on day 14. The immediate magnitude of the inhibitory effect on *E. coli* O157:H7 was not sufficient to be used as an intervention step during post-slaughter meat processing, however, the use of AH (1%) may significantly reduce levels of *E. coli* O157:H7 associated with raw beef during longer term storage.

INTRODUCTION

Typically, beef is injected with a water solution (brine) containing salt, phosphate, sodium lactate, seasonings, and flavorings. Salt and phosphates act synergistically to alter protein solubility and increase meat pH. Raising the final pH improves water holding capacity (WHC), tenderness, juiciness, and favor (*17*). The substitution of sodium phosphates by ammonium hydroxide in the formulation of brines may have several advantages for the beef industry as well as for consumers. Some of these advantages could be the exclusion of "hidden" sources of sodium (up to 50% of sodium in the brine), the elimination of phosphates in the diet, the production of a "cleaner label", and the reduction of pathogens or organisms that cause spoilage. Our studies have focused on the use of ammonium hydroxide (AH) as an alternative to replace phosphate-based ingredients in the formulation of injection brines. Initial research efforts demonstrated that although a 0.1% AH brine solution had lower aerobic and anaerobic plate counts (APC and AnPC); in terms of quality and aiding fluid retention, it was not sufficient for the replacement of 4.5% phosphates (*4*). These results raised a question about the appropriate level of AH needed to effectively replace phosphates in the brine. Thus, a subsequent study was conducted. Striploins were injected with brines containing

different AH concentrations (0.1%, 1%, 2%, 3%, 4%, 5%, and 10%). After brine injection, steaks were sliced, packaged in high-oxygen MAP (80% O_2/20 CO_2), placed at 5ºC in dark storage for 5 days (to simulate transportation), and then placed under retail lights at 5ºC. Steaks were evaluated for % purge, pH, and subjective color score (which included: muscle color, % discoloration, and overall acceptability). Additionally, sensory taste panel attributes such as initial and sustained juiciness and tenderness, connective tissue, and overall acceptability were evaluated. Results demonstrated that by increasing the concentration of AH, the % of purge was reduced. However, it was also observed that when AH concentrations were between 1% and 3% there was not a significant improvement in % of purge. Moreover, when AH levels were higher than 3% in the brine, most panelists were able to perceive an ammonia odor. Consequently, lower amounts of AH were used (i.e. 1%) in subsequent studies focusing on injecting AH containing brine into beef striploins. In one study, quality parameters as well as general microbial analysis were evaluated (*17*). No differences were found between pH, shear force, lipid oxidation, and sensory parameters of beef injected with 1% AH versus a phosphate-based control brine. However, aerobic and anaerobic plate counts (APCs and AnPCs) were lower in steaks injected with phosphates than with AH brine (*17*). A second study has focused on the microbial aspects of striploins injected with either a 1% AH brine or 4.5% phosphate brine. It was demonstrated that 1% AH brine had an antimicrobial effect against psychrotrophic, mesophilic, and Gram-negative bacteria when compared to the 4.5% phosphates brine (*5*). These results were promising and raised new questions as to whether AH brines would be effective in controlling primary beef-related pathogens such as *E. coli* O157:H7. Therefore, the objective of this research

was to examine whether a dilute solution of AH in and of itself could reduce *E. coli* O157:H7 populations on beef surfaces.

MATERIALS AND METHODS

Meat Disk Fabrication. Following the methodology of Morgan et al. (*11*), 48-hours aged beef top butts, weighing about 10-15 lbs were acquired directly from a beef fabrication facility. Beef pieces were placed into boxes in a cooler at 4.4°C and then transported to the Robert M. Kerr Food and Agricultural Product Center (FAPC) at Oklahoma State University. With the aid of a coring device of 2 inches diameter (5.08 cm) connected to a drill (Model FSX-treme™, Fire Storm Black & Decker, Towson, MD) meat core samples were obtained. Meat cores were then placed at -20°C to allow them to freeze for one hour. Using a meat slicer (Bizerba GmbH & Co. KG, Balingen, Germany) meat cores were sliced into 0.6 cm thick disks, resulting in 20.5 cm^2 by 0.6 cm meat disks. Meat disks were placed into vacuum bags (n= 25; Cryovac® Duncan, SC), vacuum packaged (Multivac Inc., Kansas City, MO), and maintained at -20°C until needed. A day before treatment, vacuum packaged meat disks were transferred to 4°C to allow them to thaw.

Bacterial Cultures. The bacterial culture used in this study was used previously by Morgan et al. (*11*). These included the following *Escherichia coli* O157:H7 strains: ATCC 43890, from a California outbreak isolated from human feces; ATCC 43894, from a Michigan outbreak isolated from human feces; ATCC 43895, from hamburger implicated in a human outbreak; and ATCC 35150, from a sporadic case of hemorrhagic colitis. All four strains were gentamycin and rifamycin resistant after passage on agar

media containing 20 µg/ml gentamycin and then 10 µg/ml rifamycin. At the BSL2-food microbiology laboratory, a bacterial cocktail was prepared by transferring 100 µl of each culture stored at -80°C into 10 ml Tryptic soy broth (TSB, TSA; Becton, Dickson and Company, Sparks, MD) and incubating overnight at 30°C. A 100 µl of the overnight cultures were transferred again into 10 ml of sterile TSB and incubated overnight at 30°C. The cells were harvested by centrifugation at 3,000 rpm for 10 min at room temperature, and the pellet was re-suspended into 9 ml of 1x phosphate buffer saline (PBS, MP Biomedicals, LLC, Solon, OH). All four strains were combined into a sterile 50-ml tube, vortexed to have a homogenous bacterial suspension of approximately 10^8 CFU/ml, and were maintained in ice until used (*11*).

Sample Inoculation. Using sterile forceps, meat disks were placed (n=8 per tray) onto deep sterile stainless steel trays (Vollrath Company, LLC, Sheboygan, WI) which were placed on ice. A 100 µl volume of the bacterial cocktail was pipetted onto each meat disk. The bacterial suspension was spread by a gently spreading the inoculum using a "gloved-finger" (*14*). Trays were covered with a piece of clear food-wrap film, and then placed at 4°C for 30 min to promote attachment. Six meat disks were selected to serve as controls (inoculated, non-sprayed). Two control disks were placed into each 6 x 9" pre-labeled sterile stomacher bag (VWR International, Sherbrooke, Quebec, Canada) and maintained at 4ºC until further microbial analysis (*11*).

Spray Solutions. Three AH solutions were prepared (1%, 2% and 3%) by weighing 100g, 200g, and 300g of AH [food grade AH, ≥ 25% as ammonia (NH_3), 35.04 M, Fisher Scientific, Fair Lawn, NJ] and then dissolved into enough Millipore water to

make a final weight of 10,000 g or 10 Kg. Additionally water containing no-AH was also used as a control spray solution (0% AH or water).

Spray Treatment Application. Inoculated samples selected to be sprayed were transported to the processing room in closed-lid coolers containing ice. The clear food-wrap film was removed, and then using flame sterilized forceps, meat disks were placed at the beginning of the conveyor of a multi-nozzle spray system machine (Model TC700M; Ross Industries Inc., Midland, VA) with the inoculated surface facing upward (*11*). Meat disks passed throughout the conveyor at a velocity of 5.5 ft/min and were sprayed with either 0%, 1%, 2% or 3% AH. The application spray rate was 1.5 gpm. Meat disks were collected individually at the end of the conveyor belt using sterile forceps. Meat disks were placed into various receiving trays containing a layer of adsorbent pad (*11*).

Sampling Procedure. Two meat disks were randomly selected from different trays and aseptically placed into each 6 x 9" pre-labeled sterile filter stomacher bag (*11*). Meat disks were placed side by side with the inoculated surface area facing the filter layer, sealed, placed on a tray containing ice, and transported to the food microbiology laboratory. A total of 24 meat disks were sprayed for each treatment (n = 12 sampling bags). The 12 sampling bags from each treatment were divided as follows: three bags were randomly selected to be analyzed on day 0, three on day 1, three on day 7, and the last three on day 14. A volume of 40.5 ml of Dey-Engley neutralizing broth (D/E broth; Neogen® Corporation, Lansing, MI) was added to the sampling bags selected to be analyzed on day 0, while the remaining bags were vacuum sealed using a vacuum

packaging machine (Hobart Corporation, Troy, OH, *11*). Sealed bags were placed at 4°C until day of analysis. On each day of analysis with the aid of a sterile scalpel a ~2 inch cut was done under the sealed line using a sterile scalpel and D/E broth was added, followed by stomaching and plating (*11*).

***E. coli* O157:H7 Analysis.** Sampling bags containing meat disks and D/E broth were pummeled using a stomacher (Laboratory Blender Stomacher 400; Tekman Company, Cincinnati, OH) at normal speed for 30 sec each side (total 1 min per sample). From the stomacher bag suspension, appropriate additional serial dilutions were prepared by using a buffered peptone solution (0.1%) as described by Morton (*12*). Duplicate samples were spread-plated onto tryptic soy agar (TSA; Becton, Dickson and Company, Sparks, MD) containing 20 µg/ml gentamycin (Sigma-Aldrich, Saint Louis, MO) and 10 µg/ml rifamycin (Sigma-Aldrich, Saint Louis, MO) to enumerate *E. coli* O157:H7. Plates were then incubated at 30°C for 48 hours (*9*). Representative colonies were counted (averages were calculated from duplicate plates) and final counts were reported as colony forming units per cm^2 (CFU/cm^2) and converted to logarithmic values.

Statistical Analysis. The log reduction value for each treatment was calculated by subtracting the final $\log_{10}$ value of treated samples from the average $\log_{10}$ value of the untreated control. Least squares means (LSM) were analyzed by the general linear model procedure (GLM) using SAS, version 9.2 (SAS Institute, Cary, NC), and a significance level of 0.05 ($P < 0.05$).

RESULTS AND DISCUSSION

The pHs for the treatment solutions were as follows: 8.28 for water, 11.0 for 1% AH, 11.28 for the 2% AH, and 11.46 for the 3% AH solution. Previously Parsons et al. (*17*) reported 10.81 for a 1% AH brine solution and 10.73 for a 1% AH plus 1% phosphates Parsons et al. (*16*). Additionally, in a previous study we obtained a pH solution of 10.66 for a 1% AH brine (*5*). It is known how the temperature and pH may affect ammonia solubility and volatility. The solubility of ammonia increases with a lower pH, while the volatility increases with a raise in pH (*7*). Thus, the observed inconsistencies in pH for 1% AH brines might be caused by variations in water temperature and water pH.

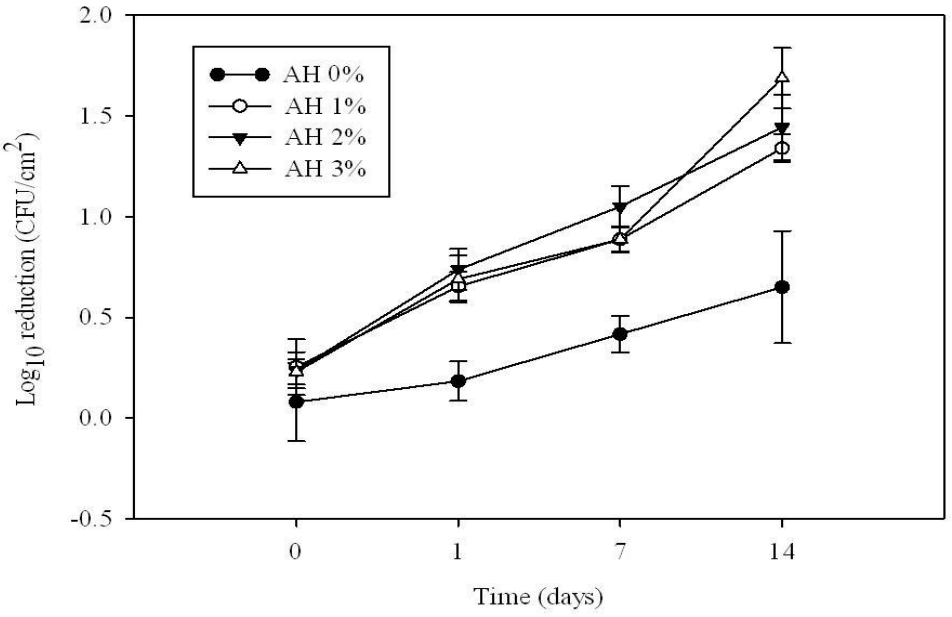

FIGURE 4.1. Reduction of populations of *E. coli* O157:H7 of inoculated meat samples caused by water, 1%, 2%, and 3% ammonium hydroxide solutions applied by a multi-nozzle spray system. Bars represent the standard deviation of the mean.

TABLE 4.1. Reduction of populations of *E. coli* O157:H7 of inoculated meat samples caused by water (0%), 1%, 2%, and 3% ammonium hydroxide solutions applied by a multi-nozzle spray system.

Day	Treatment	Repetitions			Average (CFU/cm^2)	Log Reduction (CFU/cm2)
		R1	R2	R3		
0	No inoculum + No Spray	0.00	0.00	0.00	0.00	
	Inoculum + No Spray	5.80	5.83	5.77	5.80	
	Water (0%)	5.71	5.73	5.71	5.72	-0.08
	AH 1%	5.39	5.49	5.60	5.49	-0.31
	AH 2%	5.46	5.62	5.61	5.56	-0.24
	AH 3%	5.59	5.62	5.50	5.57	-0.23
1	Water (0%)	5.64	5.62	5.60	5.62	-0.18
	AH 1%	5.23	5.11	5.10	5.15	-0.65
	AH 2%	4.99	5.18	5.02	5.06	-0.74
	AH 3%	5.20	4.98	5.15	5.11	-0.69
7	Water (0%)	5.38	5.35	5.41	5.38	-0.42
	AH 1%	4.92	4.97	4.85	4.91	-0.89
	AH 2%	4.64	4.77	4.84	4.75	-1.05
	AH 3%	4.97	4.84	4.93	4.91	-0.89
14	Water (0%)	5.18	5.16	5.11	5.15	-0.65
	AH 1%	4.53	4.46	4.39	4.46	-1.34
	AH 2%	4.46	4.17	4.44	4.36	-1.44
	AH 3%	3.99	4.07	4.28	4.11	-1.69

The results (Fig. 4.1 and Table 4.1) showed that 1%, 2%, and 3% AH solutions were significantly more effective in controlling *E. coli* O157:H7 compared to the control ($P < 0.05$). However, there were no significant differences observed between treatments, and 1%, 2% and 3% AH were found to have the same effectiveness against *E. coli* O157:H7 (Table 4.1).

Differences in concentration strength of AH may have occurred due to degassing during spraying. It is possible that some gaseous ammonia may have been released/escaped into the air upon de-pressurization (i.e., degassing) during exit from the spray nozzles as the odor of ammonia was noticeably strong during our spray trials. Thus, levels of AH that actually contacted the meat surface and/or *E. coli* O157:H7 may have been less than anticipated. For further studies utilizing pressure-type spray applications, the measurement of ammonia levels in the solutions (before and after spray application) should be considered. The initial levels of ammonia of the AH before spraying were 161.3 mM, 323.5 mM, and 485.3mM for the 1%, 2%, and 3% AH solutions, respectively. Muller et al. (*13*) reported that ammonium has a detrimental effect on bacteria only when present in high concentrations, levels of ≥ 750 mM for *E. coli*. The reductions caused by AH on *E. coli* O157:H7 increased with time. On day 0 reductions ranged from 0.23 and 0.25 $\log_{10}$ CFU/cm^2. However, by day 1 the $\log_{10}$ CFU/cm^2 reduction was increased to 0.65 to 0.74, by day 7, the reduction was 0.89 to 1.05 $\log_{10}$ CFU/cm^2 and 1.34 to 1.69 $\log_{10}$ CFU/cm^2 by day 14. It is known that alkaline solutions can damage bacterial cell walls. Mendoca et al. (*10*) demonstrated that high pH causes disruption of the cytoplasmic membrane of *E. coli* O157:H7, which leads to leakage of cytoplasmic constituents. The inhibition of bacteria, fungi, and molds caused by ammonia has been reported (*1*). The application of 1 and 2% of ammonium hydroxide inhibited the growth of fungi and reduced bacterial growth in corn (*18*). Himathogkham et al. (*6*) observed higher reduction of *E. coli* O157:H7 over time in contaminated alfalfa beans and mung beans treated with ammonia at 180 and 300 mg per liter of air space (*6*). Niebuhr and Dickson (*15*) injecting gaseous ammonia (ammoniation) to increase the pH

(to ~ 9.6) of ground beef inoculated with *E. coli* O157:H7 using the technology of Beef Products International, Inc. (BPI). The product was then frozen, chipped, and compressed into blocks. Immediately after ammoniation, populations of *E. coli* O157:H7 were reduced by 3 $\log_{10}$ cycle and after freezing these populations were reduced to below the detection limits of the study (*15*). Additionally, it has been reported that a longer exposure of ammonia increases the absorption of meat water causing higher uptake of ammonia by tissue (*8*). Thus, the reductions observed over time in this study seem to support this theory.

Although AH treatments were significantly higher than control (water, 0% AH); a progressive reduction of *E. coli* O157:H7 populations in samples sprayed with water (from 0.08 on day 0 to 0.65 on day 14) was also observed. These findings could be attributed to the fact that *E. coli* O157:H7 lacks of ability to grow at 4ºC (*3*) and their inability of synthesizing proteins at a temperature below 8°C (*2*). Thus, the over time reductions observed in control samples seems feasible.

In conclusion, AH applied in a multi-nozzle spray system is effective in reducing populations of *E. coli* O157:H7 under the conditions of this study. There were no differences between 1%, 2% and 3% AH solution on $\log_{10}$ CFU/cm^2 reduction of *E. coli* O157:H7. These findings are important for the food processing industry from the safety, economical, sensory quality, and food labeling standpoint.

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CHAPTER V

EVALUATION OF THE ANTIMICROBIAL EFFECT OF 1% AMMONIUM HYDROXIDE BRINING SOLUTION APPLIED THROUGH NEEDLE INJECTION AGAINST *ESCHERICHIA COLI* O157:H7 IN BEEF STRIPLOINS

ABSTRACT

The objectives of this study were to evaluate if ammonium hydroxide (AH) possesses an immediate (phase 1) or long term (phase 2) antibacterial effect on *Escherichia coli* O157:H7 populations. Six USDA-Select striploins were used in each phase. Striploins were pre-cored (from the fat side) prior to being subdivided. A 100 µl of gentamicin and rifamycin-resistant cocktail (10^8 log CFU/ml) of *E. coli* O157:H7 was inoculated onto the intact lean surface (25 cm^2) of pre-cores. Subdivided striploins were then injected to 110% of their initial weight with one of the following treatments: control (no injection), water (pH 4.4), 0%-AH-brine (pH 4.7), 1%-AH-brine (pH 10.08, 1% FCC grade AH), or 0.2%-NaOH-brine [pH 10.08, 0.02% FCC grade sodium hydroxide (NaOH)]. All the brines included in their formulation 3.6% NaCl and 0.5% rosemary powder. The 0%-AH-brine and 1%-AH-brine treatments were selected for phase 1. Water, 0%-AH-brine, 1%-AH-brine, and 0.2%-NaOH-brine treatments were selected for phase 2. After injection, three sub-samples were taken from each core: surface (for enumeration), medium- and bottom-core samples (for enrichment method). For

microbial analyses days 0 and 1 were selected in phase 1; while days 0, 4, and 9 were selected for phase 2. No significant differences in days or treatments were found in phase 1. A day effect was found in phase 2. The 1%-AH-brine had lower counts of *E. coli* O157:H7 than the controls used in this study, in surface samples, only when initial meat-pH falls between 5.4 and 5.7. These data suggests that 1%-AH-brine, when applied thorough needle injection, did not promote nor enhance the growth of *E. coli* O157:H7 under the conditions of this study.

INTRODUCTION

Injection of brine solutions containing salt and polyphosphate brines is widely practiced by the meat industry in North America (*8, 28*) mainly to reduce variability in tenderness and increase juiciness. However, this practice introduces safety concerns regarding the use of invasive technologies. Invasive technologies such as blade tenderization, brine injection or mechanical tenderization are known to serve as vehicles for bacteria to be internalized by the needles from a contaminated surface into the sterile deep beef tissue (*8, 15, 16, 27, 29*). Additionally, brines can accumulate bacteria and increase the risk of cross contamination as the brines are continuously re-circulating (*8, 35*). As a result, the microbiological aspects of invasive technologies has been received much attention lately. One of the most common bacteria that has been associated with the consumption of undercooked beef is the causative agent of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans known as *Escherichia coli* O157:H7 (*7*). The Food Safety and Inspection Service (FSIS) has classified *E. coli* O157:H7 as an adulterant and established a zero tolerance policy for non-intact meats (*16*). Therefore,

many physical, chemical, and biological technologies have been developed in an attempt to reduce bacterial populations of non-intact beef products. However, there are still outbreaks linked to tenderized and brine-injected meat products (*16*). One of the main difficulties is the challenge of applying control techniques in processing meat plants. Hence, new applicable technologies are needed to reduce the prevalence of pathogens such as *E. coli* O157:H7 in meat and meat products. A practical alternative may be the use of ammonium hydroxide (AH) in the formulation of brines. Ammonium hydroxide is an alkaline agent, generally recognized as safe (GRAS), and it is approved by the FDA to be used in the formulation of brines to increase brine pH up to 11.6 (*34*). It was demonstrated that replacing phosphate-based ingredients with 1% AH in the formulation of injection brines resulted in similar meat quality characteristics (*25, 26*). In addition, it was found that steaks injected with a 1%-AH-brine had lower bacterial counts than steaks injected with the conventional phosphate-based brine (*4*). The Gram-negative counts were lower > 1 log CFU/g compared with the conventional brine immediately after injection and these differences were maintained for two weeks (*4*). The objective of this study was to determine if AH possesses an immediate, and/or a long term antimicrobial effect against *E. coli* O157:H7 when used as an alkaline aid in the formulation of brines applied through needle injection to striploins.

MATERIALS AND METHODS

Bacterial Cultures. The bacterial cultures used in this study were *Escherichia coli* O157:H7 strains: ATCC 43890 (California outbreak isolate from human feces), ATCC 43894 (Michigan outbreak isolate from human feces), ATCC 43895 (hamburger

isolated implicated in human outbreak), and ATCC 35150 (sporadic cases of hemorrhagic colitis). All four strains were gentamicin (20 µg/ml) and rifamycin (10 µg/ml) resistant. At the BSL2 laboratory of food microbiology, the individual strains were maintained at -20°C in tryptic soy broth (TSB; Becton, Dickson and Company, Ss, MD) plus 15% glycerol (Sigma-Aldrich, St. Louis, MO). To prepare a cocktail, 100 µl of a thawed frozen stock was streaked onto tryptic soy agar (TSA; Becton, Dickson and Company, Sparks, MD) plates containing 20 µg/ml gentamicin (Sigma-Aldrich, Saint Louis, MO) and 10 µg/ml rifamycin (Sigma-Aldrich, Saint Louis, MO). Plates were incubated at 35°C for 22h as described previously by Byelashov et al. (*3*). A loop full of an isolated colony of each isolate was separately transferred into 10-ml TSB and incubated for 22 h at 35°C without shaking. The 10-ml volumes (one for each freshly grown culture) were combined, and the resulting suspension was harvested by centrifugation. The bacterial cells were then re-suspended into 40 ml sterile phosphate buffer solution (PBS, MP Biomedicals, LLC, Solon, OH) and kept in ice. The bacterial cocktail was quantified by preparing appropriate serial dilutions using a 0.1% buffered peptone solution (BD; Becton, Dickson and Company, Sparks, MD) and spread plating onto duplicate TSA plus 20 µg/ml gentamicin and 10 µg/ml rifamycin. Plates were incubated at 35°C for 48 h (*3*). The final concentration of the cocktail was 10^8 CFU/ml.

Brines Formulation. For phase 1, two brines were prepared: 0%-AH-brine (pH 4.7, 3.6% w/w NaCl, 0.5% w/w VIVOX 4 [Vitiva D. D., Markovci, Slovenia]) and 1%-AH-brine (pH 10.08, 1% w/w food grade AH [≥ 25% as ammonia (NH_3), 35.04 M, Fisher Scientific, Fair Lawn, NJ], 3.6% w/w NaCl, 0.5 % w/w VIVOX 4). For phase 2, three brines were prepared: 0%-AH-brine, 1%-AH-brine, and 0.2%-sodium hydroxide

(NaOH)-brine (pH 10.08, 0.02% w/w FCC grade NaOH [Food Grade, Fisher Scientific, Fair Lawn, NJ], 3.6% w/w NaCl, 0.5% w/w VIVOX 4). Brines were maintained in ice at 4°C until they were used.

Phase 1: Sampling Procedure. Every week, two 48h aged Select beef striploins were collected at a fabrication facility. A total of six striploins were used. Striploins were transported to the Robert M. Kerr Food and Agricultural Product Center (FAPC) at Oklahoma State University (OSU) and maintained in the dark at 3.8°C (39ºF) for the next 5 days to mimic transportation to processing plants. The next morning, from the fat side, each loin was marked lengthwise into 6 sections and 4 sterile stainless steel skewers 35.6 cm (14 in) long were inserted at ~1.5 cm parallel to the lean side. The skewers were meant to be a stop point for the coring device to maintain the lean side of the loin intact. Then with the aid of sterile stainless steel coring device of 2 inches diameter (5.08 cm) connected to a drill (Model FSX-treme™, Fire Storm Black & Decker, Towson, MD), two pre-cores were fabricated into each section (12 pre-cores per loin, Fig. 5.1A, 5.1B). Once all the pre-cores were fabricated, each loin was subdivided into 6 sections (each containing 2 pre-cores), and numbered from 1 to 6 (Fig. 5.1D). The two end sections (pieces 1 and 6) were assigned at random to be either a positive control (inoculated, no-injection) or a negative control (non-inoculated, no-injection). Then, two consecutive sections were selected to be injected with either a 0%-AH-brine or 1%-AH-brine. For instance, if subsections 2 and 3 were selected to be 0%-AH-brine the sections 4 and 5 were 1%-AH-brine or vice versa. Next, one of the two sections was designated to be inoculated and the remaining section to be un-inoculated. Sections were then placed into pre-labeled deep sterile stainless steel trays (Vollrath Company, LLC, Sheboygan, WI),

and then covered with a clear wrapping film, placed into a cooler containing ice, and then transported to the BSL2-food microbiology laboratory. At the laboratory, the initial weight of each meat section was recorded.

Phase 1: Ink-Stamped Templates. Four bamboo skewers (15 cm by 2 mm) were inserted from the fat side to the lean side at the four cardinal directions of each pre-fabricated-core. On the lean surface, a stainless steel template outline (25 cm^2 of area) was dipped in edible ink (Great Lakes, Kansas City, MO) and aligned inside of the four bamboo skewers to stamp a circle (exactly above each pre-core). Meat sections selected to be inoculated were stamped with red ink, while the pieces selected to be un-inoculated were stamped with purple ink (Fig. 5.1E).

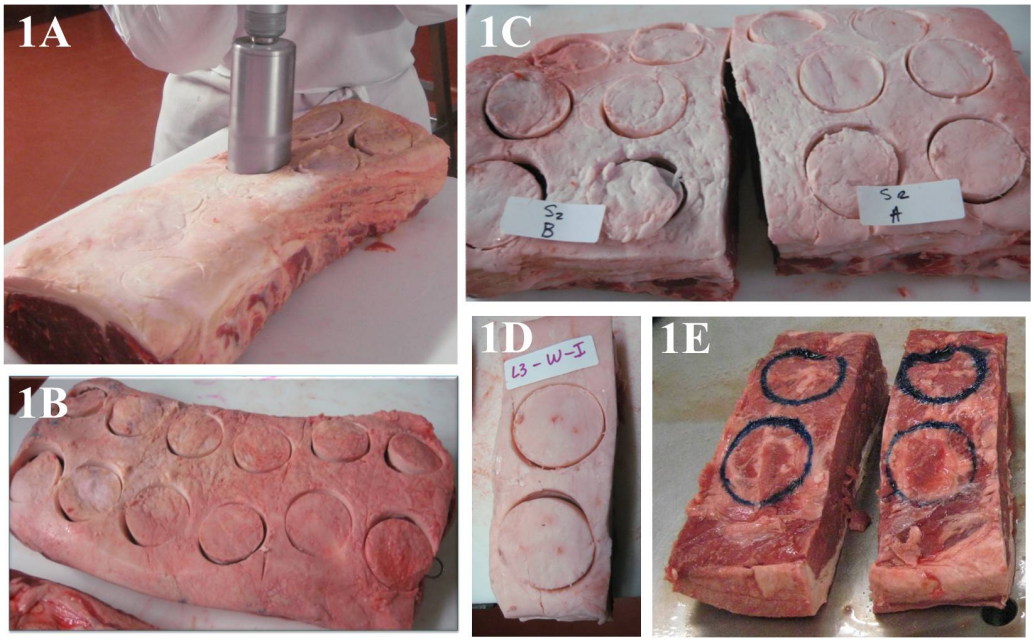

FIGURE 5.1. Photograph showing the fabrication of pre-cores (from the fat side) using a coring device attached to a drill (1A). Fat side of striploin showing 12 pre-cores (1B). Striploin cut into halves, each half contains 6 pre-cores for phase 2 (1C). The fat side of

a subdivided striploin containing two pre-cores for phase 1 (1D). Intact lean side of pieces showing the stamped ink circle to be inoculated for phase 1 (1E).

Phase 1: Bacterial Inoculation. A100 µl aliquot of a gentamicin and rifamycin-resistant multi-strain cocktail of *E. coli* O157:H7 (10^8 CFU/ml) was pipetted onto select areas in order to achieve an inoculation level of 10^6 CFU/cm^2. A sterile plastic spreader (VWR® Bacti Cell; VWR International, Inc., West Chester, PA) was used to spread the bacterial suspension inside the stamped ink-circle. Deep trays were covered with aluminum foil, avoiding any contact with the inoculated area, and then placed at 4°C for 30 min to allow bacterial attachment.

Phase 1: Meat Injection. Meat pieces were injected at 110% of initial weight using a hand-held multi-needle (5 needles of 3½ in of length) stitch pump (model 3041, Koch Equipment, Kansas City, MO) with either the 0%-AH-brine or the 1%-AH-brine. To minimize needle contamination, for each next injection a sterile set of 5 needles was used. To avoid brine contamination, the injector was set-up in the no-brine recirculation mode. Additionally, a 100 µL volume of each brine was plated onto TSA plates containing 20 µg/ml gentamicin and 10 µg/ml rifamycin before and after injection. To determine the amount of brine injected into each piece, each piece was weighed immediately after injection and then after 30 min.

Phase 2: Sampling Procedure, Ink-Stamped Templates, Inoculation, and Injection. In this case, paired USDA Select striploins (n = 3) were used, one pair per week. Striploins were obtained and transported in the same manner as previously explained for phase 1. After 5 days of cold aging, a ~1 cm thick steak was collected from

each end of both striploins (n = 4) to serve as positive and negative controls. As described previously in phase 1, 12 pre-cores were fabricated from each striploin (Fig. 5.1A, 5.1B). Loins were then halved (each half contained 6 pre-cores, Fig. 5.1C). Following a randomized scheme each half was assigned to one of the following treatments: water (control), 0 %-AH-brine, 1%-AH-brine, and 0.2%-NaOH-brine. Loin halves were placed into deep trays, covered with clear plastic wrap, and transported to the BSL2 food microbiology laboratory. After initial weights were recorded, loin halves were ink-stamped, inoculated, placed at 4ºC for 30 min to allow bacterial attachment, and then brine-injected as indicated for phase 1.

Phase 1 and 2: Collection of Surface Samples. Each meat piece was transferred to a sterile cutting board with the fat side down to collect surface samples. With a sterile scalpel, an incision of ~1 cm depth around the ink stamped circle area was made. Then using sterile forceps one edge of the surface sample was lifted to make a cut (parallel to the surface) until the entire surface sample was entirely separated from the meat piece (Fig. 5.2A, 5.2B). Each surface sample was then placed into a 6 x 9" pre-labeled sterile filter stomacher bag (VWR International, Inc., West Chester, PA) with the inoculated surface area facing the filter layer. Twenty four surface samples were collected every week (total 72 samples per phase). In phase 1, half of the samples were analyzed immediately after sample collection, while the remaining half was placed at 4ºC for 24 h. In phase 2, two samples (from each treatment) were analyzed on day 0, two on day 4, and two on day 9. In order to mimic overwrapping supermarket package conditions, the openings of the bags containing selected samples to be analyzed on days 1, 4 or 9 were folded once to allow oxygen permeability.

Phase 1 and 2: Microbial Analysis of Surface Samples. To each stomacher bag containing a meat surface sample, a volume of 100 ml of Dey-Engley neutralizing broth (D/E broth; Neogen® Corporation, Lansing, MI) was added. Stomacher bags were pummeled using a stomacher (Laboratory Blender Stomacher 400; Tekman Company, Cincinnati, OH) at normal speed for 2 min (1 min each side). From the stomacher bag suspension, appropriate additional serial dilutions were prepared by using a buffered peptone solution (0.1%) as described by Morton (*20*). Duplicate samples were spread-plated onto Tryptic Soy Agar (TSA; Becton, Dickson and Company, Sparks, MD) containing 20 µg/ml gentamicin and 10 µg/ml rifamycin to enumerate *E. coli* O157:H7. Plates were incubated at 35°C for 48 hours (*3*). Representative colonies were counted, the average was calculated from duplicates, and final counts were reported as colony form units per centimeter square (CFU/cm^2) and converted to logarithmic base 10 values for the statistical analysis. Additionally, 3 colonies from positive samples were confirmed serologically with a latex agglutination assay (RIM *E. coli* O157:H7, Remel, Lenexa, KS).

Phase 1 and 2: Collection of Core Samples. To have access to the core, while maintaining the fat side down, a vertical incision in one of the sides of the surrounding tissue of the core was done (avoiding contact to the core). Then, one of the edges of the surrounding meat layer was pulled away from the core using sterile forceps. The remaining thin layer on the upper-core area (which was still maintaining the core attached to the rest of the meat piece) was cut all along the upper-core until the core was completely free of any adjacent tissue (Fig. 5.2C). Each core was aseptically transferred to sterile butcher paper, and placed in a horizontal position (Fig. 5.2D). To avoid cross

contamination the two ends of the core were removed: the fat layer (~ 2 cm) at the bottom and a thin lean layer (~0.5 cm) at the top (Fig. 5.2D). The remaining piece was then cut into two halves, which were called: medium-core sample and bottom-core sample (Fig. 5.2E). The surrounding tissue was placed into individual sterile plastic bags (Ziploc®, S.C Johnson, Sturtevant, WI) to be used for pH analysis.

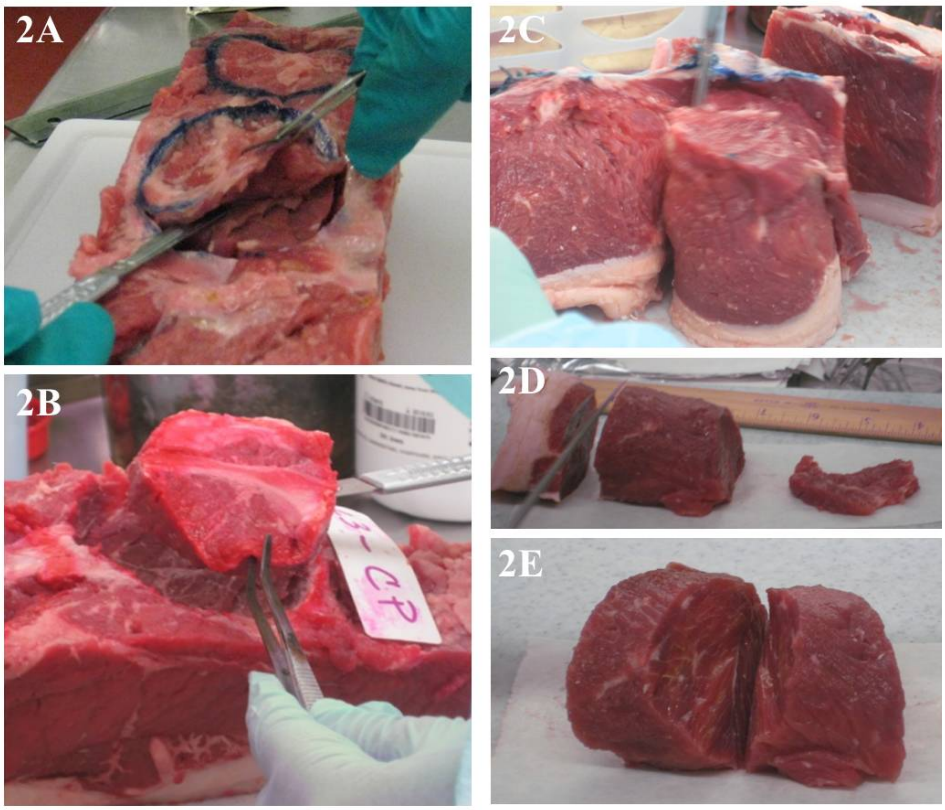

FIGURE 5.2. Photograph showing the collection of surface sample (2A, 2B), collection of core sample (2C), removal of two ends of core (2D), and division of core into two halves: medium core and bottom core (2E).

Phase 1 and 2: Microbial Analysis of Core Samples. Following the USDA/FSIS *E. coli* O157:H7 enrichment protocol (*33*), medium and bottom core

samples were weighed into sterile Petri dishes using a top balance (model XE 4100, Denver Instrument, Arvada, CO), then placed into a stainless steel blender container (Waring Products Inc., New Hartford, CT), and appropriate enrichment media to have a 1:10 dilution was added. Meat core samples were blended for 30 sec and the resulting slurry was transferred to sterile filter stomacher bags (Nasco Whirl-Pak® filter bag, model B01318, Fort Atkinson, WI), pummeled for 2 min at normal speed (60 sec each side), and then incubated at 42°C ± 1°C for 22 h. From the enrichment cultures, latex agglutination assay was performed (RIM *E. coli* O157:H7, Remel, Lenexa, KS). Cores that were selected to be analyzed on days 1, 4, or 9 were weighed, placed into pre-labeled sterile Whirl-Pak® stomacher bags, double bagged, and maintained at 4ºC until needed. Medium- and bottom-core samples were tested only for the presence or absence of the pathogen by the enrichment method.

Phase 1 and 2: Meat pH Analysis. Samples collected for pH analysis (days 1, 4 and 9) were also maintained at 4°C in Ziploc® bags. Every day of microbial analysis its corresponding meat pH sample was frozen at -20°C. To have a more consistent pH analysis all the pH samples were analyzed the same day. Meat samples were partially thawed at room temperature, cut into small pieces, and then 10 g of sample was weighed into a blender cup (Waring Products Inc., New Hartford, CT). A volume of 90 ml of distilled water was added, and then blended for 30 sec at medium high. The pH readings were taken using a pH meter model AR50 Accumet® Research (Fisher Scientific, Los Angeles, CA).

Data Analyses. Bacterial counts were converted to $\log_{10}$ CFU/cm^2. Means and standard deviation were calculated on the assumption of a normal distribution. Phase 1 was arranged as a randomized complete block design (RCBD) with 6 replications per treatment. Fixed variables were day (n=2) and treatment (n=2). Random block variable was week. Phase 2 was analyzed using a mixed model with multiple repetitions per week and with initial pH as a covariate. Data were blocked by week and animal (within week). Brine treatments, days after treatment, and the interaction between them were the fixed effects. Data were analyzed using SAS, version 9.2 (SAS Institute, Cary, NC). The least significance difference (LSD) type approach was used to determine significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Previously, striploins were injected (110% of their initial weight) with brines containing different AH concentrations (from 0.1% to 10%). These brines also included in their formulation 3.6% salt and 1% Herbalox (rosemary extract). A negative correlation between concentrations of AH and % of purge was found. When AH concentrations were between 1% and 3% there was not a significant improvement in % of purge and when the AH levels were > 3% the ammonia-off smell was perceived. Consequently, following studies were conducted using the minimal amount of AH, 1% AH (*4, 25, 26*). It was found that loins injected with 1%-AH-brine had lower Gram-negative counts (~ 1 log) compared with the ones injected with the conventional brine containing phosphates (*4*). Hence, a question was raised whether 1% AH was enough to reduce *E. coli* O157:H7 or in fact, this concentration needed to be increased to a 2% AH

or even 3% AH. To answer this question, a preliminary study was performed to evaluate differences between 1%, 2% and 3% AH solutions against *E. coli* O157:H7. In this case, lean meat disks (0.6 cm thick and 20.5 cm^2 area) were fabricated from beef top butts. Meat samples were inoculated with 10^6 log_{10} CFU/cm^2 of a gentamicin and rifamycin-resistant cocktail of *E. coli* O157:H7 (same strains used in this study). After 30 min of attachment period at 4°C, meat disks were passed throughout the conveyor (5.5 ft/min, for 18 s, and with a spray rate of 1.5 gpm) of a multi-nozzle spray system (Model TC700M; Ross Industries Inc., Midland, VA). The four spray treatments were: water (control, 0%), 1%, 2%, and 3% w/w AH solutions. Immediately after spray treatment, samples were aseptically retrieved, and randomly two samples were placed into each stomacher bag. Bags were vacuum-packaged and placed at 4ºC until microbial analysis was performed. Samples were analyzed on days 0, 1, 7, and 14. Every day of analysis, it was found a significant difference existed between the AH-treatments (1%, 2%, and 3% AH solutions) and the control (water, $P < 0.05$). The 1%, 2%, and 3% AH solutions have the same effectiveness reducing *E. coli* O157:H7 populations and these reductions increased with time. The bacterial log_{10} reductions, for the AH treated samples, increased from 0.24 ± 0.10 log_{10} CFU/cm^2 on day 0 to 1.49 ± 0.13 log_{10} CFU/cm^2 on day 14. For the water samples from 0.08 ± 0.20 on day 0 to 0.65 ± 0.28 on day 14. These results demonstrated that increasing AH concentrations from 1% to 3% did not significantly improve the antibacterial effect against *E. coli* O157:H7, therefore again the minimal concentration of 1%-AH-brine was selected for this study. Additionally, to recover our *E. coli* O157:H7 inoculum a non-selective media (TSA) containing antibiotics to which the strains were resistant was selected over the selective MacConkey Sorbitol Agar

(SMAC). The use of TSA media to recover higher populations of *E. coli* O157:H7 has been documented previously by Sharma et al. (*30*). It was found that the bile salts of SMAC are a secondary stress to the cells, causing a lower population recovery compared to TSA media (*30*).

In phase 1, the brine pH for the 1%-AH-brine was 11.59 ± 0.43 and 6.70 ± 0.27 for the 0%-AH-brine. The water pH used to prepare brines was 8.14 ± 0.39. The percentage of brines injected into subprimals was different across treatments for phase 1 ($P < 0.001$). The initial weight was increased by 12.50% ± 2.48% for the 1%-AH-brine injection and by 10.07 % ± 1.58% for the 0%-AH-brine.

No differences between treatment ($P = 0.49$), day ($P = 0.96$), or their interaction ($P = 0.49$) were found during the statistical analysis of phase 1. Reductions in bacterial numbers for day 0 were: 0.41 ± 0.31 $\log_{10}$ CFU/cm^2 and 0.32 ± 0.22 $\log_{10}$ CFU/cm^2 for the 0%-AH-brine and for the 1%-AH-brine, respectively. Similar results were observed on day 1: 0.40 ± 0.22 $\log_{10}$ CFU/cm^2 for 0%-AH-brine and 0.40 ± 0.17 $\log_{10}$ CFU/cm^2 for 1%-AH-brine (Fig. 5.3). According to Muller et al (*21*) ammonium compounds have high diffusion rate across biomembranes and high concentrations of ammonium in the cell become detrimental for bacteria due to a harmful energy-wasting futile cycle (*21*). A pre-requisite for ammonium to be cytotoxic, however, is its presence in the cell. It is also known that longer exposure to ammonia results in higher uptake of ammonia by the cells (*13*). This might explain the mechanism behind the higher bacterial reductions observed on day 1 during the spray treatment in the preliminary study compared to results from phase 1 (0.69 $\log_{10}$ CFU/cm^2 vs. 0.40 $\log_{10}$ CFU/cm^2, respectively). The spray treatment

was more effective in exposing the bacterial cells to the AH-solutions than the needle-injection.

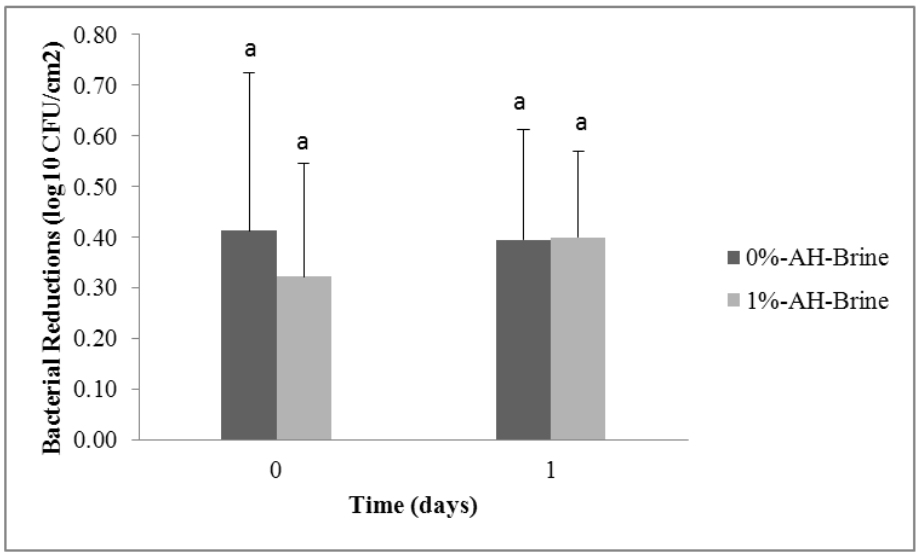

FIGURE 5.3. Reductions of *E. coli* O157:H7 populations (expressed in $\log_{10}$ CFU/cm^2) in meat samples injected with 1%-AH-brine, and 0%-AH-brine (Phase 1). [a]Means with differing letters are significantly different ($P > 0.05$).

The enrichment results for medium- and bottom-core samples revealed that 50% of the medium-core samples and 16.7% of the bottom-core samples were positive for the 1%-AH-brine. The 58.3% of medium and 41.7% of bottom core samples of the 0%-AH-brine were positive. The translocation of bacteria (4% to 8%) into deep tissues caused by invasive technologies has been reported previously (*15, 16, 29*). Interestingly, the percentages of positive samples were lower for the AH-injected samples. However, there was no evidence to affirm that AH was causing this difference. Although no significant differences were found in phase 1 (Fig. 5.3), results from this phase were useful to determine the practicability of the methodology. For each treatment, un-inoculated meat

samples were included as negative controls. As intended, *E. coli* O157:H7 were not found in un-inoculated samples, confirming that cross contamination was not occurring. However, new questions were raised about whether these no-significant differences were caused by the presence of salt and antioxidant (rosemary extract) into the brine, or it was an effect of differences in brine pHs. Therefore, two-extra treatments were included for phase 2: water and same brine-pH (11.08) using NaOH. To determine changes in meat pH caused by the injection of brines, meat pH was also measured for phase 2. Additionally, to determine more precisely if there was a day effect, a small study was achieved using the same methodology as described for phase 1. However, in this case, only one-pair of striploins were pre-cored, inoculated (10^6 CFU/cm^2), injected with a 1%-AH-brine, and then surface samples were collected and stored under aerobic conditions at 4°C. The enumeration analysis was done on duplicates (one sample from each loin) on days 0, 2, 4, 6, 8, and 10. There were no differences in bacterial reductions between day 0 and day 2 ($P = 0.65$), however days 4, 6, 8, and 10 were different from day 0 ($P = 0.0059$, $P = 0.0053$, $P = 0.0019$, $P = 0.0024$, respectively). Based on these results days 0, 4, and 9 were selected for phase 2.

In phase 2, no differences between injection rates were found ($P = 0.12$). Initial weights were increased by 11.12% ± 0.98% for 1%-AH-brine, by 9.20% ± 1.76% for 0%-AH-brine, by 10.94% ± 2.48% for 0.2%-NaOH-brine, and by 10.09% ± 0.17% for water. Brine pHs were: water 4.45 ± 0.51, 0%-AH-brine 4.75 ± 0.08, 1%-AH-brine 11.09 ± 0.10, and 0.2%-NaOH-brine was 11.08 ± 0.11. The statistical analysis of pH from phase 2 revealed that brines change pH differently ($P < 0.001$). The 1%-AH-brine was more effective increasing the meat pH than the other treatments ($P < 0.001$). The

1%-AH-brine increased the meat pH by 0.33 ± 0.13, while 0%-AH-brine declined pH (-0.35 ± 0.03). The 0.2%-NaOH-brine and water had the lowest effect on meat pH (-0.17 ± 0.04 and -0.07 ± 0.02, respectively). It was expected to have similar meat pHs in both the 1%-AH-brine and 0.2%-NaOH-brine samples after injection. However, the later brine failed to increase meat pH as expected. As opposed to AH (weak base), NaOH is a strong base which lacks of buffering capacity. The increase in meat pH caused by the injection of 1%-AH-brine was reported previously by Parsons et al. (*25*, *26*) who reported an increase in meat pH after injection of 0.31 pH units. Moreover, Karim et al. (*13*) reported high affinity of ammonia to water, and they suggested that ammonia is mainly absorbed by meat water (since 75% of the muscle weight is water), thus effectively raising the meat pH. Fernandez-Lopez (*6*) reported a reduction of pork meat pH caused by the addition of salt. It was concluded that pH decreases as salt concentration increases, and when 3% salt was added meat pH was lowered from 5.97 to 5.77.

The statistical analysis of bacterial reductions showed a significant day effect ($P < 0.001$). In general, all the samples showed a reduction in bacterial populations during storage at 4°C (Table 5.1). Previous studies reported that *E. coli* O157:H7 are not able to grow at refrigeration temperatures (< 39°F, *1*). Arnold and Kaspar (*2*) suggested that *E. coli* O157:H7 lack the ability of synthesizing proteins at a temperature below 8°C, which might impact negatively their survival at refrigeration temperatures (*2*). Hence, the over time reductions observed in all the treatments were expected. Additionally correlation analysis between bacterial log reduction and pH after injection was performed. No correlation between bacterial growth and pH after injection was found for day 0 ($P = 0.1679$). However, on days 4 and 9, there was a positive correlation between bacterial

growth and meat pH (r = 0.77472, and r = 0.75916, respectively). Which could be attributed to the amino acid metabolism during bacterial spoilage (*17*). When overall means were compared, no significant differences between treatments were found (P = 0.3905). However, if initial pH was taken into consideration as covariate, there was a treatment effect (P = 0.0003). Log reductions of *E. coli* O157:H7 were significantly higher for samples injected with 1%-AH-brine when initial meat pH was ≤ pH 5.7 (Table 5.1 and Fig. 5.4, 5.5, 5.6).

TABLE 5.1. Reductions of *E. coli* O157:H7 populations (expressed in $\log_{10}$ CFU/cm^2) in meat samples injected with 1%-AH-brine, NaOH- brine, 0%-AH-brine and water. Data are depicted by initial meat pH (before injection) and day of analysis (Phase 2). $^{a-c}$ Means appearing in the same column within day with different superscripts are significantly different (P > 0.05).

Days	**Treatments**	**Initial Meat pH**						
		5.4	**5.5**	**5.6**	**5.7**	**5.8**	**5.9**	**6.0**
0	1%-AH	0.18^{a}	0.23^{a}	0.28^{a}	0.34^{a}	0.39^{a}	0.45^{a}	0.50^{a}
	0.2%-NaOH	0.00^{b}	0.00^{a}	0.04^{ab}	0.16^{a}	0.28^{a}	0.39^{a}	0.51^{a}
	0%-AH	0.00^{b}	0.00^{ab}	0.00^{ab}	0.00^{b}	0.00^{a}	0.00^{a}	0.15^{a}
	Water	0.00^{b}	0.00^{b}	0.10^{b}	0.04^{b}	0.17^{a}	0.30^{a}	0.43^{a}
4	1%-AH	1.21^{a}	1.27^{a}	1.32^{a}	1.37^{a}	1.43^{a}	1.48^{a}	1.54^{a}
	0.2%-NaOH	0.77^{b}	0.89^{b}	1.01^{b}	1.13^{b}	1.24^{ab}	1.36^{a}	1.48^{a}
	0%-AH	0.40^{b}	0.57^{b}	0.74^{b}	0.92^{b}	1.09^{b}	1.26^{a}	1.43^{a}
	Water	0.59^{b}	0.72^{b}	0.85^{b}	0.99^{b}	1.12^{b}	1.25^{a}	1.38^{a}
9	1%-AH	1.45^{a}	1.51^{a}	1.56^{a}	1.61^{a}	1.67^{a}	1.72^{a}	1.77^{a}
	0.2%-NaOH	1.06^{b}	1.18^{ab}	1.30^{ab}	1.42^{ab}	1.53^{a}	1.65^{a}	1.77^{a}
	0%-AH	0.49^{b}	0.66^{b}	0.84^{bc}	1.01^{bc}	1.18^{b}	1.35^{a}	1.53^{a}
	Water	0.83^{b}	0.96^{b}	1.10^{c}	1.23^{bc}	1.36^{ab}	1.49^{a}	1.63^{a}

Interestingly, the log reduction of *E. coli* O157:H7 for controls (water, 0%-AH-brine, and 0.2%-NaOH-brine) progressively increased along with meat pH (Fig. 5.4, 5.5, 5.6), hence when initial meat pH was ≥ 5.8 no significantly differences between treatments were found (Table 5.1). Conner and Kotrola (*5*) reported gradual loss of viability of *E. coli* O157:H7 at 4°C in media with neutral pH and when pH of the media was ≤ 5.0, *E. coli* O157:H7 populations remained stable for 56 days of study. These results suggested that at 4°C, the increase in pH increases loss of viability of *E. coli* O157:H7.

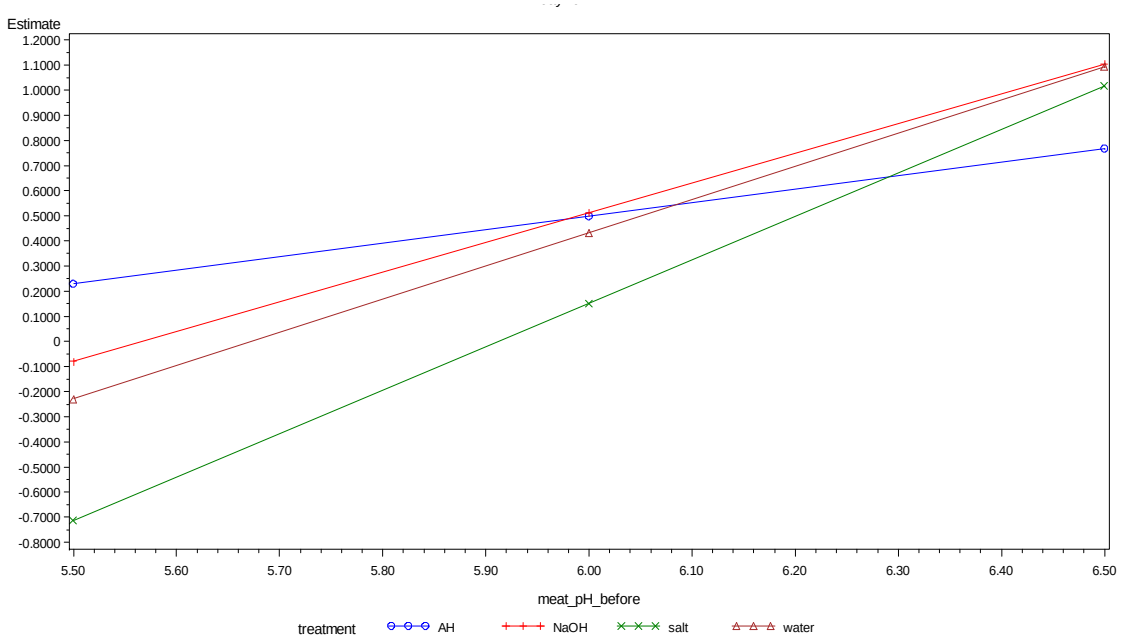

FIGURE 5.4. Reductions of *E. coli* O157:H7 populations (expressed in $\log_{10}$ CFU/cm^2) in meat samples injected with 1%-AH-brine, and 0%-AH-brine for day 0 (Phase 2).

Based on these observations and if solely pH is taken into consideration our findings are in agreement with Conner and Kotrola's study. However, the observation

that meat injected with water had lower *E. coli* O157:H7 populations than samples injected with 0%-AH-brine does not seem possible. The 0%-AH-brine contained both salt and rosemary. Previous work has demonstrated that both salt (*31*) and rosemary (*19*) have an antimicrobial effect against bacteria. Thus, it is possible meat pH is not the only factor that dictates the viability of *E. coli* O157:H7 at 4°C in meat systems, and more than only meat pH is responsible for reductions of *E. coli* O157:H7 observed in this study.

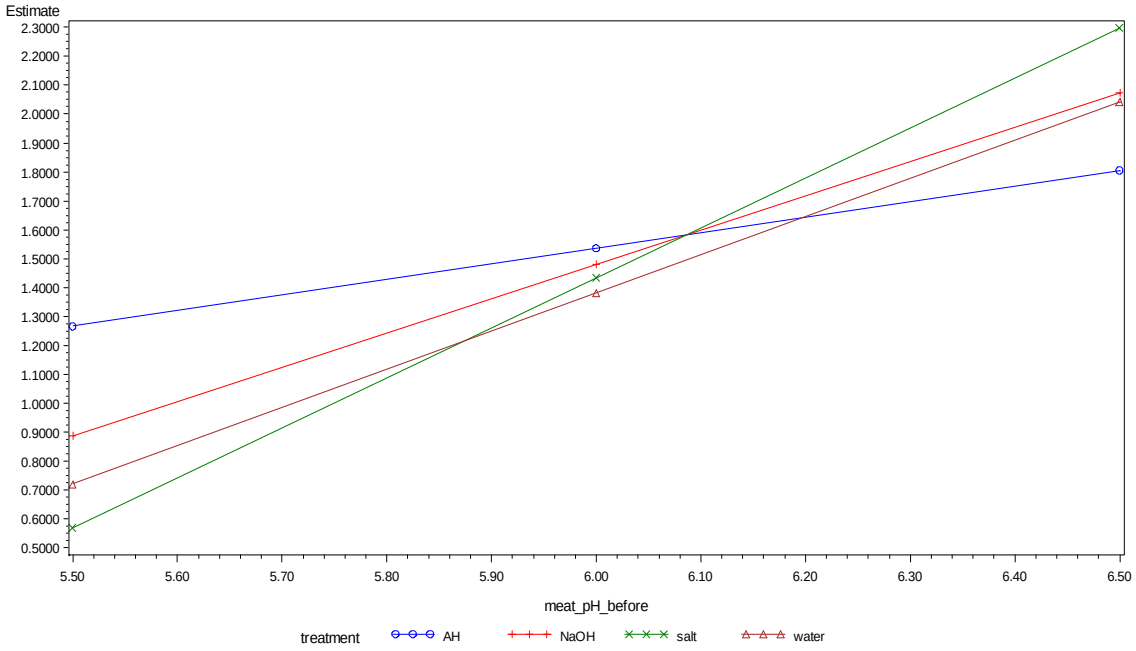

FIGURE 5.5. Reductions of *E. coli* O157:H7 populations (expressed in $\log_{10}$ CFU/cm^2) in meat samples injected with 1%-AH-brine, and 0%-AH-brine for day 4 (Phase 2).

In 2008, Knox et al. reported that spoilage bacteria grew faster in meat (pork loins) with pH > 5.85 (*14*). It is known that under aerobic conditions *Pseudomonas* spp. are the predominant spoilage bacteria in meat systems (*9*). Hence, a possible explanation for higher log reductions of *E. coli* O157:H7 observed in water injected meat samples is

these reductions were caused by spoilage bacteria (such as *Pseudomonas* spp.) competing with *E. coli* O157:H7. Then, our hypothesis is that when initial meat pH is greater than pH 5.8 *Pseudomonas* spp. are able to grow more aggressively in meat treated only with water than meat treated with salt and rosemary (0%-AH-brine). The result is, consequently, less growth of *E. coli* O157:H7 in water treated meat than in salt and rosemary treated meat.

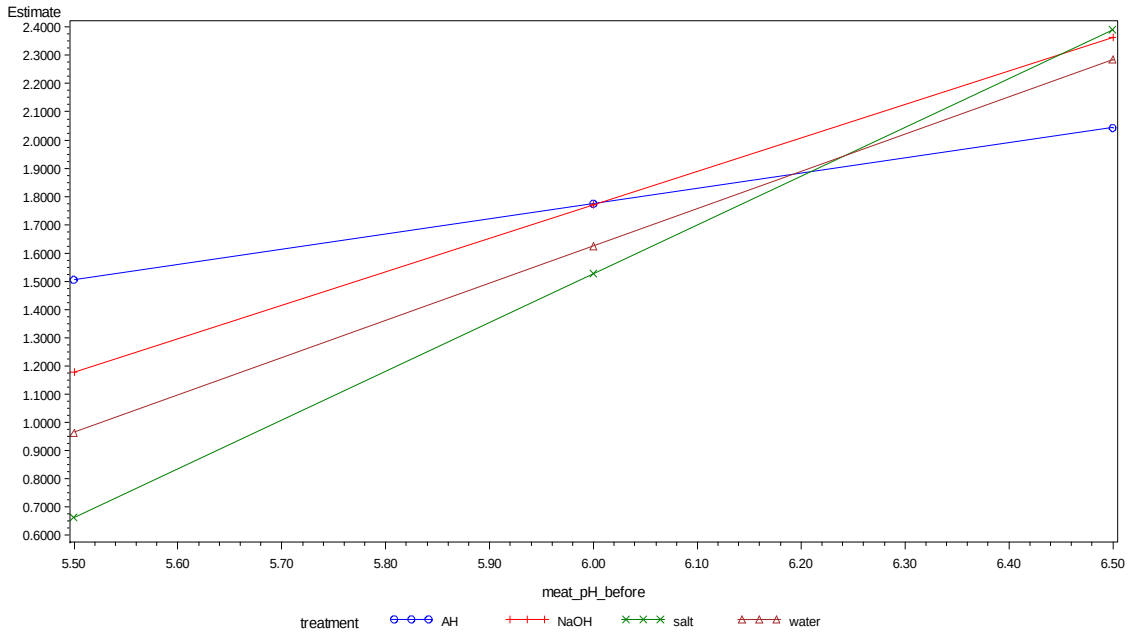

FIGURE 5.6. Reductions of *E. coli* O157:H7 populations (expressed in $\log_{10}$ CFU/cm^2) in meat samples injected with 1%-AH-brine, and 0%-AH-brine for day 9 (Phase 2).

Additionally, the observed higher reductions caused by the alkaline control (0.2%-NaOH-brine) than the water and the 0%-AH-brine were expected since the susceptibility of Gram-negative bacteria to alkaline agents is well known (*12*, *18*, *23*). In contrast to these differences in *E. coli* O157:H7 populations caused by initial meat pH, the 1%-AH-brine injected samples exhibited the same effectiveness against *E. coli*

O157:H7 regardless the initial meat pH. This might be understood since 1%-AH-brine was found to reduce Gram-negative spoilage bacteria in meats such as *Pseudomonas* spp. (*4*). Hence, the observed effect of 1%-AH-brine could be merely an antibacterial effect on *E. coli* O157:H7 rather than a pH effect and/or a bacterial competition effect, which results in an advantage from a shelf life and meat quality standpoint.

These results are in agreement with previous research that focused on the use of ammonia-based solutions to reduce bacterial growth. For example, Gupta et al (*10*) used a mixture of ground goat meat and 0.4 M-AH (1.6%, pH 9.5) to evaluate the antibacterial effect of AH on Gram-negative bacteria. The AH treatment had ~0.5 $\log_{10}$ lower Gram-negative populations than meat adjusted at the same pH using NaOH after 11 days of storage at 4ºC (*10*). Additionally, other studies reported the use of ammonia-based compounds to reduce *E. coli* O157:H7. Himathongkham et al. (*11*) showed a reduction of 2 to 3 logs in alfalfa seeds and 3 to 5 logs in mung beans experimentally inoculated with *E. coli* O157:H7 and *S.* Typhimurium (10^8 to 10^9 cfu/g) and then treated with ammonia gas. Later, Park and Diez-Gonzales (*24*) reported that the level of ammonia gas at which reductions of *E. coli* O157:H7 and *S.* Typhimurium started to be observed was ~ 5mM for inoculated cattle manure (*24*). Niebuhr and Dickson (*23*) reported the immediate reduction of *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* by 4.5, 3.0 and 0.5 $\log_{10}$ cycles, respectively, in boneless lean beef trimmings when ammonia gas was applied to raise the meat pH to ca. 9.6, and then the product was frozen. Stopforth et al. (*32*) inoculated samples of boneless beef plates with *E. coli* O157:H7 and *S.* Typhimurium and dipped then into a 0.1% AH solution (pH 10.89) for 30 s at 23ºC (*32*). In this case, the treatment raised the meat pH from ~5.7 to 7.6, however it did not

reduced pathogen populations. Hence, authors concluded, for AH solution to have an effective antimicrobial effect, meat pH must be 9 or higher (*32*). Nath (*22*) inoculated beef bottom rounds with *E. coli* O157 and then injected with the Beef Products Inc. (BPI) patented solution (Freezing Machines Inc. Dakota Dunes, SD) containing water, AH and salt at 130% of initial weight. They reported a reduction of 0.63 log cfu/cm^2 on day 0 and 1.52 log cfu/cm^2 on day 2 compared to non-injected beef bottom rounds (*22*). Differences in meat pH were also reported, 5.61 and 7.72 for non-injected and AH injected samples, respectively (*22*).

Results from the enrichment method from phase 2 for positive medium-core samples were: 11.1% for 1%-AH, 44.4% for NaOH, 77.8% for 0%AH, and 55.6% for water. Positive bottom-core samples were: 5.6% for 1%-AH, 44.4% for NaOH, 66.6% for 0%AH, and 50% for water. In general, the percentage of positive samples for both medium- and bottom-core samples were always lower in meat samples injected with the 1%-AH-brine. There was a higher level of pathogen translocated into medium samples than into the bottom samples. These findings are consistent with Phebus et al (*29*) who reported *E. coli* O157:H7 translocation of 3 to 4% of surface inoculums to the center of the subprimals. Additionally, Luchansky et al. (*15, 16*) reported the majority of the *E. coli* O157:H7 into the topmost 1 cm of mechanically tenderized subprimals. Although this study did not include enumeration of bacteria in bottom and medium samples to give an estimate of the bacterial populations that were internalized into deeper meat tissue by the needle injection, still it can be concluded that fewer bacteria are internalized into the deeper layers of meat during brine injection.

In conclusion, the use of a 1%-AH-brine by the industry is feasible without promoting microbial growth and without negatively impacting meat quality (*4, 25, 26*). The replacement of phosphate-based ingredients with 1%-AH-brine has several advantages for the industry. Some of these advantages are: the production of a clearer label, the reduction of up to 50% of the sodium content in the brine formulation, the production of a phosphate-free brine, and an antibacterial effect against *E. coli* O157:H7. It is also important to point out the observation that AH has same effect on *E. coli* O157:H7 populations regardless the initial pH (Fig. 5.4, 5.5, 5.6). This is another advantage because initial meat pH is highly variable.

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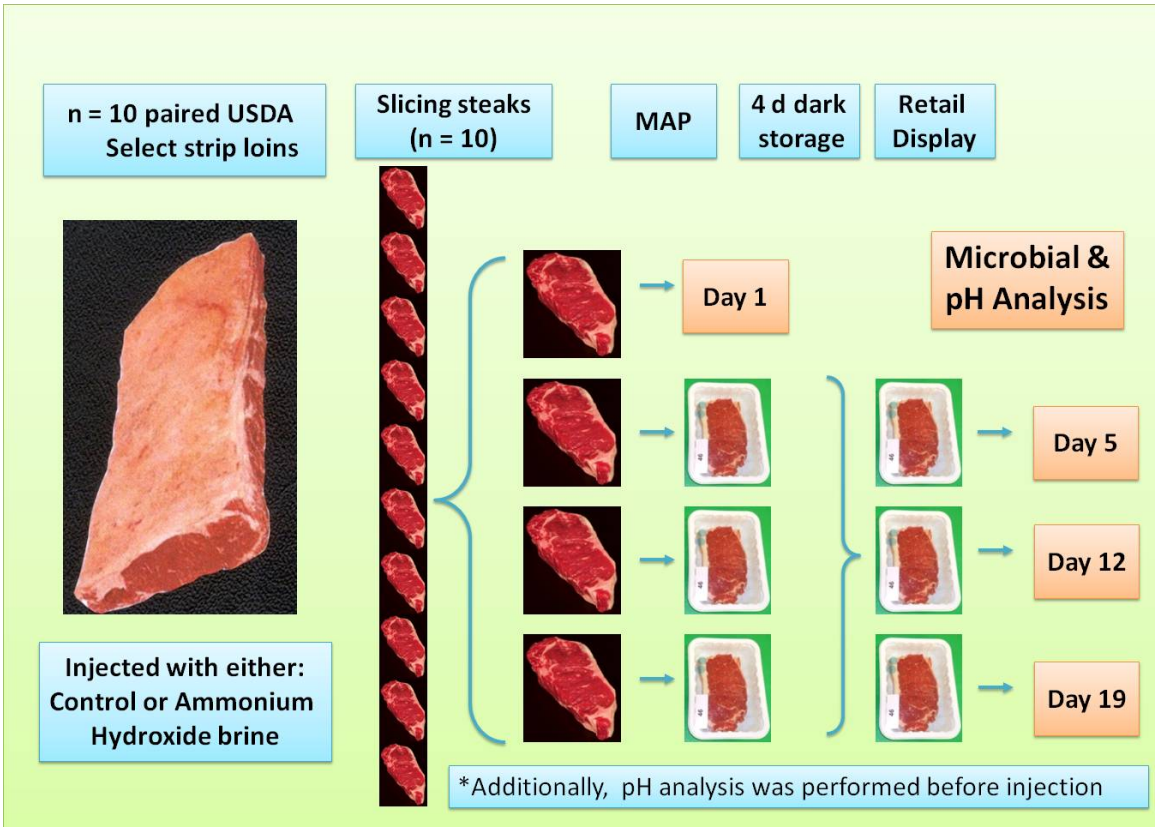
APPENDIX 1:

ANTIMICROBIAL EFFECT OF AMMONIUM HYDROXIDE WHEN USED AS AN ALKALINE AGENT IN THE FORMULATION OF INJECTION BRINE SOLUTION

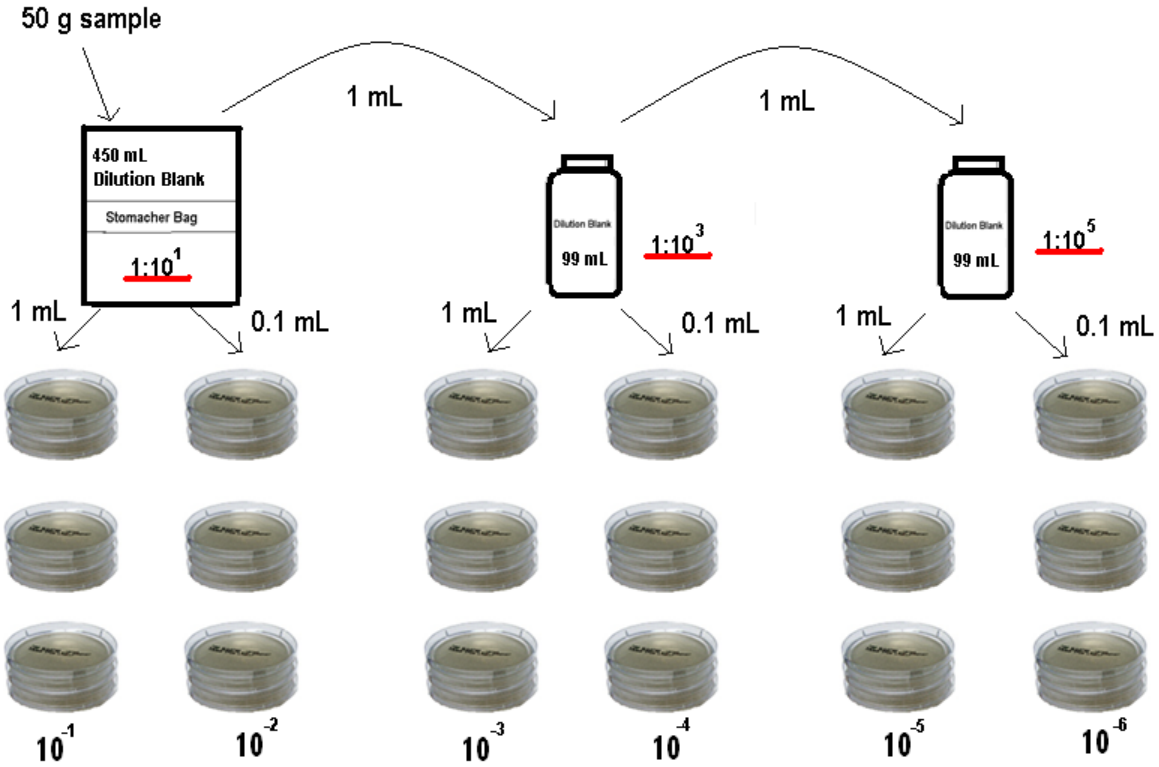
MATERIALS AND METHODS



APPENDIX 2:

ANTIMICROBIAL EFFECT OF AMMONIUM HYDROXIDE WHEN USED AS AN ALKALINE AGENT IN THE FORMULATION OF INJECTION BRINE SOLUTION

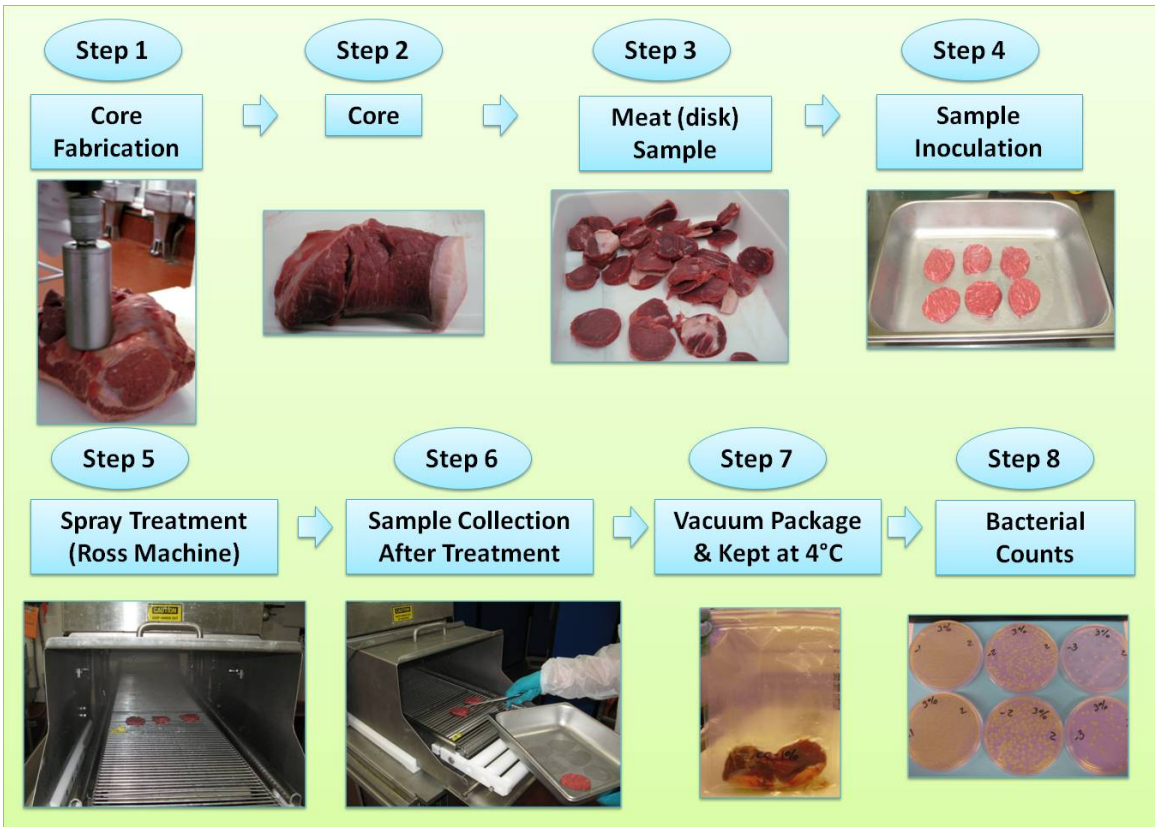
DILUTION SCHEME



APPENDIX 3:

COMPARISON OF 1%, 2% AND 3% AMMONIUM HYDROXIDE SOLUTIONS TO CONTROL *ESCHERICHIA COLI* O157:H7 ON BEEF LEAN SURFACES

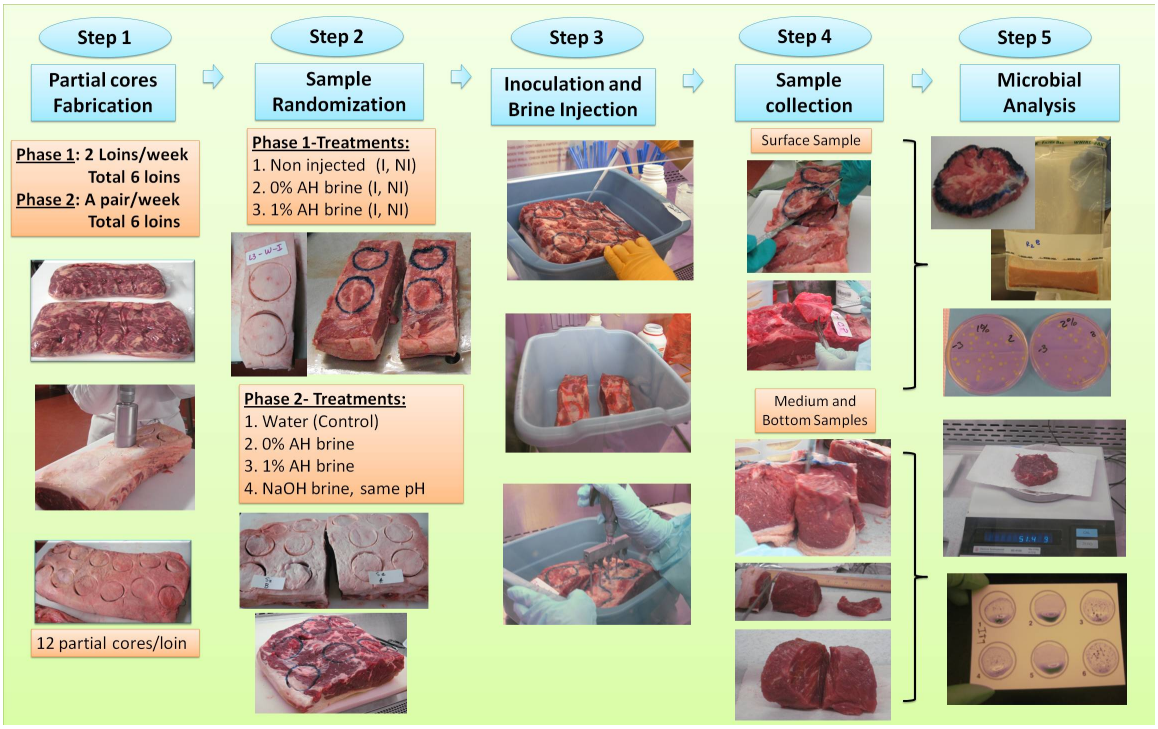
MATERIALS AND METHODS



APPENDIX 4:

EVALUATION OF THE ANTIMICROBIAL EFFECT OF 1% AMMONIUM HYDROXIDE BRINING SOLUTION APPLIED THROUGH NEEDLE INJECTION AGAINST *ESCHERICHIA COLI* O157:H7 IN BEEF STRIPLOINS

MATERIALS AND METHODS



VITA

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Title of Study: EVALUATION OF THE MICROBIALASPECTS OF AMMONIUM HYDROXIDE WHEN USED IN BRINE SOLUTIONS

Pages in Study: 121 Candidate for the Degree of PhD of Science

Major Field: Food Science

Scope and Method of Study: Ammonium hydroxide (AH) is considered a safe and suitable ingredient as a pH agent in brines for meat products up to final brine pH 11.6. However, its impact on microbial aspects of meats injected with a brine containing AH was not fully addressed previously. Four studies were conducted to determine whether AH possesses an antimicrobial effect when injected into meats. First, striploins were injected with 1% AH-brine or a phosphate-based-brine, and then MAP-packaged to evaluate APCs, Gram(-), LAB, and coliform counts. Subsequent studies focused on the pathogen *Escherichia coli* O157:H7. A multi-nozzle spray system was used to spray water, 1%, 2%, and 3% AH solutions onto inoculated meat-disk-samples with *E. coli* O157:H7. Next, striploins were pre-cored and inoculated with *E. coli* O157:H7 before they were injected with 1% AH-brine and then stored aerobically. The final study evaluated *E. coli* O157:H7 reductions over time.

Findings and Conclusions: AH-steaks had lower psychrotrophic, mesophilic, and Gram-negative counts than control (phosphate-steaks). All 1%, 2%, and 3% AH-solutions were effective in reducing *E. coli* O157:H7 compared to the control (water) and its effectiveness increased with time. When 1% AH-brine was injected into inoculated striploins with *E. coli* O157:H7, no-differences were found between AH-brine and control (no-AH brine) on days 0 and 1. The long term effect showed that the 1%-AH-surface samples had lower counts of *E. coli* O157:H7 than controls only when initial meat pH was ≤ 5.7. These data suggests that 1%-AH-brine, when applied thorough needle injection, did not promote nor enhance the growth of *E. coli* O157:H7 under the conditions of these studies. The use of 1%-AH-brine may have several advantages for the industry such as: production of a clearer label, reduction of up to 50% of the sodium content in the brine, production of a phosphate-free brine, and an antibacterial effect against *E. coli* O157:H7. It is also important to point out the observation that AH has same effect on *E. coli* O157:H7 populations regardless the initial pH and this is an additional advantage because initial meat pH is highly variable.

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