Reduction of *Salmonella* Embedded in Lymph Nodes During Grinding Using Lactic Acid and Peracetic Acid Treatments on Beef Trim, Combined with Acidified Sodium Chlorite

by

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ABSTRACT

Salmonella within lymph nodes can be a source of contamination for beef trim and ground beef products. Organic acids and acidified sodium chlorite are commonly used as post-harvest interventions to reduce microbial contamination on the surface of the carcass or trim. However, the use of these interventions during grinding to reduce pathogens protected within lymph nodes has not been previously evaluated. The objectives of this study were as follows: 1) Determine the effectiveness of lactic acid (LA, 4.5%) and peracetic acid (PAA, 220 ppm) treatments combined with acidified sodium chlorite (ASC, 1000 ppm) to reduce *Salmonella* at high (4.8 log CFU/g) and low (2.31 log CFU/g) concentrations embedded in lymph nodes in beef trim during grinding. 2) Conduct sensory analysis to evaluate the impact of the chemical interventions on organoleptic properties of ground beef. 3) Evaluate the induction of a possible stress response on *Salmonella* Montevideo to the chemical intervention. We hypothesized the chemical intervention with organic acid combined with acidified sodium chlorite as secondary intervention during grinding process will reduce *Salmonella* introduced through contaminated lymph nodes on ground beef products. In addition, differences in flavor, color, and texture among treated samples are not expected. Finally, differences in expression of housekeeping genes could provide insight into how *Salmonella* Montevideo adapt and survive in the presence of chemical intervention tested.

Subiliac lymph nodes were inoculated with a cocktail containing rifampicin resistant derivatives of 6 *Salmonella* strains recognized as the most frequent of clinical significance or isolated from cattle lymph nodes. Organic acids were applied to the

trim/node mixture using a six-nozzle sanitizing CHAD spray cabinet. After the first application, the sample was coarsely ground using a meat grinder. The product was then fine ground and an ASC solution was applied through a spray application. Samples were stored at 2-4°C in the dark, and three subsamples for each treatment combination were obtained at 1, 24, and 72 h after grinding. Resultant *Salmonella* population were enumerated on selective medium supplemented with rifampicin with a thin layer of a non-selective medium to allow for recovery of injured cells.

All treatments significantly reduced *Salmonella* introduced through contaminated lymph nodes on ground beef products ($P < 0.05$). When *Salmonella* was present at high concentrations (4.8 $\log_{10}$ CFU/g), LA + ASC, PAA + ASC, and ASC alone reduced rifampicin-resistant non-typhoidal *Salmonella* significantly ($P < 0.05$) by 0.59, 0.47, 0.50 log cycles after 1 hour and by 0.67, 0.51, 0.59 log cycles after 72 hours, respectively. No significant difference between ASC and peracetic acid + ASC was observed ($P=0.23$). In the ground beef containing low concentrations of *Salmonella,* the same treatments showed similar reductions of 0.66, 0.42, 0.52 log cycles and 0.76, 0.51 and 0.64 log cycles after 1 and 72 hours, respectively. No significant difference between LA and PAA + ASC was observed ($P = 0.90$).

To conduct the sensory analysis, ground beef was obtained in the same manner described in the chemical treatment methodology with the exception that subiliac lymph nodes were not included, hence trims were not inoculated with *Salmonella*. The Triangle test indicated that panelists detected a significant difference ($P < 0.05$) in beef patties between the control and the LA + ASC, PAA 220 ppm, and ASC1000 ppm within treatment after

24 and 72 hours. Panelists were unable to identify a significant difference ($P > 0.05$) between control, LA 4.5%, and PAA + ASC beef patties samples at 24 and 72 hours.

The instrumental color results indicated that the five individual chemical treatments compared with the control were significant different in L*, a*, and b* values. There were statistically significant differences ($P < 0.05$) for L* (luminance) values between the control and lactic acid 4.5%, peracetic acid (220 ppm), peracetic acid + acidified sodium chlorite and ASC alone (1000 ppm) at the three sampling points (1, 24 and 72 hours). Regardless to the analysis of a* (redness) values indicated a statistically significant difference ($P < 0.05$) between control beef patties and LA, LA + ASC, PAA, and PAA + ASC. All the treated samples and the control became less red through the time. The b* (yellowness) values indicated no statistically significant differences when comparing the control beef patties with LA + ASC ($P = 0.18$), PAA ($P = 0.70$), PAA + ASC ($P = 0.65$) and ASC ($P = 0.65$). Beef patties treated with lactic acid had a slightly significant difference with control beef patties treated with lactic acid ($P = 0.045$).

A difference was observe in the expression level of the housekeeping genes *rpoS* and *dnaK* when *S*. Montevideo was exposed to stress condition by the acid shock of LA, PAA and ASC for 5, 15 and 30 min. The highest expression of *rpoS* (6.374-fold) was induced by the lactid acid at 30 min. There was not significant difference ($P > 0.05$) among the expression level of *rpoS* in the presence of ASC. When the *dnaK* gene expression was evaluated, the maximun level reached was 3.310-fold after the 30 min. of exposure in the lactic acid.

The result of this study can be used by the meat industry to improve the safety of ground beef that may be contaminated with *Salmonella* within lymph nodes incorporated during process.

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CHAPTER I

INTRODUCTION

Salmonella subspecies *enterica* consists of more than 2500 serotypes (typhoidal and non-typhoidal) and has been recognized as a human pathogen since 1888 when, in Germany, 58 people developed gastrointestinal infections with one death due to the consumption of beef contaminated with Gärtner's bacillus (now known as *Salmonella Enteritidis*); this episode is considered the first laboratory confirmed outbreak of non-typhoidal *Salmonella* (Bell and Kyriakides, 2007). To date, non-typhoidal *Salmonella* is one of the foodborne pathogens that cause a vast number of morbidity and mortality worldwide (Mani-López et al., 2012; Coburn et al. 2007).

According to the World Health Organization (2013), every year approximately ten million of non-typhoidal *Salmonella* infection (salmonellosis) occur every year around the world, resulting in more than one thousand deaths. A global review elaborated by Ao et al. (2015), indicated that in 2010, salmonellosis was as associated with 70 disability-adjusted life years/100,000 population and 81,300 deaths by diarrhoeal disease. Hoffmann et al. (2015), reported that in the United States salmonellosis is the first cause of hospitalization (35%, 19,000 cases) and deaths (28%, 378) related to foodborne illnesses. Moreover, the authors indicated that in terms of economic burden, this foodborne illness represents an annual cost of $3.7 billion, where 90% (3.3. billion) are due to deaths, 8% ($296 million) due to hospitalization, and 2% ($74 million) due to causes that do not require hospitalization.

Non-typhoidal *Salmonella* infection in humans is characterized by gastrointestinal disorder (diarrhea and abdominal cramp) and fever, sometimes, bacteremia and focal infection may develop, especially in people with weak immune systems (Hohmann 2001; Coburn et al. 2007; Gal-Mor et al., 2014). The gastrointestinal symptoms persist in the host for about 2 – 7 days (CDC, 2015). In some cases, an individual remains in the carrier state for the foodborne pathogen for several months after the recovery infection time (Ray and Bhunia, 2008).

The consumption of raw or improperly cooked foods of animal origin like eggs, poultry, meat, and milk is commonly associated with non-typhoidal *Salmonella* infection (Anon 2016; Majowicz et al., 2010). Also, vegetables, fruits, and other plant products have been linked to the infection (Hohmann 2001; Jackson et al., 2013). Moreover, direct contact with infected animals or their environment may be a source of non-typhoidal *Salmonella* transmission to humans (CDC, 2015).

In the United States, between 1998 and 2008 there were 1,491 outbreaks of salmonellosis reported to the Foodborne Disease Outbreak Surveillance System of the CDC. Of these reported outbreaks, 403 outbreaks could be attributed to a single food source. Beef was implicated in 8% of these cases as a confirmed source of non-typhoidal *Salmonella* infection in the human outbreaks (Jackson, et al., 2013). Ground beef has also been specifically implicated as one mode of transmission in food-borne outbreaks.

In a study conducted in 2009, non-typhoidal *Salmonella* was recovered in 4.2% of the 4,136 raw ground beef samples tested from commercial ground beef producers (Bosilevac et al., 2009). In 2014, the USDA FSIS reported that 1.6% of the 7,314 raw

ground beef samples tested were positive for non-typhoidal *Salmonella*. Typically, ground beef is obtained by grinding the less tender cuts and/or higher fat portions of the beef carcass. Also, adipose trim containing lymph nodes can be included in the product if it is labeled as hamburger. Gragg et al. (2013), reported that non-typhoidal *Salmonella* has the ability to invade and survive within lymphoid tissue. When the pathogen is embedded in lymph nodes it is protected from post-harvest interventions applied onto the carcass surface. Hence, the non-typhoidal *Salmonella* can be a source of contamination for beef trim and further be incorporated in the ground beef product (Arthur et. al., 2008).

Because contamination of non-typhoidal *Salmonella* and other foodborne pathogens is inevitable to occur in meat products, different technologies, and treatments with approved chemical agents have been implemented in slaughter and meat processor facilities to reduce or eliminate the presence pathogens on beef carcasses, subprimal cuts and beef trim (Ellebracht et al. 2005; Smulders and Greer, 1998). However, these treatments are not commonly applied in ground beef facilities. Mohan et al. (2012), suggested that antimicrobial interventions will be more effective in reducing bacterial population if it is applied to the final product. Ground beef processors generally use cold temperatures to prevent the growth of bacteria as a critical control point, but this approach will not reduce the pathogen from the final product (Harris et al., 2006).

Chemical interventions to reduce pathogens in meat products include the use of organic acids such as lactic, acetic, citric, and peracetic, they can be applied as a single intervention or combined with other oxidizing agents (acidified sodium chlorite and sodium metasilicate). Acid solutions inhibit microbial activity by the disruption of the

cell membrane, thus altering the intracellular pH homeostasis (Mani-López et al., 2012; Hirshfield et al., 2004). Despite the lethal action of chemical interventions, bacteria can survive the acid stress condition and other alterations by the expression of alternative sigma factors encoded by *rpoS, rpoH,* and *dnaK* housekeeping genes (Bang et al., 2005).

Although chemical interventions have been effective for improving beef safety, they can impact the organoleptic properties of the meat product. Harris et al. (2006), concluded the color of beef patties was no affected by the chemical intervention. However, in a study conducted by Mohan et al. (2012), differences in color were found. Pohlman et al. (2007), addressed that changing color, flavor and texture can lead to declined consumer demand of ground beef.

The objectives of this study were as follows: 1) Determine the efficacy of lactic acid (4.5 %) and peracetic acid (220 ppm) applied to trim in combination with acidified sodium chlorite (1000 ppm) during grinding to reduce *Salmonella* embedded in lymph nodes that are incorporated into ground beef during processing. 2) Conduct sensory analysis to evaluate the impact of the chemical interventions on organoleptic properties of ground beef. 3) Evaluate the induction of a possible stress response on *Salmonella* Montevideo to the chemical intervention.

We hypothesized the chemical intervention with organic acid combined with acidified sodium chlorite as secondary intervention during grinding process will reduce *Salmonella* introduced through contaminated lymph nodes on ground beef products. In addition, differences in flavor, color, and texture among treated samples are not expected.

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CHAPTER II

REVIEW OF LITERATURE

Salmonella enterica

Salmonella cells are rod-shape bacteria, members of the *Enterobacteriaceae* family, Gram-negative and facultative anaerobic; most of them are motile via peritrichous flagella (Adams and Moss, 2008). The bacterium has the ability to grow in a temperature range of 5 to 47 ºC, with an optimum of 37 ºC, its pH requirement for growth goes from 4 to 9 with an optimum between 6.5 and 7.5; concentrations of salt at 3 – 4% inhibit the growth of the pathogen. Moreover, temperature of 70 ºC or above kills the bacteria; however, it has been concluded that low water activity (a_w) increases the heat resistance of *Salmonella* (Mattick et al., 2001; Pui et al., 2011). In addition, *Salmonella* can survive in frozen and dry environments (Montville et al., 2012).

In 1934, Fritz Kauffmann and P. Bruce White proposed the characterization of *Salmonella* enterica by the unique combination of the immune reaction of two major antigens in the bacterial structure: Antigen O, the outermost portion of the cell surface, and antigen H, the filamentous portion of the bacterial flagellum (McQuiston et al., 2011). A third antigen is also involved in the characterization of *Salmonella*, antigen K also called Vi (virulence) capsular antigen; however, this is only present in few serotypes (Ray and Bhunia, 2008). The antigen O is a polysaccharide composed of four to six sugars; the major differences among O antigens are the sugar components, the nature of the covalent bond, and the linkages between sugars (CDC, 2011). On the other hand, the

antigen H is a subunit of protein also known as flagellin, where two types of antigens H (phase 1 and phase 2) have been identified in *Salmonella*. This characteristic is unique to *Salmonella* among enteric bacteria (Su and Chiu, 2005). According to the characterization of Kauffmann and White, today, more than 2,500 serotypes have been identified from the *Salmonella* subspecies *enterica* (CDC, 2011).

Moreover, *Salmonella* subspecies *enterica* serotypes can be characterized as host generalist, host adapted or host restricted (Waldner et al., 2012). *Salmonella* host adapted or restricted serotypes like *Salmonella* Typhoidal (Typhi and Paratyphi A, B, or C) which are common in developing countries, have developed strategies for persisting inside of the host and evading immune defenses (Gal-Mor et al., 2014; Waldner et al., 2012). According to Waldner et al. (2012) approximately up to 6% of the people who have been infected with *Salmonella* Typhi become chronic and asymptomatic carriers. On the other hand, *Salmonella* host generalist or non-typhoidal *Salmonella* that include the majority of the serotypes that cause the most incidence of foodborne diseases worldwide, leads to gastroenteritis, and infected patients shed *Salmonella* for a relatively short period of time (WHO, 2013; Waldner et al., 2012). Furthermore, non-typhoidal *Salmonella* are natural residents of the intestinal tracts of domestic and wild animals, reptiles, and insects (Ray and Bhunia, 2008). As non-typhoidal *Salmonella* live in the gastrointestinal tracts of these asymptomatic carriers, the pathogen may be transmitted through feces to soil and water, thus contaminating food and feed sources, and hence humans and animals (Andino and Hanning, 2014).

Among non-typhoidal serotypes, *Salmonella* Typhimurium and *Salmonella* Enteritidis are the two most commonly laboratory confirmed isolates from human infections (Adams and Moss, 2008; NIAID, 2016; WHO, 2013). Other common serotypes are *S.* Newport, *S.* Javiana, *S.* Heidelberg, *S.* Infantis, and *S.* Virchow (Adams and Moss, 2008; Boore et al., 2015). Furthermore, *Salmonella* Montevideo, Anatum, and Reading have been reported as the most frequent serotypes isolated in bovine lymph nodes (Davis and Russell, 2011; Gragg et. al., 2013). In addition, *S.* Montevideo and *S.* Anatum are in the top list of the National Antimicrobial Resistance Monitoring Program (NARMS) (Ayala et. al., 2013).

Non-typhoidal *Salmonella* Associated with Food

Pokharel et. al., (2016), indicated that *Salmonella* has the ability to survive in food matrices for a long time despite the challenges of pH, heat, acid and other common environments it can encounter. Non-typhoidal *Salmonella* infections (salmonellosis) have been mostly attributed to foods of animal origin, however in the last decade, an increase of non-typhoidal *Salmonella* outbreaks has been observed from vegetables, fruits, and low a_w food products (CDC, 2016). Surveillances concluded that four factors contribute to food borne outbreaks incidence: 1) improperly handled food (temperature, time), 2) environmental contamination, 3) cross-contamination (from food to food, equipment), and 4) worker (hand) contamination (Adams and Moss, 2008; Laufer et. al., 2015). Batz et al., (2012) analyzed non-typhoidal *Salmonella* outbreaks in the United States from 1999 through 2008 and attributed non-typhoidal *Salmonella* outbreaks to twelve types of foods, poultry being the first vehicle of transmission 20.9%, followed by complex foods

18.7%, produce 17.6%, eggs 11.8%, beef 6.9%, pork 6.6%, dairy 5.6%, seafood 5.3%, deli and other meats 2.9%, baked goods 2.6%. and beverages 1.1%.

Specifically, in foods of animal origin, *Salmonella* Enteritidis has been frequently associated with poultry products and eggs, while *Salmonella* Typhimurium has been linked with beef meat products (Clark, n.d; Jackson et al., 2013). In research conducted by Laufer et al., (2015), *S.* Typhimurium and *S.* Newport have been the most common isolates in more than 50% of ground beef outbreaks.

Another finding indicated that in the United States during 1973 – 2011 beef products were associated with 96 (4.9%) of non-typhoidal *Salmonella* outbreaks. Ground beef was attributed to 23% (22/96) of the outbreaks; from those 77% (17/22) occurred between 2002 – 2011 (Laufer et. al., 2015). In the United States, ground beef represents 42% of the beef share market, it is consumed as ground beef granulated, meat balls, and hamburger patties (Davis et al., 2005). The American Meat Institute estimated that in 2014, each American consumed 29.5 pounds of hamburger patties per year (NAMI, 2016).

By USDA definition, when hamburger patties are produced, fat trimmings can be added until a maximum of 30%. This fatty tissue may include deep tissue lymph nodes that are inevitable to remove from the beef carcasses. Different studies have documented that lymph nodes are reservoirs of non-typhoidal *Salmonella* (Li et al., 2015; Gragg et al. (2013); Brichta-Harhay et al. 2012; Arthur et al. 2008). Thus, the incorporation of contaminated lymph nodes represents a potential source of contamination of non-typhoidal *Salmonella* in ground beef products. Li et al. (2015) indicated that subiliac,

iliofemoral, and superficial cervical lymph nodes are frequently included in ground beef. Gragg et al. (2013) analyzed lymph nodes from beef cattle raised in different regions of the United States and Mexico, the results indicated that prevalence of non-typhoidal *Salmonella* was found higher in mesenteric lymph nodes (91.2%), followed by subiliac lymph nodes (76.5%). However, mesenteric lymph nodes usually are removed from the carcasses and do not represent a real hazard to meat products (*3*).

Antimicrobial Interventions in the Meat Industry

Federal agencies, research institutes, and the meat industry have implemented different post-harvest practices and technologies to minimize the microbial contamination in meat products. In a study conducted in 598 abattoirs and meat fabrication facilities in the United States, Cates et al., (2006) found that the two most common carcasses antimicrobial decontamination technologies are organic acid rinse and hot water carcass rinse/wash. Among several organic acids that have been used to reduce microbial contamination, lactic and acetic acid are the most commonly utilized. Scientific literature indicated that the mechanism of the organic acids is not completely understood, their antimicrobial activity has been attributed to the undissociated molecule that facilitate the infiltration on the cell membrane following the accumulation on the cytoplasm of the microorganism (Van Immerseel et al. 2006; Huffman 2002; Hirshfield et al. 1982). Nevertheless, Sofos (2005) mentioned that in Gram-negative bacteria (*Salmonella, E. coli, Campylobacter)*, lactic acid acts as an antimicrobial agent because pH decreases rather than of the undissociated molecule.

Theron and Lues (2007), commented that to have an ideal antimicrobial activity of organic acids on meat products it is important to consider the acid dependence of the organic acid, type of tissue it will be applied on, type of bacteria, slaughter technology, and decontamination technique. Similarly, other studies suggested the multiple bacterial toxicity mechanisms may exist and thus it would explain the differences on the antimicrobial effect on the target microorganism (Sofos, 2005).

Furthermore, Dorsa et al., (1998) concluded the use of organic acids as intervention on beef carcasses may decrease the pathogen presence in ground beef products by the residual effects of the substances. However, based on the fact that some tissue, for instance, lymph nodes, are incorporated in the grinding process during hamburger patty production, a second chemical intervention may be necessary to achieve the safety of the product.

Although the effectiveness of chemical interventions has been well documented, every day new initiatives emerge to improve the safety of the products. For instance, since 2006 the Food Safety Inspection Service (FSIS) has improved the monitoring activities to control non-typhoidal *Salmonella* in ground beef; one of the actions has been the publication of the results from the non-typhoidal analysis in samples from beef meat facilities, which promote the accomplishment of safety practices and reinforce the improvement of the traceability process in the processor facility. In addition, Bosilevac et al., (2009), indicated that incidence of non-typhoidal *Salmonella* in ground beef has decreased by 30% with the implementation of Hazard Analyzes Critical Control Point (HACCP) plans in beef production facilities.

Common Chemical Agents Used in Meat Industry

Lactic acid (LA)

Lactic acid is a non-toxic organic acid with an acid dissociation constant (pKa) of 3.8 and low hydrophobicity, and these properties confer the advantage compared with other organic acids to be highly miscible with water. Lactic acid can be produced by synthetic process or by microbial fermentation (Boomsma et al., 2015; Wee et al., 2006). Lactic acid bacteria, specifically, *Lactobacillus* and *Rhizopus oryzae* fungi, are the most common strain used in industrial production (Mirdamadi et al., 2002).

Lactic acid, like other organic acids, has demonstrated bactericidal and bacteriostatic properties (Theron and Lues, 2007; Hirshfield et al. n.d.). The Food and Drug Administration (FDA) categorized lactic acid as generally recognized as safe (GRAS) to use as antimicrobial agent, flavor enhancer, pH control, among other applications (FDA, 2015). Under the approval of the FSIS, lactic acid is commonly used with a maximum concentration of 5%, during the production of beef products (FSIS, 2015).

Lactic acid exists in two optical isomers (D- and L-); according to Theron and Lues (2010), penetration of cell membrane does not differ between the two isomers. Scientific literature mentioned that L-lactic acid is more effective as antimicrobial on pathogen microorganisms than D-lactic acid (*49, 71*). Based on research studies Theron and Lues (2010) reported that L-lactic acid was more effective on *E. coli* (Gram-negative bacteria) than D-lactic acid, while the latter had better inhibition results on *Listeria monocytogenes* (Gram-positive bacteria). In the same way, Boomsma et al., (2015) indicated the effectiveness of L-lactic acid differs between Gram-negative and Gram-

positive bacteria. On the other hand, a study conducted by Conner et al., (1997) concluded no significant difference in the reduction of *E. coli and L. monocytogenes* when lactic acid (2 and 4%) was applied on beef trim. Furthermore, Castillo et al. (2001) mentioned that the temperature of the lactic acid solution may have an effect in the antimicrobial activity. Additionally, Pokharel et. al., (2016) mentioned the method of application may interfere with the antimicrobial effectiveness of lactic acid in meat products, for instances dip method or spray method.

In the beef industry lactic acid is commonly applied on carcasses at pre and post-evisceration steps, as well as on beef trimming and ground beef to control the presence of *E. coli* O157:H7 and non-typhoidal *Salmonella*. Winkler and Harris (2009), cited *Enterobacteriaceae* and aerobic plate count were reduced up to 1.0 $\log_{10}$ CFU/cm^2 on beef carcasses before evisceration process, moreover, when lactic acid was combined with hot water (74 ºC) treatment, the *Enterobacteriaceae* load reduced in 2.5 $\log_{10}$ CFU/$cm^{2.}$ Harris et al., (2006) achieved a reduction of approximately 2 $\log_{10}$ of *E. coli* O157:H7 and non-typhoidal *Salmonella* by spraying lactic acid on trim surfaces. Moreover, Hughes et al., (2010) concluded that lactic acid was effective reducing Multidrug Resistant non-typhoidal *Salmonella* in ground beef.

Peroxyacetic Acid (PAA)

PAA, also called peracetic acid, is an equilibrium mixture of acetic acid and hydrogen peroxide with a dissociation constant (pKa) of 8.2 (AMS, 2013). Peroxyacetic acid is used in different food industries because its acts at low temperature and does not leave of toxic residues. Moreover, the presence of organic material does not interfere

with its antimicrobial effectiveness (Kitis, 2004; Bauermeister et. al., 2008). The Peroxyacetic acid has antimicrobial properties because it oxidizes the outer cell membrane of bacteria, endospores, yeast and mold spores. Additionally, it helps the block of the enzymatic and transport systems of microorganisms (Vandekinderen et al., 2009). According to Kitis (2004) its antimicrobial activity can be ranked from highest to lowest as follow: bacteria, viruses, bacteria spores, and protozoan cyst.

Researchers showed that the peroxyacetic acid is more effective than chlorine in reducing bacteria in equipment and utensils (Pfuntner, 2011). Peroxyacetic acid is identified in the FSIS Directive 7120.1 to use as a safe ingredient in the meat, poultry and egg products. In beef carcasses, whole cuts, and trims, it can be applied at maximum concentration of 220 ppm (FSIS, 2016). Ellebracht et al. (2005) reported that after peroxyacetic acid dip treatment (200 ppm) on inoculated beef trim there was a reduction of 0.7 $\log_{10}$ CFU/cm^2 in *E. coli* O157:H7 and 1.01 $\log_{10}$ CFU/cm^2 in *Salmonella* Typhimurium. Similarly, Geornaras et al., (2012) found a reduction of 0.5 to 0.7 of *Salmonella* Typhimurium and *Salmonella* Newport in beef trims after a peroxyacetic acid treatment at 200 ppm. Furthermore, a study evaluated the effect of different concentrations of peroxyacetic acid (200, 600, and 1000 ppm) on beef carcasses surface showed that a reduction higher than 1 $\log_{10}$ CFU/cm^2 on *E. coli* O157:H7 and *S.* Typhimurium was only achieved when PAA was applied at 1000 ppm (King et al., 2005).

Acidified Sodium Chlorite (ASC)

It is an aqueous solution obtained by mixing of sodium chlorite with any generally-recognized-as-safe (GRAS) acid component, such as citric acid, sodium sulfate,

phosphoric acid, or hydrochloric acid (Rao, 2007). Acidified sodium chlorite (ASC) was approved by the FDA in 1996 as a secondary additive for use as an antimicrobial in poultry, beef, seafood products, vegetables and fruits. In meat products, ASC may be applied by dipping or by a spay system, in which cases the allowed concentration is between 500 to 1,200 ppm at a pH of 2.5 to 2.9 (21CFR173.325; Bosilevac et al., 2004).

The antimicrobial activity of acidified sodium chlorite is attributed to the conversion into chlorous acid which has an acid dissociation constant (pKa) of 1.8 at 25 ºC. Its effect kills bacteria, fungi, viruses and algae by acting on the cellular membrane (*62*). ASC is commonly used as an integrated agent of multiple hurdle intervention to reduce pathogens in beef carcasses, meat parts, organs, beef trim, and comminuted meat product (21CFR173.325; Rao, 2007)

The literature indicates that the antimicrobial properties of ASC depends on the acid used, the procedures of preparation, the application method, the temperature and the contact time (Echeverry et al. 2010; Rao 2007; Kim et al., 2014). One study reported that when sodium chlorite was activated with citric acid, it was more effective in the reduction of *E. coli* O157:H7 and non-typhoidal *Salmonella* inn fresh beef cuts than when it was acidified with phosphoric acid (Echeverry et al., 2007). Another study by Kim et al. (2014), addressed the differences in the antimicrobial effect of ASC on *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes* and. *S. aureus* related to the preparation method (conventional, acidification or dilution after reaction). The authors concluded that a better antimicrobial effect is achieved when ASC is prepared using a high concentration of the reactants (sodium chlorite and acid component) and is diluted to the final

concentration. In addition, Geornaras et al. (2012) found a reduction of 0.6 $\log_{10}$ CFU/ cm^2 of multidrug resistant *S.* Newport and *S.* Typhimurium and 0.5 $\log_{10}$ CFU/ cm^2 of *E. coli* O157:H7 when beef trims were immersed for 5 s in a solution of ASC at 1000 ppm.

Although multiple studies have evaluated the antimicrobial effects of ASC on beef, there is not enough information about its application and efficacy in ground beef products. Harris et al. (2006) applied acidified sodium chlorite to beef trim (inoculated with *E. coli* O157:H7 and *S.* Typhimurium) prior to grinding. The results indicated a reduction of 0.5 and 0.63 $\log_{10}$ CFU/g in the ground beef respectively after 24 h of treatment application. Similarly, Bosilevac et al. (2004) conducted a study to evaluate the antimicrobial effects of two different concentrations of ASC (300 and 600 ppm) applied to beef trim before grinding to reduce the aerobic plate count (APC) and *Enterobacteriaceae* count (EBC) of ground beef (73/27 lean to fat ratio) stored in chubs at 2 ºC for 20 days. The results indicated a reduction of 1.0 $\log_{10}$ CFU/g in APC and 1.6 $\log_{10}$ CFU/g in EBC when ASC was applied at 600 ppm. On the other hand, when ASC was applied at 300 ppm, similar reduction was observed (0.6 $\log_{10}$ CFU/g in APC and 2.0 $\log_{10}$ CFU/g in EBC).

Effects of acid treatment on quality characteristic in meat products

Chemical interventions are commonly applied in the meat industry to reduce microbial load. Some researchers concluded that although these acids have decontamination effects, they may changes in quality characteristics (color, odor, texture, and appearance) may occur in the meat product (beef, poultry, and seafood). Theron and Lues (2007) argued that when lactic or acetic acid are applied to beef carcasses, changes

in color and odor are minimal or may not happen. However, when the acid solutions are applied directly to meat cuts, irreversible changes in the appearance can occur. Quilo et al. (2009) cited that meat color is a critical factor in the purchasing decision of the consumer. Moreover, they indicated that changes in color can result in redder and more color stable or in a discoloration of the meat.

Changes in the quality properties on beef products can be determined by objective and subjective measurements. Objective tests include instrumental analyses, which measure one particular attribute of a product. Objective measures are repeatable and not subject to human variation. They must be correlated with sensory analysis in order to provide an overall evaluation of the product. Conversely, subjective measures may utilize more than one test. Usually, those tests are analytical (descriptive and discriminative) and are performed by trained panels, and affective test are conducted by non-trained consumer panel (Birwal et al., 2015).

Color measurement is one of the most common attributed assessed when chemical intervention on beef products are evaluated. Mohan et al. (2012) evaluated the effect of peroxyacetic (200 ppm) and other acids (octanoic, malic, and potassium lactate) applied on beef trim to produce ground beef and concluded the color characteristic was not affected by the chemical treatments. Usually, color is measured using a colorimeter device, Bauermeister et al. (2008) cited when colorimeter is used some limitations can occur, especially when opaque material is measured, in that, much of the light is either transmitted or absorbed versus reflected to the detector. Color values are commonly expressed in the Hunter Lab scale, where L indicated the level of lightness or darkness

with a range of value of 100 (light) to 0 (dark). The values of a and b do not have any numerical limit, a values are related to the redness (positive values) or greenness (negative values), and the b values which indicated the yellowness (positive values) and blueness (negative values) of the product (Hunt et al., 2012).

From the subjective measurements, triangle test is commonly used as a discriminative method to identify whether a sensory difference exists between two products. According to (Meilgaard et al., 2007) this technique is useful to evaluate the consumer sensory perception of meat products which have received a chemical intervention that may produce changes in the product that cannot be evaluated by one or two attributes. Harris et al. (2012) concluded that panelists of a triangle test did not identify any organoleptic differences on ground beef treated with acetic acid, lactic acid, or acidified sodium chlorite.

Bacteria Stress Condition

In general, stress is a condition caused by affecting at least one of the basic requirements that bacteria need to grow and survive; the affection can be caused by physical (heat, pressure, or osmotic shock), chemical (acids or detergents) or biological (bacteriocins) factors. Altered environments may cause damage in the cellular structures of bacteria, such as the cell wall, cell membrane, proteins, RNA and DNA (Begley and Hill 2015). When encountered with stressful conditions bacteria express specific genes in response of the affection of its surrounding environment. (Wesche et al., 2009). Begley et al. (2015) enumerated eleven environmental conditions that may induce stress responses in foodborne pathogens, those are: low and high temperature, low pH, osmolarity,

starvation, oxidative stress, drying, high hydrostatic pressure, radiation, chemical sanitizers, and preservatives.

Ray (2008) indicated that depending on the time of exposure to stressful events and the severity of those, the bacteria can present three different independent states: sublethal injury, viable but non culturable state, and stress adaptation.

Sublethal Injured cells

Sublethal injury of bacteria refers to damage in structure within the cells which may result in the loss of essential function, it may be transient or permanent (Wesche et al., 2009). Ray (2008) mentioned two states may be observed in injured bacterial cells: 1) reversibly injured, cells that present an inflammatory or disrupted condition. The level of these abnormal conditions differ between cells, if the environment does not become less hostile, bacteria will eventually die. However, if the cell is able to recover, it will behave as a normal cell including the ability to multiply. 2) Irreversible injured cells, these are unable to survive the stressful conditions in the environment and die immediately. Independently of the type of injury, Wu (2008), cited that the bacterial cell wall damage is more observed after freezing or drying exposure, and DNA and rRNA damage occurs by high heat conditions.

Viable but Non Culturable Cells

Viable but non culturable (VNC) cells are those that lose the ability to multiply. According with Ramamurthy et al. (2014), this state is an exceptional stress response mechanism that bacteria possess. In addition, Monteville et al. (2012) cited that a rod

shaped bacteria changes its morphological characteristics, becoming spherical bodies. Moreover, despite that they cannot be cultured, it is possible to show its viability through cytological methods.

Stress Adaptation

It is the ability of bacteria to resist altered environmental conditions. Ray (2008) defined stress adaptation as the process where the bacteria is exposed to an unfavorable environment and becomes resistant in order to survive. During stress adaptation many physiological changes occur in the bacteria such as protein regulation, modification of the cell membrane composition, and alteration in morphology. Besides physiological changes, stress adaptation can induce modifications in bacteria's pathways of colonization and virulence. Furthermore, adaptation process may lead to cross-protection or cross-adaptation, this phenomenon is an attribute that bacteria acquire of adapting to a second stressful condition once exposed and adapted to a first stressful condition. (Leyer and Johnson 1993; Dubois-Brissonnet, 2012).

Two of the most stress adaptations that have been studied are the heat and acid adaptations. These two factors have been implemented as main interventions in food processing. According to Ray (2008), depending on the time of exposure of bacteria to low pH, the following events can occur: 1) Acid resistance which involves long time exposure to mild pH (5.0 – 5.8), this resistance enhances the ability of bacteria to survive strong acid conditions at pH < 2.5. 2) Acid tolerance which is the resistance to short-term exposure of bacteria to mild pH. This tolerance enables bacteria to survive at pH between 2.4 to 4.0. 3) Acid shock which is the response of bacteria to low pH without previous

interaction. These mechanisms of defense need to be considered at the moment of organic or inorganic acid intervention in food processing to guarantee the success in killing foodborne pathogens. Moreover, Sharma et al. (2005) specified that acid adaptation or resistance may lead to enhanced bacterial thermal tolerance. However, Alvarez et al. (2008) stated that thermal tolerance is still not well understood, the authors addressed that high presence of fat may be another factor related with thermal stress response. In addition, Foster et al. (1995), mentioned that cells exposed to heat shock may develop a cross-protection to heat, oxidative and osmotic stress.

Shen and Fang (2012), stated that bacteria have different pathways to survive stress conditions, these are: alternative sigma factors, two components regulatory system, and transcriptional regulator system. Begley et al. (2015) declared that the ability of bacteria to survive stress factors varies among strains. In this review, the alternative sigma factor approach is addressed.

Alternative Sigma Factor

Sigma factors (σ-factors) are polypeptide subunits of RNA polymerase that are essential in the initiation of transcription in the process of RNA synthesis during DNA template strand (Kazmierczak et al. 2005). Bacterial sigma factors are involved in different biochemical pathways such as core binding, activation of promoter recognition, DNA melting, and inhibition of nonspecific transcription (Helmann and Chamberlin, 1988). Wösten (1998), indicated that bacteria synthesize several different sigma factors in response to altered environment. These sigma factors give the opportunity to the bacteria to maintain basal gene expression and the regulation of specific gene expression.

McMeechan et al. (2007), cited that *Salmonella* Typhimurium requires specific and coordinated responses in order to survive environmental stress.

According to their identification, characterization and sequence analysis, sigma factors can be grouped in two families: sigma factor 70 and sigma factor 54 (Paget 2015; Wösten 1998; Lonetto et al.,1992). Sigma factor 70 is larger and more diverse than sigma factor 54. Sigma factor 70 (σ^{70}) is responsible for transcription during the bacteria's growth phase, while sigma factor 54 (σ^{54} or σ^{N}) is involved in the transcription process during environmental signals, and requires the input of enhancer proteins and ATP hydrolysis to drive DNA melting (Paget, 2015). Based on the gene structure and function sigma factor 70 is divided into four phylogenetic groups (G1, G2, G3, and G4). The structure size among groups goes from a range of 70 kDa to 20 kDA. Group 1 is associated with the transcription of genes that allow the growth and metabolism of the bacteria. In Group 2, the produced proteins are related to the primary sigma factor but are expendable for bacterial growth. In group 3, sigma factors are activated in response to a specific signal, also it can be divided into several clusters with roles in sporulation, flagella biosynthesis or heat shock response. The last group (4), is involved in extracytoplasmic function (Paget and Helmann 2003).

Bagn et al. (2005) mentioned that the mechanism of action of alternative sigma factors is different between Gram-positive and Gram-negative bacteria, in the first, the interaction is recognized as hierarchical behavior, in the latter it is activated by direct expression. Different alternative sigma factors have been identified during stressful conditions to preserve cellular homeostasis. In addition, sigma factors have been related

to the virulence pathways of the bacteria. Five different alternative sigma factors (sigma E (σ^{24}), sigma F (σ^{28}), sigma H (σ^{32}), sigma S (σ^{38}) and sigma N (σ^{54})) are involved in stress condition during growth phase (Shen and Fang, 2012). In this review the alternative sigma factor *rpoS* and *rpoH* are addressed.

Alternative Sigma Factor *rpoS*

The sigma factor S (*rpoS*) also known as katF sigma 38 which belong to the group 2 of sigma factors, is required for maximal survival during stationary phase of enteric pathogens (Wong et al., 2014; Paget, 2015). Chen et al. (1995), cited that the sequence of the *rpoS* is 99% identical in *S.* Typhimurium and *E. coli*. Monot & Le (2014) mentioned that the *rpoS* helps to the biofilm formation and virulence of *Salmonella* Typhimurium. Wesche et al. (2009), indicated that the *rpoS* controls more than 50 genes in the general stress response of *E. coli*. The expression and activation of the sigma factor S are highly complex and occur at the transcriptional, post-transcriptional, translational and post-translational level (McMeechan et al., 2007).

In a study conducted by Bang et al., (2005) concluded that maximal expression of the alternative sigma factor S involved the interaction with alternative sigma factor E. Moreover, the authors indicated that both sigma factor S and sigma factor E are essential for *Salmonella* virulence. The sigma factor E which is activated by the presence of unfolded proteins in the cell envelope is considered as minor sigma factor that participates in the response to heat shock and other stresses that occurred in the membrane and periplasmic protein (Ades et al., 2003; Bang et al., 2005).

Alternative Sigma Factor *rpoH*

The *rpoH o*r sigma factor 32 (ϭ32) which belongs to the group 3 of sigma factors, is required to the response of bacteria to heat stress conditions. It is activated by unfolded cytoplasmic proteins (Wösten, 1998; Bang et al., 2005). It has been recognized that the *rpoH* reach higher expression during the stationary phase. According to Wösten (1998), the concentration of the *rpoH* is low because of its short life-time (less than 1 min), this sensitivity is due to the interaction of the DnaK chaperone. Furthermore, the sigma factor 32 (*rpoH*) is transcribed from four promoters that belong to the sigma factors D and E (Shen and Fang 2012). Foster et al. (1995) concluded that in addition to the *rpoH* some others promoters are involved in bacteria heat tolerance, those are P1, P4, and P5, moreover, the induction htrA (periplasmic protease) is required to overcome high heat exposure. Furthermore, Perlin (2012) stated that the *rpoH* manages the transcriptions of genes that induce the removal of misfolded protein into the cytoplasm. In addition, the authors stated that the most important function of this sigma factor (ϭ32) is to mediate the regulation of sigma factors S and E.

CHAPTER III

REDUCTION OF *SALMONELLA* EMBEDDED IN LYMPH NODES DURING GRINDING USING LACTIC ACID AND PERACETIC ACID TREATMENTS ON BEEF TRIM, COMBINED WITH ACIDIFIED SODIUM CHLORITE

Introduction

Non-typhoidal *Salmonella* is one of the foodborne pathogens that cause a vast number of morbidity and mortality worldwide (Mani-López et al., 2012; Coburn et al. 2007). In the United States salmonellosis is the leading cause of hospitalization (35%, 19,000 cases) and deaths (28%, 378) related to foodborne illnesses (Hoffmann et al., 2015).

The consumption of raw or improperly cooked foods of animal origin like eggs, poultry, meat, and milk is commonly associated with non-typhoidal *Salmonella* infection (Anon 2016; Majowicz et al., 2010). In a study conducted in 2009, non-typhoidal *Salmonella* was recovered in 4.2% of the 4,136 raw ground beef samples tested from commercial ground beef producers (Bosilevac et al., 2009). In 2014, the USDA FSIS reported that 1.6% of the 7,314 raw ground beef samples tested were positive for non-typhoidal *Salmonella*. Typically, ground beef is obtained by grinding the less tender cuts and/or higher fat portions of the beef carcass. Also, adipose trim alone containing lymph nodes can be included in the product if it is labeled as hamburger and adipose trim

containing small amounts of lean can be added to ground beef. Gragg et al. (2013), reported that non-typhoidal *Salmonella* has the ability to invade and survive within lymphoid tissue. When the pathogen is embedded in lymph nodes it is protected from post-harvest interventions applied onto the carcass surface. Hence, the non-typhoidal *Salmonella* can be a source of contamination for beef trim and further be incorporated in the ground beef product (Arthur et. al., 2008).

Different technologies, and treatments with approved chemical agents have been implemented in slaughter and meat processor facilities to reduce or eliminate the presence pathogens on beef carcasses, subprimal cuts and beef trim (Ellebracht et al. 2005; Smulders and Greer, 1998). However, these treatments are not commonly applied in ground beef facilities. Mohan et al. (2012), suggested that antimicrobial interventions will be more effective in reducing bacterial population if it is applied to the final product.

Chemical interventions to reduce pathogens in meat products include the use of organic acids as a single intervention or combined with other oxidizing agents. Acid solutions inhibit microbial activity by the disruption of the cell membrane, thus altering the intracellular pH homeostasis (Mani-López et al., 2012; Hirshfield et al., 2004). Despite the lethal action of chemical interventions, bacteria can survive the acid stress condition and other environmental alterations by the expression of alternative sigma factors encoded by *rpoS, rpoH,* and *dnaK* housekeeping genes (Bang et al., 2005).

Although chemical interventions have been effective for improving beef safety, they can impact the organoleptic properties of the meat product. Pohlman et al. (2007),

addressed that changing color, flavor and texture can lead to declined consumer demand of ground beef.

The objectives of this study were as follows: 1) Determine the efficacy of lactic acid (4.5 %) and peracetic acid (220 ppm) applied to trim in combination with acidified sodium chlorite (1000 ppm) during grinding to reduce *Salmonella* embedded in lymph nodes that are incorporated into ground beef during processing. 2) Conduct sensory analysis to evaluate the impact of the chemical interventions on organoleptic properties of ground beef. 3) Evaluate the induction of a possible stress response on *Salmonella* Montevideo to the chemical intervention.

We hypothesized the chemical intervention with organic acid combined with acidified sodium chlorite as secondary intervention during grinding process will reduce *Salmonella* introduced through contaminated lymph nodes on ground beef products. In addition, differences in flavor, color, and texture among treated samples are not expected. Finally, differences in expression of housekeeping genes could provide insight into how *Salmonella* Montevideo adapt and survive in the presence of chemical intervention tested.

Materials and methods

Bacterial strain selection

S*almonella* serovars associated with human illnesses (Typhimurium, Enteritidis, and Newport) and the serovars isolated from cattle lymph nodes (Montevideo, Anatum, and Reading) were used in this study. In order to measure the "worst-case scenario" for pathogen survival, four strains of each serovar (Table 1) were tested to select strains that were the most resistant to lactic acid (LA), peracetic acid (PAA), acidified sodium

chlorite (ASC), and the combination of LA+ASC, and PAA+ASC. Each strain was grown in a solution (50%) of Brain Heart Infusion (BHI, Becton, Dickinson and Company) mixed with 50% of the acid solutions at the specified concentrations (LA 4.5%, PAA 220 ppm, ASC 1000 ppm), and incubated at 10 °C in 96-well plates for 1, 24 and 72 hours. Serial dilutions were plated with an automated spiral plater (Autoplate® 4000, Spiral Biotech, Norwood, MA) onto BHI agar to determine the surviving populations at each period of incubation (1, 24 and 72 hours). The most resistant strain of each serovar was individually adapted to be rifampicin-resistant by growing them in BHI supplemented with 100 µg/ml of rifampicin (Sigma-Aldrich) and incubating at 37 °C for 12 hours. After incubation, 1 ml was transferred to 9 ml of fresh BHI + rifampicin media and this step was repeated for 8 consecutive periods until resistance was obtained. Resistant colonies were frozen into stock cultures for future use.

Inoculum preparation

A colony from each frozen *Salmonella* rifampicin-resistant culture was activated with two consecutive passes into 9 ml of BHI and incubated at 37 °C for 12 -18 hours. After incubation, cells of each culture were harvested by centrifugation at 5,000 rpm for 10 min at 4 °C (AG 5804R, Eppendorf, Hamburg, Germany). Cell pellets were re-suspended in BHI. An equal volume of the six cultures was mixed to obtain two cocktails of 9 log CFU/ml and 6 log CFU/ml as our base cocktail.

Sample preparation and inoculation

A combination of beef trim (14 kg) and cattle subiliac lymph nodes (14 nodes) was prepared as the original sample to be inoculated. The trim was obtained from a local beef fabrication plant and the lymph nodes were collected in a local slaughter facility. Lymph nodes were selected and trimmed to a standardized weight of 100 g/node. Two different concentrations (high and low) of *Salmonella* were used in this study. The rifampicin resistant *Salmonella* cocktail was injected in the lymph nodes reaching a final concentration in the trim/node mixture of 4.8 log CFU/g (high) and 2.3 log CFU/g (low). The inoculated mixture was separated into seven equal portions to be individually subjected to six different treatments (described below) while one portion was used as a control.

Chemical treatments

Solutions of lactic acid (LA 4.5%) (Corbion Purac, East Rutherford, NJ), peracetic acid (PAA 220 ppm) (Craft-Chem Inc, Lawrenceville, GA), and acidified sodium chlorite (ASC 1000 ppm) (Zee Company, Chattanooga, TN) were prepared following the manufacturers' instructions. The pH of the ASC was measured to ensure a range of 2.5 – 2-9 to be in accordance with the 21CFR173.25. Six treatments were individually evaluated to determine pathogen reductions as follows:

1) Lactic acid applied to trim only (LA)

2) Peracetic acid applied to trim only (PAA)

3) Acidified sodium chlorite applied during fine grinding only (ASC)

4) Lactic acid applied to trim + acidified sodium chlorite applied during fine grinding (LA+ASC)

5) Peracetic acid applied to trim + acidified sodium chlorite applied during fine grinding, and (PAA+ASC)

6) Water applied to trim only.

An untreated portion of the inoculated trim/node mixture was used as a control. Lactic acid, peracetic acid, and water treatments were separately applied to the trim/node mixture using a commercial six-nozzle trim sanitizing spray cabinet with a conveyor belt system (series 800, Intralox Inc., Harahan, LA) at room temperature. The acidified sodium chlorite was prepared with water at room temperature and applied using a manual spray bottle in a posterior step described below. After each intervention, the sample was coarsely ground using a meat grinder (grinder disc 32 x 3/8 in). The coarse ground product was then fine ground (grinder disc 32 x 3/16 in) and the solution of solution of acidified sodium chlorite (1000 ppm) was applied as the meat was leaving the grinder. To improve the distribution of the ASC on the meat, a manual mixer (Weston, model 36-2001-w) was used to homogenize (30 sec) the ground beef and the chemical solution. The process of grinding was done in a room held at 8 – 10 °C simulating industrial conditions. All of the processing equipment was cleaned and sanitized between treatments.

Equal portions of the treated ground beef were made and sealed into vacuum packaging using a vacuum system. Samples were stored at 2 – 4 °C in the dark for three days (72 hours). All the treatments were replicated three times in the Texas Tech University BSL II Pathogen Processing Laboratory.

Microbial Analysis

The effect of each chemical intervention was determined by analyzing samples over time (1, 24, and 72 hours). More specifically, three samples for each treatment on each sampling day were weighed (25 g) and placed into a filter bag (VWR, model 11216-515). Buffered Peptone Water (BPW, Becton, Dickinson and Company) was added (225 ml) and processed in a stomacher (Model 400 circulator, Seward, West Sussex, United Kingdom) at 250 rpm for 2 min. From the homogenized bag, serial dilutions were made and plated onto *Salmonella*-selective agar (Xylose Lysine Deoxycholate. Becton, Dickinson Co., Sparks, MD) overlaid with a layer of Tryptic Soy Agar (TSA; Merck KGaA, Darmstadt, Germany) both media were supplemented with rifampicin solution (100 µg/ml). Plates were incubated for 24 ± 2 hours at 37 °C.

Sensory Analysis

Ground beef was obtained in the same manner described in the chemical treatment methodology with the exception that subiliac lymph nodes were not included, hence none of the trim was inoculated with *Salmonella* cocktail. The sample preparation and the sensory evaluation were conducted in the Texas Tech University Gordon W. Davis Meat Science Laboratory (a separate facility from the Texas Tech University BSL II Pathogen Processing Laboratory). An approval from the Texas Tech University Institutional Review Board was obtained to conduct the sensory evaluation using the triangle test methodology.

From each of the six individual treated beef trim portion, ground beef was formed into 1/3-pound patties using a manual beef patty maker. Ground beef patties were

packaged and labeled with a random three-digit number according to chemical treatment, then were refrigerated at 4 ºC until sensory evaluation (24 and 72 hours). Raw beef patties were cooked on non-stick electric grills (George Foreman, Wilkes Barre, PA) to an internal temperature of 74 ºC, a thermocouple thermometer (Cole-Parmer Instrument Company, Vernon Hills, IL) was used to monitor the temperature. Immediately after cooking, each patty was cut into eight pieces and held in a food warmer prior to sensory evaluation.

Beef patty samples from the five chemical treatments and water treatment as control were placed on pre-numbered and tri-sectioned Styrofoam plates and served under red lights to mask any color differences. Each section of the plates was labeled with the random three-digit number corresponding to treatment to evaluate. Panelist were provided a pre-numbered answer form corresponding to the panelist number and sample numbers.

A non-trained panel (n = 33) was served five rounds of three samples, each round corresponding one chemical treatment and water treatment as a control. Following the principle of the triangle test (Meilgaard et al., 2007) panelists were asked to determine the different sample among the three presented samples and record their answer in the form provided. If the panelist could not discern a difference among samples, he or she was asked to guess. Water and cracker were served as a palate cleanser among samples.

Additionally, raw beef patty samples from each treatment were compared with raw beef patties treated with water as control to determine color change over three periods of time (1, 24, and 72 hours). Specifically, six beef patty samples from each

chemical treatment (LA 4.5%, PAA 220 ppm, ASC 1000 ppm, LA + ASC, PAA + ASC) and six beef patty samples treated with water as control were placed separately on Styrofoam tray and sealed with Reynolds 914, PVC wrap film (Richmond, VA) and stored at 4 ºC for the mentioned period of times. The average of three random readings on each beef patty took using a spectrophotometer with D_{65} primary illuminant (CM-600d, Konica Minolta, Inc. Japan) was used to obtain L* (luminance), a* (redness), and b* (yellowness) values. These attributes were used to determine the color differences among the chemical treatments (*38*).

Genetic Analysis to Evaluate the Amount of Stress Response

From all the serovars used in this study only *Salmonella* Montevideo was used for the stress response analysis because it is an emergent serovars that has been found associated with *Salmonella* contamination in cattle lymph nodes.

The genetic analysis of the stress response was evaluated on *Salmonella* Montevideo 11TTU382B rifampicin resistant by quantifying the expression level of two housekeeping genes (*rpoS* and *dnaK*) after different exposure times to acid solutions (LA, PAA, and ASC). From the frozen stock culture of the mentioned strain, a loop (1 µl) was streaked onto TSA and incubated at 37 °C for 18 -24 hours. An isolated colony was subcultured into a test tube containing 9 ml of Tryptic Soy Broth (TSB; Merck KGaA, Darmstadt, Germany) and incubated at 37 °C with shaking at 130 rpm for 12 -18 hours (Eppendorf™ Scientific, Excella™ E 24, USA). From the overnight culture, 500 µl were transferred to 50 ml of fresh TSB and incubated at 37 °C with shaking at 130 rpm for 12 - 18 hours (Eppendorf™ Scientific, Excella™ E 24, USA). Aliquots of 9 ml from the

culture were transferred to three conical tubes (Falcon™, 15 ml conical centrifuge tubes) containing lactic acid (4.5%), peracetic acid 220 ppm, and acidified sodium chlorite 1000 ppm, respectively.

From each tube of the mixture (culture and treatment) 1 ml was collected into micro-centrifuge tubes (VWR, Radnor, PA) at 5, 15 and 30 minutes. Immediately after each harvest time the 1 ml aliquot was centrifuge at 12,000 rpm by 5 minutes at 4 °C (AG 5804R, Eppendorf, Hamburg, Germany), the supernatant was decanted and the pellet was resuspended in 300 µl of RNAprotect® Cell Reagent (Qiagen, Venlo, The Netherlands). RNA extraction was conducted using the TRIzol® Max™ Bacterial RNA Isolation kit (Thermo Fisher Scientific, USA) with modifications. RNA samples weren quantified using the Nanodrop 2000c Spectrophotometer (Thermo Scientific, USA) and stored at -80 °C.

Expression level of the genes *rpoS* and *dnak* was quantitatively determined by real-time reverse transcription PCR (qRT-PCR) in Mx3005p qPCR System (Agilent Technologies, Santa Clara, CA) with the following thermal conditions: normal two step program with annealing temperature of 60 °C for 50 cycles with 2 plateaus (30 minutes 50 °C and 10 minutes 95 °C). Forward (F) and reverse (R) primers, and probe (P) (Table 2) for each of the genes were design using sequence from the genBank website. Final primer sequences were processed on the NCBI website in order to determine cross-reactivity with other species of bacteria. The reactions were performed using the Brilliant II QRT-PCR 1-step master mix (Agilent Technologies, Santa Clara, CA). Relative

quantification method ($2^{-\Delta\Delta CT}$) was used to describe the change in expression level of the target genes compared with the control gene *ttrC*.

Statistical Analysis

Linear model followed by a pairwise comparison test was conducted using Procedures of R (v.3.3.2) to determine the effects of the acid treatments in *Salmonella* population and the effect of the acid in the expression level of the housekeeping genes. Changes in the sensory characteristic of beef patties, and differences in the genes expression. *P*-values < 0.05 were considered statistically significant different.

Results and discussion

Microbiological

The six non-typhoidal *Salmonella* serovars used in this study were selected based on the clinical significance associated with beef outbreaks reported by Jackson et al., 2013 (Typhimurium, Enteritidis, and Newport) and those most frequently isolated from subiliac lymph nodes reported by Gragg et al., 2013 (Montevideo, Anatum, and Reading).

All treatments (water, lactic acid 4.5%, peracetic acid 220 ppm, acidified sodium chlorite 1000 ppm, lactic acid + acidified sodium chlorite, and peracetic acid + acidified sodium chlorite) significantly reduced rifampicin-resistant non-typhoidal *Salmonella* (Figures 1 to 6) introduced through contaminated lymph nodes on ground beef products ($P < 0.05$) compared to the control but the reductions were less than 1 log cycle.

Efficacy of chemical treatments at high inoculum concentrations

When *Salmonella* was present at high concentrations (4.8 $\log_{10}$ CFU/g), LA + ASC, PAA + ASC, and ASC alone reduced rifampicin-resistant non-typhoidal *Salmonella* significantly ($P < 0.05$) by 0.59, 0.47, 0.50 log cycles after 1 hour and by 0.67, 0.51, 0.59 log cycles after 72 hours, respectively (Table 3). Reductions were significantly greater than the water treatment. The reduction at 1 hour with PAA alone treatment was not greater than the treatment with lactic acid alone on trim. After 72 hours, the LA treatment alone applied to trim reduced the pathogen significantly less than the ASC alone treatment applied at fine grinding and the LA+ASC treatment which resulted in more reductions after 72 hours. The PAA + ASC treatment did not statistically differ from the acidified sodium chlorite treatment alone at any sampling point ($P = 0.23$).

Efficacy of chemical treatment at low inoculum concentration

Similarly, when rifampicin-resistant non-typhoidal *Salmonella* populations was at 2.3 $\log_{10}$ CFU/g on the ground beef, LA + ASC, PAA + ASC, and ASC alone showed similar reductions of 0.66, 0.42, 0.52 log cycles and 0.76, 0.51 and 0.64 log cycles after 1 and 72 hours, respectively (Table 4). Similarly, to the results of the efficacy of the chemical treatments at high inoculum level, the reductions were greater than the ones observed by treating with water alone. The lactic acid treatment to trim only resulted in a 0.40 and 0.52 log cycle reduction after 1 and 72 hours, respectively and it was not statistically different ($P = 0.90$) from the reductions observed in the combined PAA+ASC treatments. Overall, after water treatment, PAA showed less reduction of the pathogen when comparing to the others treatments.

A limited number of studies have been conducted to evaluate the effectiveness of acid organic interventions combined with other acids during the grinding process to reduce food pathogens on ground beef products. Most of the chemical treatments are applied individually to beef carcasses and trim prior to grinding. In a study conducted by Harris et al. (2006), concluded that when acids organics (lactic and acetic) and acidified sodium chlorite (1000 ppm) were applied on beef trims prior to grinding, *Salmonella* Typhimurium was reduced by 1.0 to 1.5 log cycles in the ground beef product just after grinding; the acidified sodium chlorite did not show a significant reduction in the pathogen population. However, after 24 hours of storage, there were no a significant difference among treatments. Quilo et al. (2010), concluded that when peracetic acid (200 ppm) followed by 3% of potassium lactate were applied to beef trim reduce 0.76 CFU/g of *Salmonella* Typhimurium in ground beef. On the other hand, Mohan et al. (2012), reported a reduction of 3.4 log (CFU/g) of *Salmonella* in ground beef when trimmings were treated with peracetic acid at 200 ppm after grinding process. These data indicate that during grinding, the embedded Salmonella can be reduced to lower concentrations but probably not eliminated depending on the concentration of the *Salmonella* in the lymph node.

Sensory evaluation

A significance level of $P < 0.05$ was stablished to determine the correct responses in the sensory evaluation. With 33 panelists, the minimum number of correct responses was established at 17 to show a statistically significant result (Meilgaard et al., 2007). In

the triangle test, a correct response is defined by correctly identifying the different sample (Meilgaard et al., 2007).

Panelists detected a significant difference ($P < 0.05$) in beef patties between the control and the lactic acid + acidified sodium chlorite, peracetic acid 220 ppm, and acidified sodium chlorite 1000 ppm within treatment after 24 and 72 hours. Panelists were unable to identify a significant difference ($P > 0.05$) between control, lactic acid 4.5%, and peracetic acid + acidified sodium chlorite beef patties samples at 24 and 72 hours (Table 5).

Other studies have evaluated the effects of chemical treatments applied to beef trim before grinding, and the results indicated that panelists could not detect a significant difference in the sensory attributes of the ground beef product. Harris et al. (2012), reported no significant differences between control beef patties and those treated with 5% of lactic acid and acidified sodium chlorite at 1000 ppm. In the same way, Quilo et al. (2009) concluded that beef patties treated alone with peracetic acid (200 ppm) or acidified sodium chlorite (1000 ppm) were similar to the control. Bosilevac et al. (2004), compared the effect on organoleptic properties of cooked ground beef product when it was produced from beef trim treated with ASC at 300 and 600 ppm; samples treated with 600 ppm were significantly different from the control, and the samples treated with 300 ppm of the acid. Also, the study concluded that samples treated with ASC at 300 ppm had a typical or better flavor compared to the control, this characteristic was observed after 8 and 10 days of the chemical treatment.

While some treatments did not cause a change in organoleptic properties, some did. Further and more detailed sensory analysis should be conducted to determine if the sensory changes can be overcome by adjusting the treatments.

Instrumental color measurements

The effect of the all antimicrobial treatments on L* a* and b* values is summarized in Table 6. There were statistically significant differences ($P < 0.05$) for L* (luminance) values between the control and lactic acid 4.5%, peracetic acid (220 ppm), peracetic acid + acidified sodium chlorite and ASC alone (1000 ppm) at the three sampling points (1, 24 and 72 hours). Beef patties treated with peracetic acid and acidified sodium chlorite alone were lighter in color compared to the control. Beef patties samples treated with lactic acid + acidified sodium chlorite did not show a statistically significant difference ($P = 0.09$) when comparing to the control at 1, 24, and 72 hours.

The analysis of a* (redness) values indicated a statistically significant difference ($P < 0.05$) between control beef patties and lactic acid 4.5%, lactic acid + acidified sodium chlorite, peracetic acid (220 ppm), and peracetic acid + acidified sodium chlorite. There was no a statistically difference ($P = 0.36$) between the control and acidified sodium chlorite samples. All the treated samples and the control became less red through the time (Table 7).

The b* (yellowness) values showed in the Table 8, indicate no statistically significant differences when comparing the control beef patties with lactic acid + acidified sodium chlorite ($P = 0.18$), peracetic acid ($P = 0.70$), peracetic acid + acidified sodium chlorite ($P = 0.65$) and acidified sodium chlorite ($P = 0.65$). Beef patties treated

with lactic acid had a slightly significant difference with control beef patties treated with lactic acid ($P = 0.045$).

Similarly, Jimenez-Villarreal et al. (2003) concluded that when beef trimmings were treated with 2% of lactic acid, the ground beef product was lighter and less red compared to the control. On the other hand, Quilo et al. (2009) reported, that when peracetic acid (200 ppm), acidified sodium chlorite (1000) and other acids (potassium lactate, sodium metasilicate) were applied separately to beef trim prior grinding, the ground beef from trimming treated with the peracetic acid (200) showed the highest L* value (lightest color) compared to the control and the other treatments. The acidified sodium chlorite treatment showed similar L* values to the other treatments, but was lower than the control. Regardless of the a* and b* values, samples treated with peracetic acid did not significantly differ from the redness and yellowness of the samples treated with acidified sodium chlorite. However, they were significant different compared to the control.

Stress response

The relative expression of the housekeeping genes *rpoS* and *dnaK* on *Salmonella* Montevideo 11TTU382B are presented in figures 7 to 12. All the acids (lactic acid 4.5%, peracetic acid 220 ppm, and acidified sodium chlorite 1000 ppm) increased at least 0.5-fold in the expression level of the housekeeping genes on *S.* Montevideo compared to the control.

When *S.* Montevideo was exposed for 5, 15 and 30 minutes to acid shock using lactic acid (4.5%) the *rpoS* showed an induction of 0.886, 1.533, and 6.374-fold

respectively. There was a significant difference ($P = 0.0009$) between the level expression at 5 min compared to the expression level at 30 min. On the other hand, when *S.* Montevideo was under acid stress condition due to the acid shock of the peracetic acid (220) the highest fold expression (3.344-fold) of the *rpoS* gene was observed at 15 minutes. A significant difference ($P < 0.05$) in the expression level was observed between 15 and 30 minutes. The latter time of exposure presented a decrease to 1.445-fold in the expression level of the *rpoS* gene. Similarly, when *S.* Montevideo was exposed to acidified sodium chlorite the highest fold change was at 15 minutes. However, there was not a significant difference ($P > 0.05$) among the expression level of the gene at the three different times of exposure (5, 15, and 30 min).

The results of the expression level of the *dnaK* gene exhibited a maximum of 3.310-fold independently of acid and time of exposure. The fold induction of the *dnaK* gene due to the lactic acid was 0.890, 0.774 and 3.310-fold at 5, 15 and 30 minutes respectively. A significant difference ($P = 0.045$) was observed between 5 and 30 minutes. The effect of the peracetic acid (220 ppm) in the induction of the expression level of *dnaK* on *S.* Montevideo was 1.830, 0.441, and 3.129 at 5, 15 and 30 minutes respectively. A significant difference ($P = 0.021$) was observed in the expression level at 15 minutes compared to the results at 5 and 30 minutes. The *dnaK* gene was highly induced (2.590-fold) at 5 minutes when acidified sodium chlorite was used. However, after that time a decrease to 0.5000-fold occurred. In a similar way as the other acids (LA, and PAA) the expression level at 30 minutes showed an increase to 3.180-fold in

the expression level of the *dnaK* gene when the acid stress was due to the acidified sodium chlorite.

CHAPTER IV

CONCLUSIONS

The data from this study indicate that all antimicrobial interventions of lactic acid (4.5%), peracetic acid (220 ppm), acidified sodium chlorite (1000 ppm), lactic acid + acidified sodium chlorite, peracetic acid + acidified sodium chlorite, and water during grinding can reduce non-typhoidal *Salmonella* embedded in lymph nodes in ground beef products under certain conditions. In both scenarios, low and high non-typhoidal *Salmonella* concentrations, the intervention of lactic acid applied on trim and a combination of acidified sodium chlorite applied during grinding was the most effective treatment consistently at all time point.

Data were variable in that the peracetic acid + acidified sodium chlorite was equivalent to the lactic acid when non-typhoidal *Salmonella* level was low but when non-typhoidal *Salmonella* level was high, the peracetic acid + acidified sodium chlorite was equivalent to the acidified sodium chlorite. Comparing the effects when LA, PAA and ASC were applied alone, the acidified sodium chlorite showed a better reduction of non-typhoidal *Salmonella* log CFU/g. This effect may be related the suggestion of that the antimicrobial interventions will be more effective in reducing bacterial population if it is applied to the final product (Mohan et al., 2012). It is also important to note that the selected isolates are more resistant to the interventions in laboratory media in order to effectively assess the impact under “worse case scenario” conditions. Additional reductions could occur using less hearty strains of the pathogens.

Results from the triangle test indicated that some of the antimicrobial interventions (lactic acid + acidified sodium chlorite, peracetic acid + acidified sodium chlorite and acidified sodium chlorite) after 24 and 72 hours of application changed the organoleptic properties of cooked beef patties compared to the control. Similarly, the spectrophotometer values (L*, a*, and b*) showed that the acids used in this study had an effect in the instrumental color of the beef patties.

The preliminary evaluation of the differences in the relative expression of housekeeping genes *rpoS* and *dnaK* may explain how *Salmonella* Montevideo like other bacteria adapt and survive in the presence of the chemical intervention (LA, PAA and ASC) at 5, 15 and 30 minutes.

Because little information exists on the application of multiple interventions and the impact on reducing non-typhoidal *Salmonella* embedded in lymph nodes during the ground beef process, and the effects in the organoleptic characteristic of the cooked product, this study serves as part of the initial research to the challenge of the meat industry to process safety products.

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Table 1. *Salmonella* serovars tested to select the more resistant to the acid treatments

***Salmonella* serovar**	**Strain**	**Origin**
Typhimurium	**CSU FSL R1-089***	**Human**
Typhimurium	CSU FSL R1-091	Human
Typhimurium	CSU FSL R1-097	Human
Typhimurium	CSU FSL R1-103	Human
Enteritidis	T1-493	Human
Enteritidis	**T1-496***	**Human**
Enteritidis	T1-500	Human
Enteritidis	T1-505	Human
Newport	T1-472	Human
Newport	**T1-473***	**Human**
Newport	T1-474	Human
Newport	T1-476	Human
Montevideo	11TTU1694B	Cattle Lymph nodes
Montevideo	**11TTU382B***	**Cattle Lymph nodes**
Montevideo	12TTU1271X	Cattle Lymph nodes
Montevideo	11TTU2834B	Cattle Lymph nodes
Anatum	11TTU769B	Cattle Lymph nodes
Anatum	11TTU1482B	Cattle Lymph nodes
Anatum	11TTU1577B	Cattle Lymph nodes
Anatum	**11TTU158B***	**Cattle Lymph nodes**
Reading	**MesTIF 4x1***	**Cattle Lymph nodes**
Reading	SubTIF 15x1	Cattle Lymph nodes
Reading	ManTIF 18x1	Cattle Lymph nodes

Reading	MesTIF 32x1	Cattle Lymph nodes

*Serovars used in this study (more resistant to acid treatments LA 4.5%, PAA 220 ppm, ASC 1000 ppm, LA + ASC, PAA + ASC)

Table 2. Primers sequences used for real-time PCR in this study

Gene	**Primer Sequence (5' – 3')**
RpoS	F: TTGGTGAGATTGGGTATTCACC
	R: AGGTTACTCTCAATCATGCGAC
dnaK	F: AGATTATCTGGGCGAACCGG
	R: AACCGCGATAGTACGGTTGC

F: forward, R: reverse

Table 3. Reduction of rifampicin resistant *Salmonella (4.8 Log CFU/g)* on ground beef after 1, 24 and 72 h of chemical treatment during grinding process

Treatment	*Salmonella* Log CFU/g reduction after treatment		
	1 h	24 h	72 h
Water	0.20 ± 0.03^{ay}	0.27 ± 0.04^{ay}	0.29 ± 0.13^{ay}
Lactic Acid (4.5%)	0.42 ± 0.05^{az}	0.47 ± 0.09^{az}	0.48 ± 0.01^{az}
Lactic Acid + Acidified Sodium Chlorite	0.59 ± 0.06^{ac}	0.62 ± 0.07^{ac}	0.67 ± 0.08^{ac}
Peracetic acid (220 ppm)	0.28 ± 0.01^{ad}	0.41 ± 0.08^{ad}	0.45 ± 0.06^{ad}
Peracetic acid + Acidified Sodium Chlorite	0.47 ± 0.05^{ax}	0.52 ± 0.11^{ax}	0.51 ± 0.06^{ax}
Acidified Sodium Chlorite (1000 ppm)	0.50 ± 0.06^{ax}	0.49 ± 0.13^{ax}	0.59 ± 0.03^{ax}

^{ab} Reduction values within the same row with different superscripts are significantly different ($P < 0.05$).
^{cdxyz} Reduction values within the same column with different superscripts are significantly different ($P < 0.05$).

Table 4. Reduction of rifampicin resistant *Salmonella* (2.3 Log CFU/g) on ground beef after 1, 24, and 72 h of chemical treatment during grinding process

Treatment	Salmonella Log CFU/g reduction after treatment		
	1 h	24 h	72 h
Water	0.12 ± 0.01^{ay}	0.15 ± 0.02^{ay}	0.22 ± 0.04^{ay}
Lactic Acid (4.5%)	0.40 ± 0.01^{ax}	0.47 ± 0.02^{ax}	0.52 ± 0.05^{ax}
Lactic Acid + Acidified Sodium Chlorite	0.66 ± 0.01^{ad}	0.71 ± 0.03^{ad}	0.76 ± 0.02^{ad}
Peracetic Acid (220 ppm)	0.21 ± 0.01^{ac}	0.34 ± 0.04^{ac}	0.41 ± 0.08^{ac}
Peracetic acid + Acidified Sodium Chlorite	0.42 ± 0.00^{ax}	0.46 ± 0.03^{ax}	0.51 ± 0.06^{ax}
Acidified Sodium Chlorite (1000 ppm)	0.52 ± 0.05^{az}	0.57 ± 0.03^{az}	0.64 ± 0.03^{az}

* No statistically significant difference between LA and PAA+ASC was observed ($P = 0.90$)

^{ab} Reduction values within the same row with different superscripts are significantly different ($P < 0.05$).
^{cdxyz} Reduction values within the same column with different superscripts are significantly different ($P < 0.05$).

Table 5. Results of the triangle test conducted on beef patties treated with antimicrobial acids

Acid treatments	Correct Responses	
	24 h	72 h
Lactic Acid 4.5%	14	16
Lactic Acid 4.5% + ASC (1000 ppm)	17*	21*
Peracetic Acid (220 ppm)	20*	16
Peracetic Acid (220 ppm) + ASC (1000 ppm)	10	14
Acidified Sodium Chlorite (1000 ppm)	22*	16

Number of panelist (n = 33)
Critical number or correct response = 17 with significant level of $P < 0.05$

Table 6. The effects of acid treatments on L* (luminance) value in beef patty samples

Acid treatments	L* value		
	1 h	**24 h**	**72 h**
Control	59.39 ± 0.85[a]	59.23 ± 0.55[a]	59.71 ± 1.21[a]
Lactic Acid 4.5%	57.64± 1.29[b]	58.43 ± 1.12[b]	58.22 ± 1.32[b]
Lactic Acid 4.5% + ASC (1000 ppm)	58.28 ± 0.89[a]	59.03 ± 0.89[a]	59.10 ± 0.78[a]
Peracetic Acid (220 ppm)	62.55 ± 1.65[d]	63.22 ± 1.78[d]	63.91 ± 1.07[d]
Peracetic Acid (220 ppm) + ASC (1000 ppm)	57.69 ± 0.82[e]	57.74 ± 0.86[e]	58.39 ± 1.38[e]
Acidified Sodium Chlorite (1000 ppm)	62.04 ± 1.04[f]	61.92± 0.70[f]	63.01 ± 1.69[f]

L*: 0 = pure black and 100 = pure white.

Means with different superscripts within a column differ significantly ($P < 0.05$) from the control.

Table 7. The effects of acid treatments on a* (redness) value in beef patty samples

Acid treatments	a* value		
	1 h	**24 h**	**72 h**
Control	20.14 ± 0.84[a]	16.42 ± 0.93[a]	13.64 ± 0.63[a]
Lactic Acid 4.5%	17.74 ± 1.04[b]	15.19 ± 0.93[b]	13.05 ± 0.68[b]
Lactic Acid 4.5% + ASC (1000 ppm)	15.80 ± 1.09[c]	14.62 ± 0.47[c]	12.93 ± 0.26[c]
Peracetic Acid (220 ppm)	17.11 ± 1.08[d]	15.16 ± 0.62[d]	12.43 ± 0.42[d]
Peracetic Acid (220 ppm) + ASC (1000 ppm)	18.28 ± 1.19[e]	15.91 ± 0.75[e]	12.63 ± 0.39[e]
Acidified Sodium Chlorite (1000 ppm)	19.76 ± 1.15[f]	16.83 ± 0.47[f]	12.00 ± 0.49[f]

a* value represent the amount of red to green color. An a* higher value indicates a redder color.
Means with different superscripts within a column differ significantly ($P < 0.05$) from the control.

Table 8. The effects of acid treatments on b* (yellowness) value in beef patty samples

Acid treatments	b* value		
	1 h	**24 h**	**72 h**
Control	17.96 ± 0.40[a]	16.91 ± 1.27[a]	16.29 ± 0.92[a]
Lactic Acid 4.5%	15.86 ± 0.65[b]	16.33 ± 0.87[b]	15.67 ± 0.77[b]
Lactic Acid 4.5% + ASC (1000 ppm)	15.79 ± 0.31[a]	16.57 ± 0.55[a]	16.37 ± 0.63[a]
Peracetic Acid (220 ppm)	17.21 ± 0.83[a]	16.91 ± 0.86[a]	16.04 ± 0.51[a]
Peracetic Acid (220 ppm) + ASC (1000 ppm)	16.92 ± 0.50[a]	16.60 ± 0.68[a]	16.40 ± 0.62[a]
Acidified Sodium Chlorite (1000 ppm)	17.84 ± 0.93[a]	17.76 ± 0.40[a]	17.08 ± 0.88[a]

b* value represent the amount of blue to yellow color. An b* higher value indicates a more yellow color.
Means with different superscripts within a column differ significantly ($P < 0.05$) from the control.

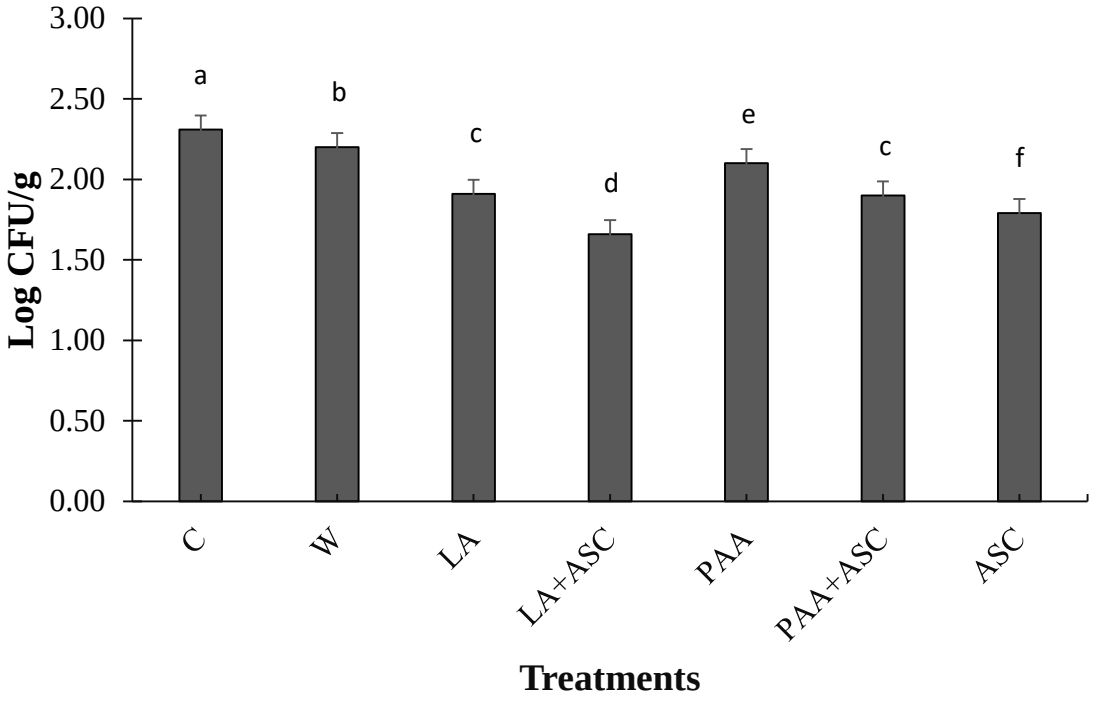

Figure 1. Reduction of *Salmonella* (2.3 log CFU/g) in ground beef after 1 h of treatment with water (w), lactic acid (LA 4.5%), lactic acid (4.5%) combined with acidified sodium chlorite (1000 ppm) (LA+ASC), peracetic acid (PAA 220 ppm), peracetic acid (220 ppm) combined with acidified sodium chlorite (1000 ppm) (PAA+ASC), and acidified sodium chlorite (ASC 1000 ppm). Control samples differed from treated samples ($P < 0.05$)

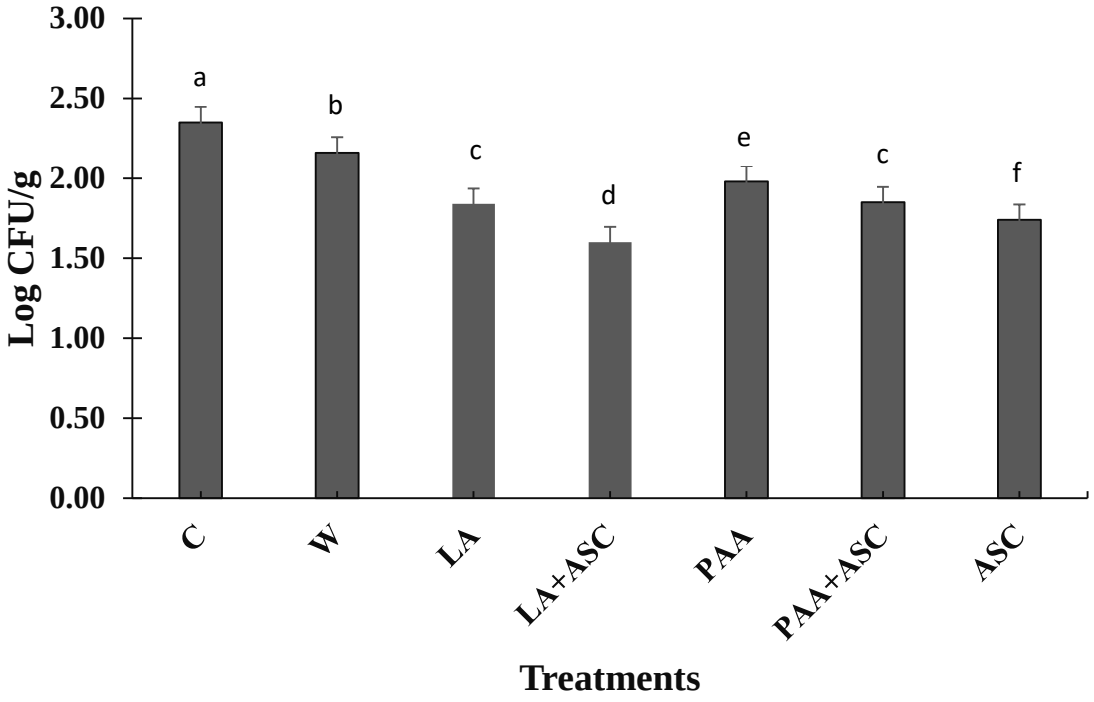

Figure 2. Reduction of *Salmonella* (2.3 log CFU/g) in ground beef after 24 h of treatment with water (w), lactic acid (LA 4.5%), lactic acid (4.5%) combined with acidified sodium chlorite (1000 ppm) (LA+ASC), peracetic acid (PAA 220 ppm), peracetic acid (220 ppm) combined with acidified sodium chlorite (1000 ppm) (PAA+ASC) and acidified sodium chlorite (ASC 1000 ppm). Control samples differed from treated samples ($P < 0.05$)

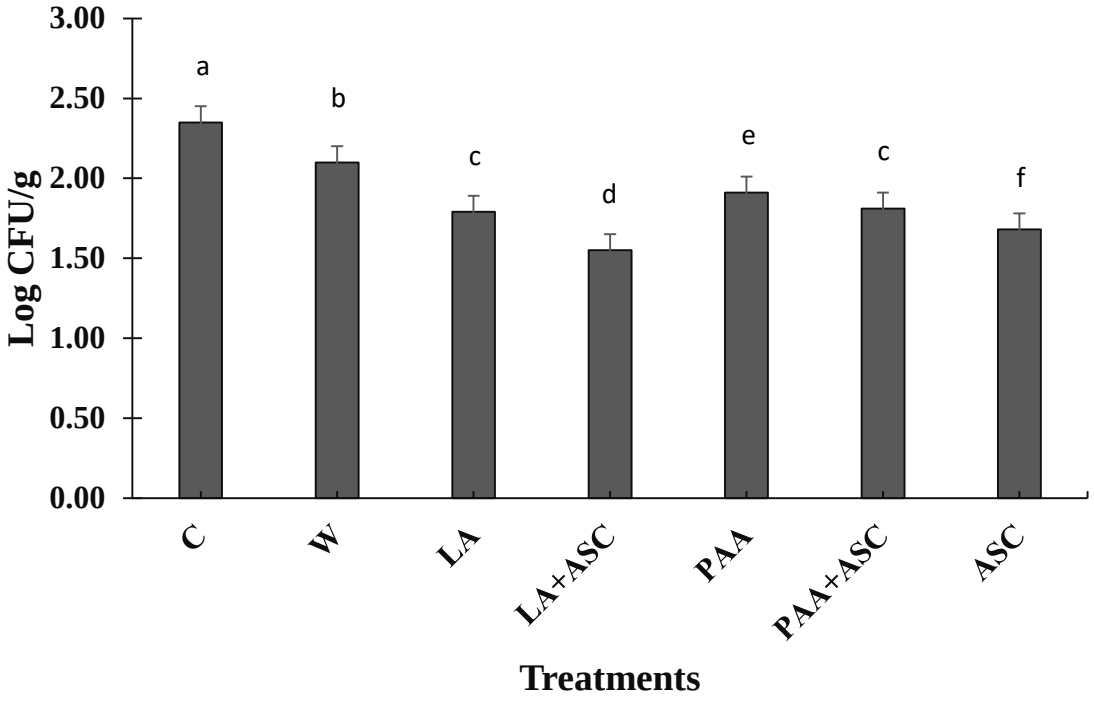

Figure 3. Reduction of *Salmonella* (2.3 log CFU/g) in ground beef after 72 h of treatment with water (w), lactic acid (LA 4.5%), lactic acid (4.5%) combined with acidified sodium chlorite (1000 ppm) (LA+ASC), peracetic acid (PAA 220 ppm), peracetic acid (220 ppm) combined with acidified sodium chlorite (1000 ppm) (PAA+ASC), acidified sodium chlorite (ASC 1000 ppm). Control samples differed from treated samples ($P < 0.05$)

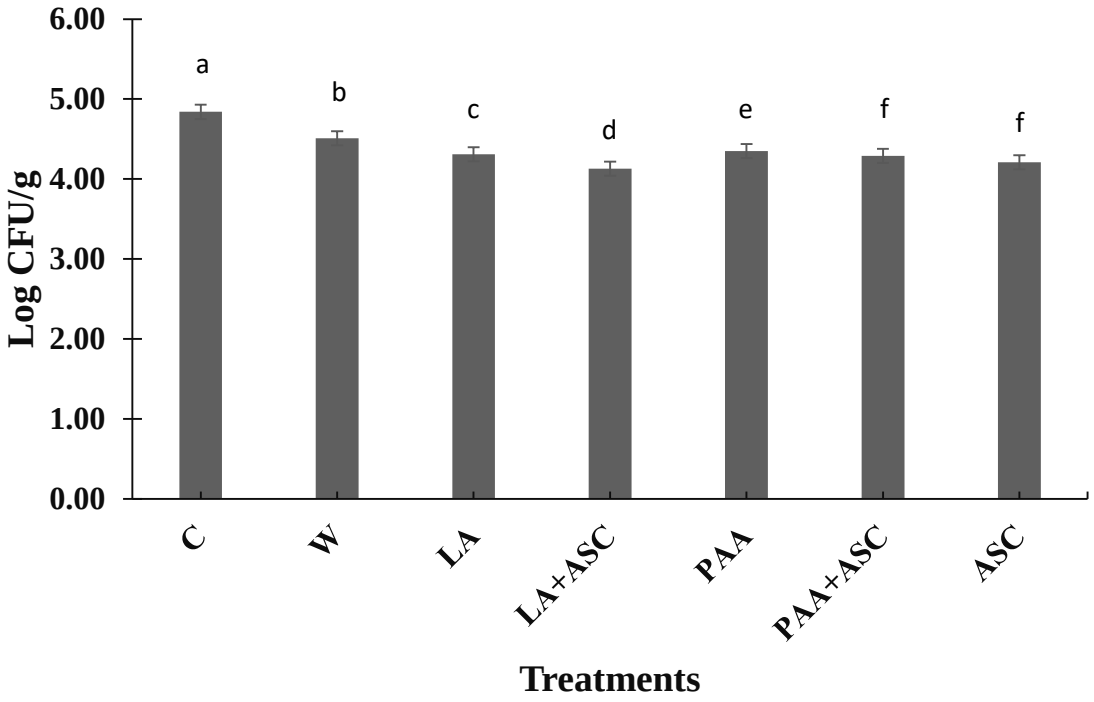

Figure 4. Reduction of *Salmonella* (4.8 log CFU/g) in ground beef after 1 h of treatment with water (w), lactic acid (LA 4.5%), lactic acid (4.5%) combined with acidified sodium chlorite (1000 ppm) (LA+ASC), peracetic acid (PAA 220 ppm), peracetic acid (220 ppm) combined with acidified sodium chlorite (1000 ppm) (PAA+ASC), and acidified sodium chlorite (ASC 1000 ppm). Control samples differed from treated samples ($P < 0.05$)

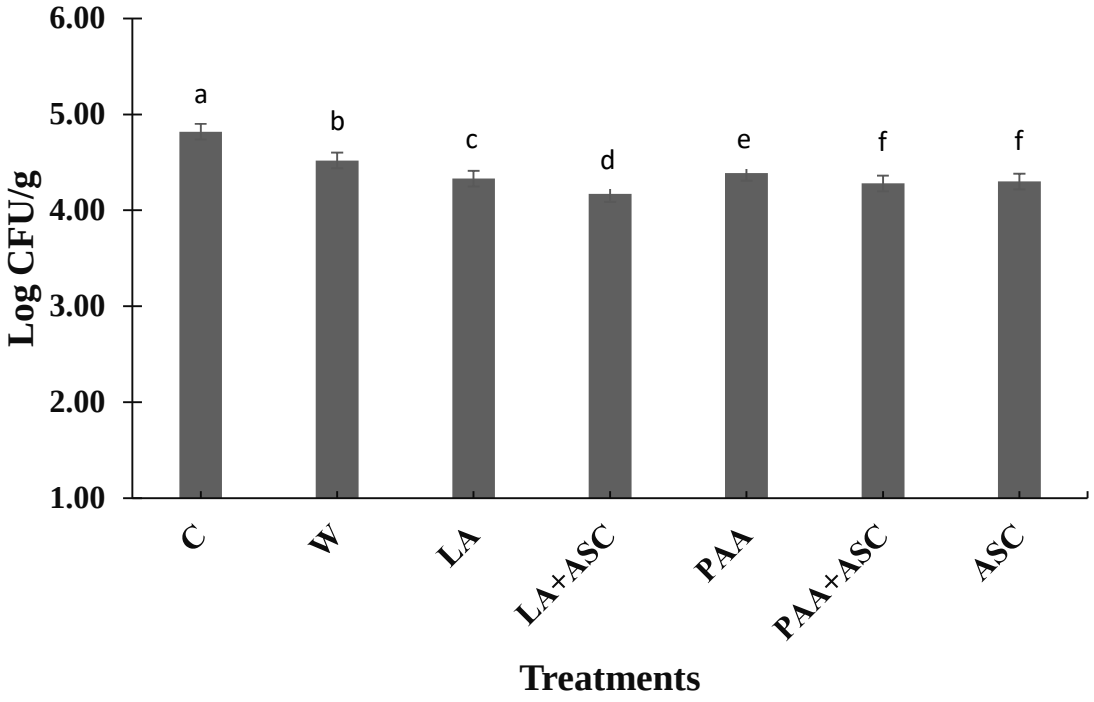

Figure 5. Reduction of *Salmonella* (4.8 log CFU/g) in ground beef after 24 h of treatment with water (w), lactic acid (LA 4.5%), lactic acid (4.5%) combined with acidified sodium chlorite (1000 ppm) (LA+ASC), peracetic acid (PAA 220 ppm), peracetic acid (220 ppm) combined with acidified sodium chlorite (1000 ppm) (PAA+ASC), and acidified sodium chlorite (ASC 1000 ppm). Control samples differed from treated samples ($P < 0.05$)

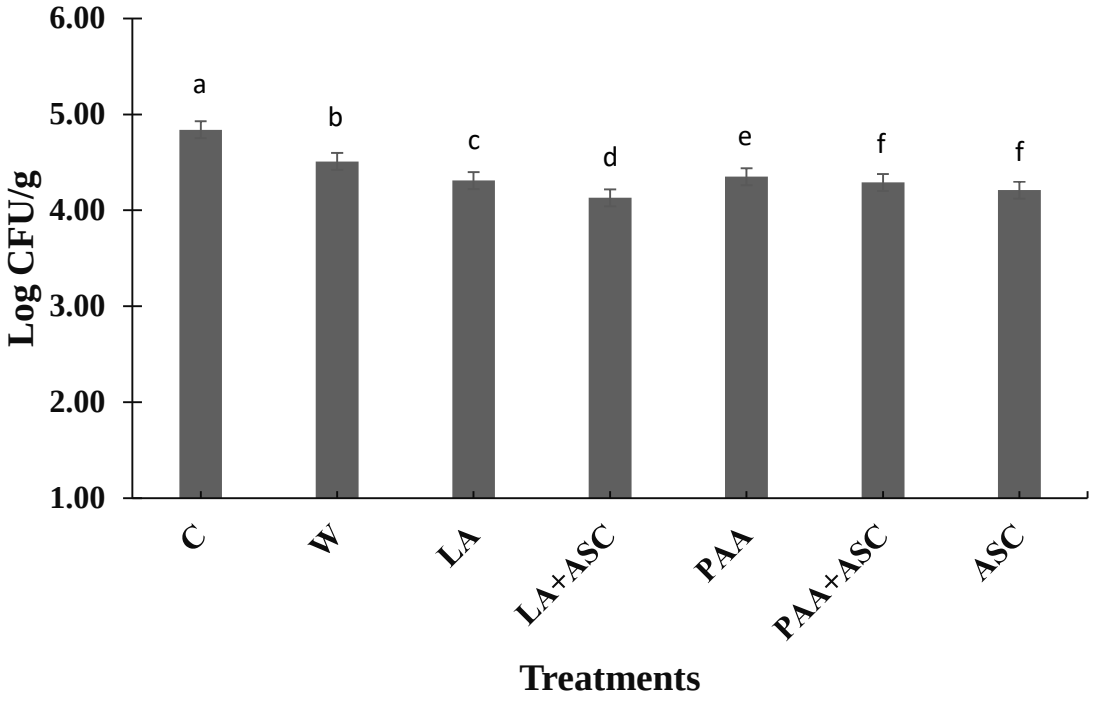

Figure 6. Reduction of *Salmonella* (4.8 log CFU/g) in ground beef after 72 h of treatment with water (w), lactic acid (LA 4.5%), lactic acid (4.5%) combined with acidified sodium chlorite (1000 ppm) (LA+ASC), peracetic acid (PAA 220 ppm), peracetic acid (220 ppm) combined with acidified sodium chlorite (1000 ppm) (PAA+ASC), and acidified sodium chlorite (ASC 1000 ppm). Control samples differed from treated samples ($P < 0.05$)

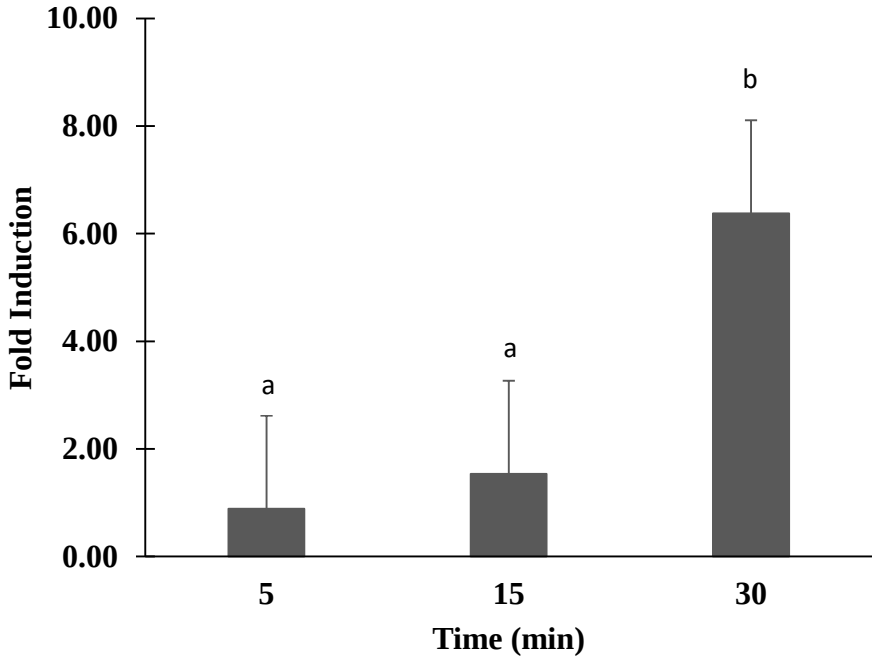

Figure 7. Relative expression of the *rpoS* housekeeping gene on *Salmonella* Montevideo. Fold induction represents relative gene expression during stress condition in comparison to reference gene (ttrC). The stress was induced by acid shock with lactic acid (4.5%). [ab] Least square means with different superscripts are significant different ($P < 0.05$).

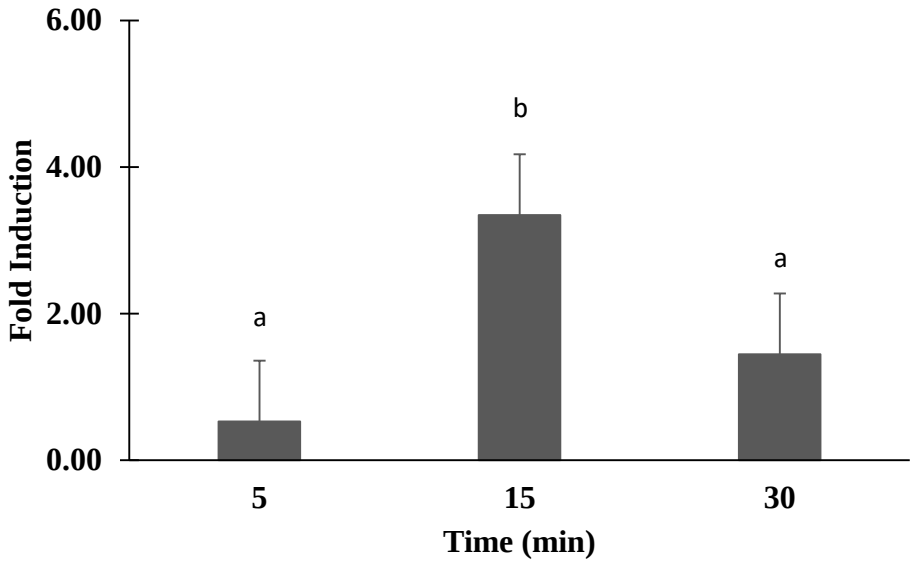

Figure 8. Relative expression of the *rpoS* housekeeping gene on *Salmonella* Montevideo. Fold induction represents relative gene expression during stress condition in comparison to reference gene (ttrC). The stress was induced by acid shock with peracetic acid (220 ppm).
[ab] Least square means with different superscripts are significant different ($P < 0.05$).

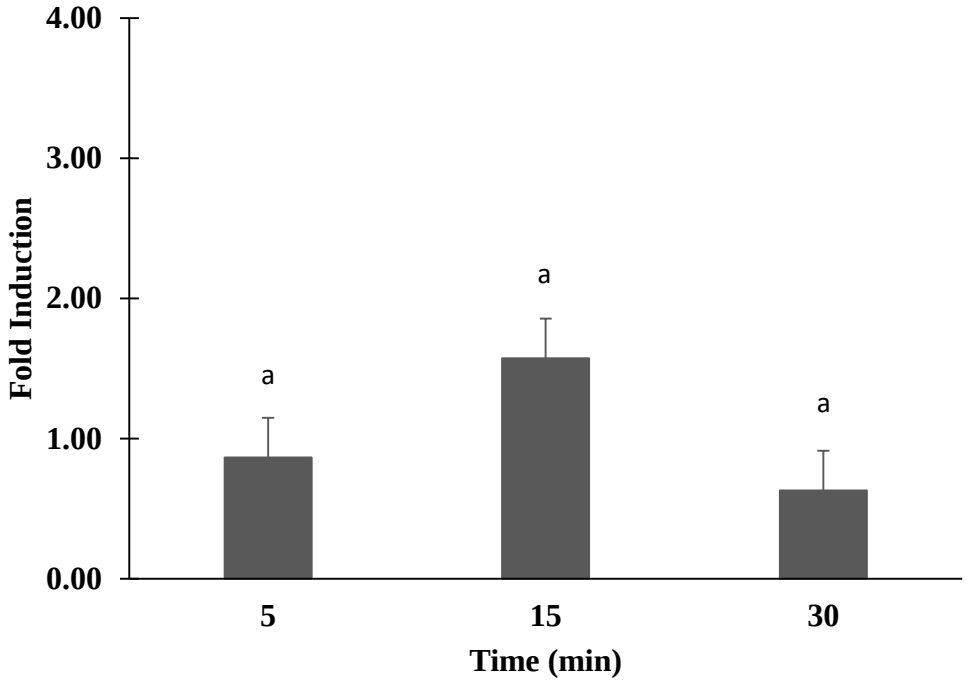

Figure 9. Relative expression of the *rpoS* housekeeping gene on *Salmonella* Montevideo. Fold induction represents relative gene expression during stress condition in comparison to reference gene (ttrC). The stress was induced by acid shock with acidified sodium chlorite (1000 ppm).
[ab] Least square means with different superscripts are significant different ($P < 0.05$).

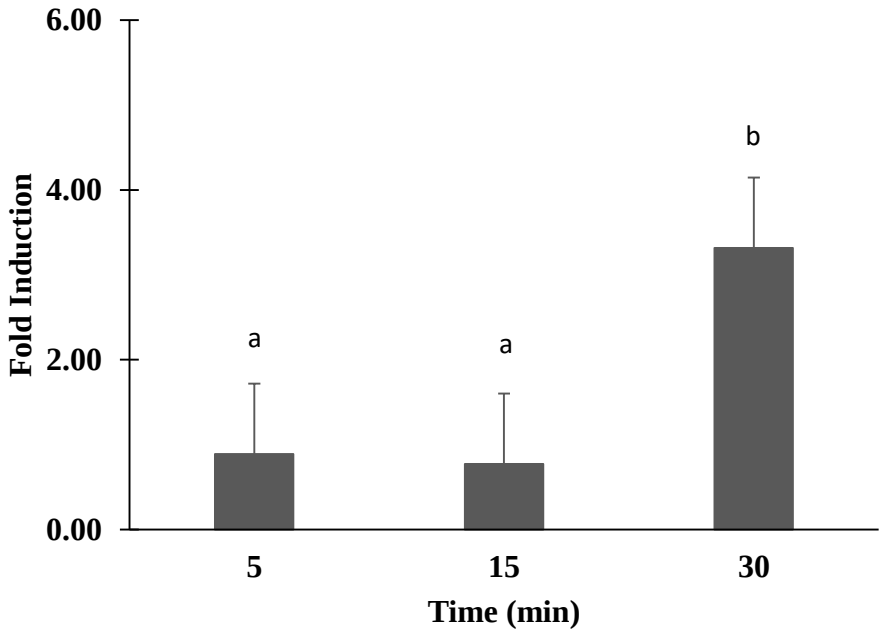

Figure 10. Relative expression of the *dnaK* housekeeping gene on *Salmonella* Montevideo. Fold induction represents relative gene expression during stress condition in comparison to reference gene (ttrC). The stress was induced by acid shock with lactic acid (4.5%).
[ab] Least square means with different superscripts are significant different ($P < 0.05$).

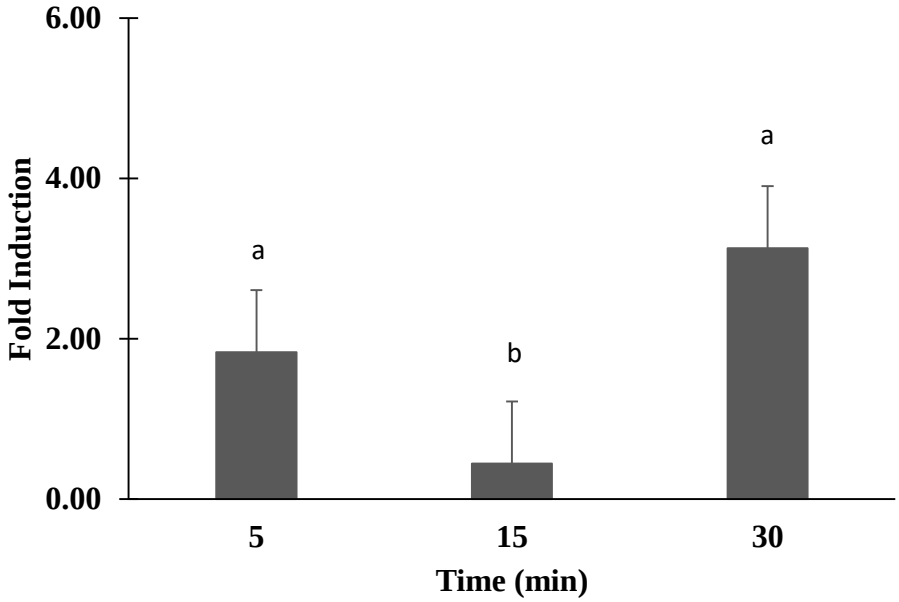

Figure 11. Relative expression of the *dnaK* housekeeping gene on *Salmonella* Montevideo. Fold Induction represents relative gene expression during stress condition in comparison to reference gene (ttrC). The stress was induced by acid shock with peracetic acid (220 ppm).
[ab] Least square means with different superscripts are significant different ($P < 0.05$).

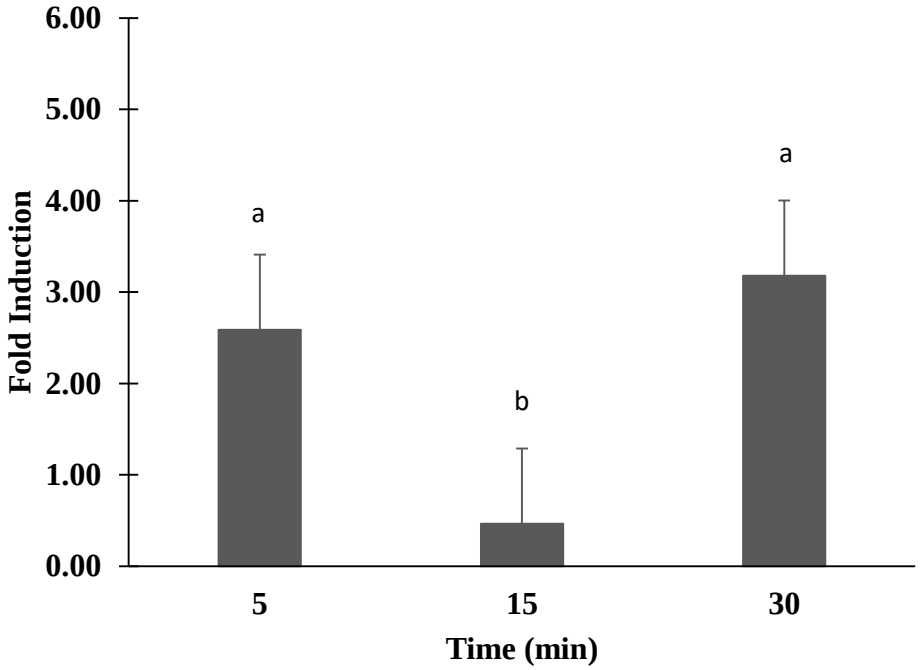

Figure 12. Relative expression of the *dnaK* housekeeping gene on *Salmonella* Montevideo. Fold induction represents relative gene expression during stress condition in comparison to reference gene (ttrC). The stress was induced by acid shock with acidified sodium chlorite (1000 ppm).
[ab] Least square means with different superscripts are significant different ($P < 0.05$).