EFFICACY OF ELECTROSTATICALLY SPRAYED ACTIVIN™ (ACTIVATED LACTOFERRIN), HOT WATER, HIGH PRESSURE WATER AND LACTIC ACID INDIVIDUALLY AND USED IN A MULTI-HURDLE APPROACH TO REMOVE *ESCHERICHIA COLI* O157:H7 FROM BEEF TISSUES

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EFFICACY OF ELECTROSTATICALLY SPRAYED ACTIVIN™ (ACTIVATED LACTOFERRIN), HOT WATER, HIGH PRESSURE WATER AND LACTIC ACID INDIVIDUALLY AND USED IN A MULTI-HURDLE APPROACH TO REMOVE *ESCHERICHIA COLI* O157:H7 FROM BEEF TISSUES

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FORMAT OF THESIS

This Thesis is presented in the Journal of Animal Science style format, as outlined by the Oklahoma State University graduate college style manual. The use of this format allows for independent chapters to be prepared suitable fo submission to scientific journals.

CHAPTER I

INTRODUCTION

Contamination of *Escherichia coli* O157:H7 is the main microbiological food safety concern of the beef industry. At the 2003 National Meat Association's annual meeting, Dr. Dell Allen (Vice President of Technical Services and Food Safety at Cargill Meat Solutions) stated, "There is no silver bullet. Get that idea through your head. We are going to have this bug (*E. coli* O157:H7) forever" (Meat News, 2005). The meat industry has accepted the ever presence of *E. coli* O157:H7 and has implemented Hazard Analysis Critical Control Point (HACCP) plans, and introduced interventions to reduce the occurrence of this elusive and troublesome pathogen (NCBA, 2005c).

Every outbreak of *E. coli* O157:H7 causes sickness, hospitalization and/or even death (NCBA, 2005b). This pathogen continues to plague the beef industry, and has emerged as a major public health concern (Padhye and Doyle, 1991). Outbreaks result in loss of trust and confidence in beef products and the industry (NCBA, 2005a). Economists report, every outbreak and product recall results in a decline in beef demand, and boneless beef prices to decrease two to two and one half percent in value (NCBA, 2005b). Furthermore, agricultural economists estimated food safety recalls from 1991 to 1999 cost the beef industry a staggering $1.6 billion as a result of decrease consumer (NCBA, 2005b). The NCBA (2005a,b) has funded over $20 million dollars from the

check-off over the past decade to attack this persistent food pathogen problem. Additionally, the NCBA (2005b) estimated over $500 million have been spent by the 30 largest beef packers in the United States on food safety research, not included is an increase in operating cost of $250 million due to compliance with food safety regulations and plant improvement for food safety (NCBA, 2005a). The estimated total cost of *E. coli* O157:H7 the beef industry is in excess of $2.6 billion during the past ten years (NCBA, 2005a).

The overwhelming financial impact of *E. coli* O157:H7 has led to reforms by the meat industry and beef cattle suppliers. During the past decade, industry leaders have developed guidelines, clarified critical control points (CCP) and interventions for every segment of the beef industry to reduce and eliminate *E. coli* O157:H7. The industry ha explored and added new interventions to eliminate the chance of microbial contamination. Despite the industry's best efforts, consumers often fail to accept their role in the equation of food safety (Doores, 1999). Smith (2000) reported shoppers fail to maintain proper temperatures of beef products. In extreme cases, products remained unrefrigerated in excess of 2 hours. Everyone involved, rancher to consumer, must take responsibility to minimize foodborne illnesses.

In early 2005, the USDA's Food Safety and Inspection Service (FSIS) reported, the beef industry's efforts against *E. coli* O157:H7 are beginning to pay off. Positive ground beef samples have decreased by 43.3% from 2003 to 2004, and more than 80% from the year 2000 (NCBA, 2005c). Furthermore, the number of *E. coli* O157:H7 recalls has declined with twenty-one in 2002, twelve

in 2003, and six in 2004 (NCBA, 2005c). Elimination of *E. coli* O157:H7 or other pathogens from the beef supply is an unattainable goal. Nonetheless, through implementation of innovative ideas, pathogens have been greatly reduced and outbreaks have occurred less frequently than in previous years.

The beef industry's most common microbial interventions for beef carcasses include organic acid rinses, hot water washes, high pressure water washes, and steam pasteurization. In 2001, Dr. A. S. Naidu developed a all natural microbial spray based upon lactoferrin's antimicrobial properties. Dr. Naidu's patented formula optimizes lactoferrin's antimicrobial effectiveness (Naidu, 2002). Currently, this technology is marketed by aLF Ventures as Activin. National Beef Packing Company applies Activin to every beef carcass processed in their facilities. Activin has been proven in laboratory settings, yet Activin's antimicrobial effectiveness has not been tested under commercial conditions on beef tissues.

This research had three objectives. The first evaluated the efficacy of Activin as a post-harvest intervention against a hot water treatment, high pressure treatment and a lactic acid treatment on *E. coli* O157:H7 inoculated on adipose tissue. The second objective determined if the National Beef Packing Company's sequential multi-hurdle intervention sequence containing Activin was superior in reducing *E. coli* O157:H7 on adipose and lean tissue to the identical intervention process without Activin. The concluding objective examined the effect Activin in a multi-hurdle sequence has on total plate counts, Coliform counts, *Enterobacteriaceae,* and Lactic acid bacteria on lean and adipose tissue

over a 7 d refrigerated (7° C) storage period compared to the identical sequence without Activin.

CHAPTER II

REVIEW OF LITERATURE

***Escherichia coli* O157:H7**

Escherichia coli (*E. coli)* of the bacterial family *Enterobacteriaceae* is commonly found in the intestines of healthy animals as well as in humans. Non-pathogenic *E. coli* has been found to suppress the growth of harmful bacteria species and synthesize vitamins in the intestines (US FDA, 2005). However, a few strains of *E. coli* commonly isolated in the environment are pathogenic, the most well known being *E. coli* O157:H7 (Johnson et al., 1983; Padhye et al., 1986; Reed, 1994; Kassenborg et al., 2004; USFDA, 2005). All *E. coli* strains are gram negative, rod shaped, facultative anaerobes that ferment lactose and do not produce spores. The optimum growth temperature of *E. coli* O157:H7 is 37° C, yet studies have shown survival in extremely cold environments (-20° C) for up to 9 months, as well as survive in pH's as low as 4.5 (Gorman et al., 1995; Glass et al., 1992; Griffin and Tauxe, 1991; Doyle, 1991, Raghubeer and Matches, 1990; Wells et al., 1983; Jay, 1986; Doyle and Schoenei, 1984). Furthermore, *E. coli* O157:H7 has been reported as being able to survive on surfaces and in soils for extended periods of time (>6 months) (Varma et al., 2003). Despite being able to survive in these extreme cases, *E. coli* O157:H7 cannot survive in conditions above 60° C, and cooking recommendations for

ground beef require an internal temperature to exceed 71° C (Line et al., 1991; USDA, 2005a).

When ingested, *E. coli* O157:H7 attaches to the epithelial lining of the intestinal tract, and produces a verotoxin (verotoxin 1, 2, or 3), closely related to the toxin produced by *Shigella dysenteriae* (US FDA, 2005; Johnson et al., 1983; Padhye et al., 1986). The resulting illness is hemorrhagic colitis (US FDA, 2005). Very little is known about the actual dose needed to produce the illness, but it is thought to be similar to *Shigella ssp.,* requiring 10 organisms (US FDA, 2005). Hemorrhagic colitis has the following symptoms:

Severe abdominal cramping

Diarrhea which is initially watery and can become bloody

Vomiting may occur

Low fever or none at all

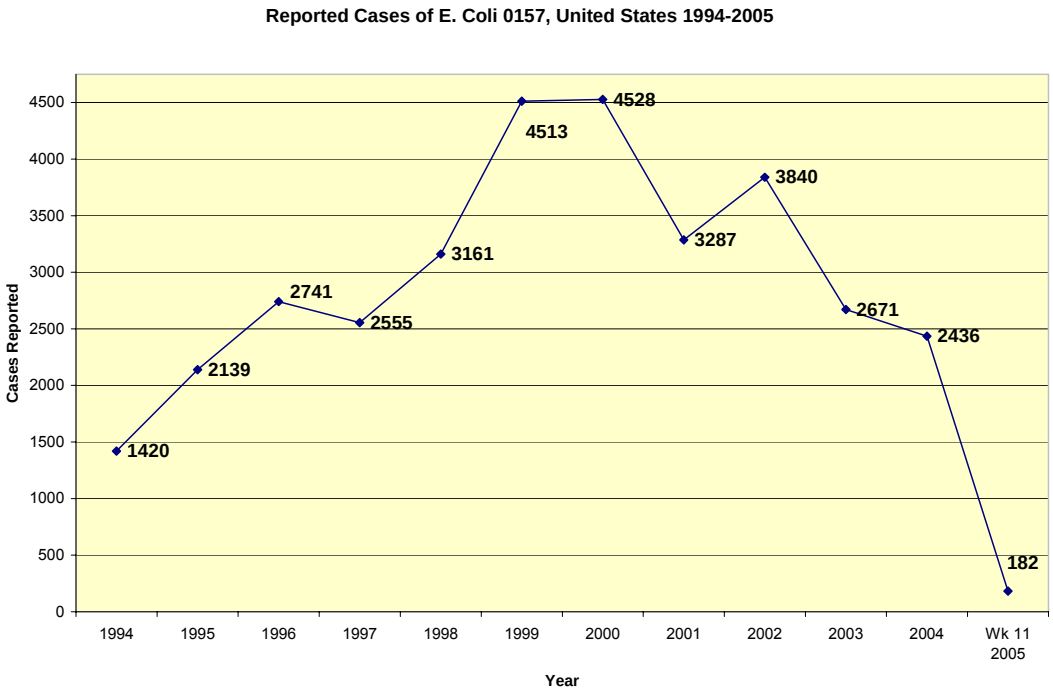
Usually lasts 2 to 9 days.

The illness is self-limiting lasting an average of eight days. The most susceptible individuals are the very young, elderly and those whom have a compromised immune system (US FDA, 2005). In these individuals hemorrhagic colitis may develop into hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), or result in acute renal failure (US FDA, 2005; Padhye and Doyle, 1991, Riley, 1987).

The true frequency of illness caused by *E. coli* O157:H7 is not known because most cases are unreported. Nonetheless, the Centers for Disease Control and Prevention (CDC, 2005) estimate that 73,000 cases of *E. coli*

O157:H7 occur every year in the United States. Of those, 2,100 people are hospitalized, and 61 people die as a direct result of *E. coli* O157:H7 infections and complications. Recently, the CDC (2005) concluded that *E. coli* O157:H7 infections declined by 36% in the past year (Figure1). The annual cost of *E. coli* O157:H7 related foodborne illnesses is estimated at $659.1 million (USDA ERS, 2005). This doesn't include monetary value of death or the value of not being able to return to work (USDA ERS, 2005). Moreover, the value also doesn't account for the costs associated with product testing, facility upgrades, recalls and loss of product value due to positive results in the product and decline in value associated with outbreaks

Figure 1: Reported Cases of *E. coli* O157:H7, United States 1994-2005. (MMWR, 2005)



According to the CDC, *E. coli* O157:H7 illness cases were not nationally notifiable until 1994 (CDC, 2005). Nonetheless, the pathogen has been a concern to the meats industry for over 2 decades. The first of over one hundred outbreaks occurred in 1982 (Arthur et al., 2004). This includes the most known "Jack in the Box" outbreaks of 1992 and 1993 which resulted in hundreds of illnesses and four deaths related to *E. coli* O157:H7 (Arthur et al., 2004; Tuttle et al., 1999; Riley et al., 1983). Only 52% of the outbreaks have been linked to beef, yet beef is characterized as the primary reservoir of *E. coli* O157:H7 (Elder et al., 2000). In 1993, *E. coli* O157:H7 was declared as an adulterant in meat products and control of the pathogen was to be implemented into every HACCP plan (USDA FSIS, 1996).

E. coli O157:H7 has been researched extensively in commercial beef production from farm to table during the past decade. The majority of the research conducted related to prevention of contamination, source of contamination and possible indicator organisms to predict the presence of *E. coli* O157:H7 with other bacteria (Arthur et al., 2004; Allen, 2004; Elder et al., 2000). *E. coli* O157:H7 peak shedding rates occur during the summer and early fall resulting in a high number of positives in lots, on hides, carcasses and illness cases reported to the CDC (Elder et al., 2000; CDC, 2005). According to Dr. Dell Allen (2004), retired Vice-President of Quality and Training Cargill Meat Solutions (Wichita, KS), in June 2003, over one million dollars of product value was lost due to *E. coli* O157:H7 positives in ground beef at one Cargill Meat Solutions beef plant. *E. coli* O157:H7 positives vary between lots of animals and plants for

several reasons: cleanliness of cattle, the incidence of cattle actually carrying the organism, skill of employees, microbial interventions in placed, number of microbial interventions, region, sampling technique and design of facility (Elder et al., 2000; Arthur, et al., 2003; Allen, 2004).

In the past ten years the beef industry has became self-motivated to prevent the problem. They have accepted the never ending presence of *E. coli* O157:H7 and other pathogenic micro-organisms. However, there has been no "silver bullet" discovered and outbreaks cause negative publicity the beef industry cannot afford. Economic costs have been estimated for the treatment of those who acquire the disease, but it is hard to estimate the economic loss of individuals not consuming beef due to fear of illness.

Bacterial Attachment

The mechanism of attachment of bacteria to animal tissues is very complex and not well understood (Firstenberg-Eden, 1981). Attachment is believed to occur in two stages. First, cells become associated through a loose reversible absorption, using London-van-der-Waals interactions. London-van-der-Waals binding is a weak interaction due to the electrical repulsive energies between two surfaces and electrical repulsive energies resulting from overlapping ionic atmospheres around the surface (Firstenberg-Eden, 1981). The second phase is irreversible attachment of bacteria to the surface. Irreversible attachment is achieved through fimbriae, pili, hydrogen bonds, ionic bonds or formation of extra cellular polysaccharides (Firstenberg-Eden, 1981). Costerton et al. (1978) claims bacteria attach strongly to a surface with a mass of

tangled fibers of polysaccharides forming the “glycocalyx” that surrounds the cell or the colony. The glycocalyx is formed by cells which have been stressed. The glycocalyx channels nutrients and aids in the regulation of the cell’s digestive enzymes (Firstenberg-Eden, 1981). Attachment of bacteria stops 20-30 minutes following inoculation of surfaces (Notermans and Kampelmacher, 1975; Firstenberg-Eden, 1981).

Attachment of microorganisms depends on many known and unknown factors. The attachment of microorganisms depends on bacterial species (Chung et al., 1989), inoculum concentration (Notermans and Kampelmacher 1975; Butler et al., 1979; Firstenberg-Eden, 1981), temperature of attachment medium (Butler et al., 1979), type of meat or tissue (Firstenberg-Eden et al., 1978), structure and morphology (Butler et al., 1979). Conflicting research adds to complexity of bacterial attachment, for example studies show that *E. coli* O157:H7, *Listeria Monocytogenes, Salmonella spp.* and Staphylococcus aureus have similar attachment rates to both lean and fat tissues (Chung et al., 1989; Cabedo et al., 1997). However, Firstenberg-Eden et al., (1978) reported difference in attachment due to species of bacteria and tissue types. Furthermore, there is a positive linear relationship between the inoculum concentration and, the number of bacteria that attach. After an attachment period, some bacteria are loosely attached and wash away due their association with the aqueous solution (Firstenberg-Eden, 1981). The tissue type has shown to effect the attachment of bacteria, but Cabedo et al., (1997) found that *E. coli* O157:H7 attached at the same rate to both beef muscle and adipose tissues.

However, they reported an unexplained variation in the detachment of the microorganism from the two surfaces. in which bacteria seemed to have a stronger attachment to adipose tissue. However, Dickson (1998) reported *Listeria monocytogenes, S. tryphimurium, S. aureus and S. marcescens* were effectively removed when washed from adipose tissue with solutions, than from beef muscle. Dickson (1998) also, suggested collagen caused a stronger attachment to muscle than to adipose tissue. Motile species of bacteria which have flagella and fimbriae more readily attach than that of non-motile species of bacteria (Butler et al., 1979). Several studies have found motility helps move the bacteria to the surface for attachment to occur. Notermans and Kampelmacher (1975) concluded that flagella had a critical role in attachment, while McMeekin and Thomas (1978) disagreed and claimed flagella played no role in bacterial attachment. Costerton et al. (1981) reported that the polysaccharide structure, glycocalyx, formed only in bacteria which were stressed for nutrients. However, Cabedo et al. (1997) stated there was no difference in attachment due to restriction of nutrients. Differing opinions occur over almost every issue of attachment. Bacteria attach at differing rates and strengths for unexplainable reasons.

Sources of Contamination

The main source for microbial contamination of beef products is the harvest floor, with the primary source being the hide (Huffman, 2002). Dr. Dell Allen (2004) observed that high microbial counts on hides resulted in increased microbial counts on carcasses, which increased the risk of pathogen

contamination (Allen, 2004). Higher fecal and ingesta contamination results in higher risks of having pathogenic bacteria on meat. Facilities use several common indicators to determine the safety, wholesomeness and storage quality of meat; and they are total plate count, *Enterobacteriaceae* count and total fecal coliform count (Gorman et al., 1995; Goepfert et al., 1975).

Most contamination of carcasses on the harvest floor occurs when an incision is made through the hide, such as sticking or opening the hide for removal. Improper evisceration can result in fecal or ingesta contamination. In addition to the obvious slaughter procedures, contamination can occur from the environment through the air, soil, water, feed, lymph nodes, improperly sanitized equipment, humans and carcass to carcass contamination (Ayres, 1955,1960; Gorman et al., 1995). Carcasses which are contaminated at high levels (6 log bacteria per square centimeter) can possibly contaminate many of the following carcasses which are touched by equipment, personnel or the carcass (Roberts and Pharm, 1980). Therefore, the level of contamination is more likely to increase with increased handling of the carcasses. Charlebois et al. (1991) reported higher counts of microbial contamination on the forequarter of the animal, compared to the hindquarter; this occurrence is due to handling, carcass to carcass contamination or contaminated runoff water from the hindquarter of the carcass. Contamination during the harvest process is unavoidable; however, good manufacturing practices and microbial interventions can reduce or eliminate microbial contamination.

Lactoferrin

Background. Sorensen and Sorensen in 1939 first reported a protein, lactoferrin, which was salmon pink in color containing iron from bovine milk (Masson et al., 1996). However, a method to isolate the red milk protein described in 1939 from bovine (Groves, 1960) and human milk (Johansson, 1960) was not refined until 1960. Groves (1960) observed a conformational change took place when iron was added or subtracted from lactoferrin. Masson et al. (1966) extracted lactoferrin from bronchial mucus, and reported its antimicrobial properties against a wide variety of micro-organisms. Furthermore, Masson et al. (1966) detected lactoferrin in various mucosal surfaces and biological fluids, and later hypothesized the function of lactoferrin was to metabolize iron and serve as a natural immune defense mechanism (Farnaud and Evans, 2003; Naidu and Bidlack, 1998). Since its isolation lactoferrin has been extensively researched by the medical society as an antimicrobial for natural immune defense (Locke, 2002). Lactoferrin is derived from milk, both human and bovine, and is most widely used for research purposes due to its concentration and ease of separation.

When lactoferrin's role in biological systems and its antimicrobial effects were defined, methods were developed to harvest lactoferrin from bovine milk. Lactoferrin is separated from milk whey. Milk whey is a liquid separated during the manufacturing of cheese and casein, and has long been considered to be a major waste and disposal problem (Smithers et al., 1996). Less than 62% of

world's whey production is used, the remaining is dumped into waterways, fields or raw sewage. Lactoferrin can be isolated from whey by the use of cation-exchange column chromatography at a neutral pH. This is easily achieved due to the isoelectric point (pI) of major proteins in whey being 7.0 or less, compared to the pI of lactoferrin being 8-9 (Law and Reiter, 1977). Recovery of lactoferrin from whey ranges from 12%, to 62% depending on the method used (Smithers et al., 1996). The methods of extraction of lactoferrin from whey have been refined to handle high volumes and obtain a purer product for numerous applications

Lactoferrin is a fist line of defense for the body (Masson et al., 1966b; Naidu, 2000). Its actual role and understanding of its specific mode of action is extremely controversial (Brock, 2002). Lactoferrin's roles include anti-inflammatory, immunomodulator, anti-tumor, iron absorption, anti-fungal, anti-parasitic, anti-bacterial, procoagulant, promicrobial, and auto-antibody as well as many more roles that are not clearly defined nor understood in the immune cascade of reactions (Farnaud and Evans, 2002; Naidu and Bidlack, 1998).

Bovine derived lactoferrin was not used as an antimicrobial in meats and food systems until the late 1990's (Naidu, 2000). The Food and Drug Administration recognized bovine derived lactoferrin as generally recognized as safe (GRAS) and allowed the use as an antimicrobial on meats (USDA, 2005). Following approval by the FDA National Beef Packaging Company, L.P. (National Beef; Kansas City, Mo) began to use the spray as an intervention step in its multi-hurdle approach to reduce microbial loads on meats. Furthermore, DMV International (Netherlands; 2005) gained approval for the use of lactoferrin

in sports drinks, infant formula, foods, personal care products, tablets for nutritional supplementation, veterinary use, animal feeds as well as meats.

Sources and Safety. Lactoferrin is found in most mucosal surfaces, numerous biological fluids as well as in blood (Masson et al., 1966). Lactoferrin has been successfully detected in bronchial mucus, milk, tears, saliva, nasal secretions, heptic bile, pancreatic fluid, seminal fluids, cervical mucus, urine, synovial fluid, blood and secondary granules of neutrophils (Masson et al., 1966; Naidu, 1991; Yeet al. 2000; Farnaud and Evans, 2003).

Table1. Sources and Amounts of Lactoferrin Present in Biological Fluids. (Masson et al., 1966; Taylor et al., 2004; DMV International, 2005)

Biological Fluid	**Amount of Lactoferrin**
Colostral Human Breast milk	7 mg/ml
Mature Human Breast Milk	1-2 mg/ml
Tear Fluid	2.0 – 4 mg/ml
Seminal plasma	0.5-1.0 mg/ml
Nasal Secretions	0.1 mg/ml
Hepatic bile	0.01-0.04 mg/ml
Cervical Mucus	0.5-1.0 mg/ml
Bronchial mucus	0.001-0.01mg/ml
Saliva	0.0007-0.01 mg/ml
Colostral cow milk	2.0-5.0 mg/ml
Mature Cow Milk	0.02-0.3 mg/ml
Blood	1-200 µg/ml
Pancreatic Fluid	0.5 mg/ml

Lactoferrin is present in all mammals, but the concentration varies among species. The amount of lactoferrin in milk depends on the stage of lactation (Sanchez et al., 1988). Additionally, Sanchez et al. (1988) reported that concentrations of lactoferrin were extremely elevated in milk derived from mastitic cows, than milk from cows showing no symptoms of mastitis. Moreover,

Masson et al. (1969) found when inflammation, disease, was present inside the body, blood Lactoferrin concentrations raised from 1 µg/ml to a high of 200 µg/ml. Most of the lactoferrin in blood was found to be from the degranulation of neutrophils (Taylor et al., 2004).

Human and bovine derived lactoferrin is the most abundant researched forms of lactoferrin. Peirce et al. (1991) compared bovine and human milk derived lactoferrin, finding that their amino acid sequence is 69% indentical. Furthermore, Taylor et al. (2004) reviewed past research concluding lactoferrin isolated from different biological fluids were produced from the same gene. Therefore, Taylor et al. (2004) concluded that bovine and human derived lactoferrin was similar enough in structure as well as amino acid sequence, and should be comparable in all aspects.

The FDA considered lactoferrin derived from bovine milk generally recognized as safe as an antimicrobial spray in concentration of 2% by weight in 2001 (US FDA, 2005). In the response letter National Beef Packing Company, the FDA did not question the safety of lactoferrin derived from bovine milk, as long as levels did not exceed 3.26 mL of spray per kg of beef or 65.2 mg of lactoferrin per kilogram of beef. The FDA and FSIS requested that National Beef Company, display a statement on all beef treated with lactoferrin be labeled “treated with lactoferrin from milk” or “treated with lactoferrin, a milk protein” (US FDA, 2005). The label would fulfill the Federal Food, Drug and Cosmetic Act and 21 CFR § 101.4, which requires that all food consisting of two or more ingredients, must list all ingredients the food contained (Taylor et al., 2004).

National Beef Company accepted the ruling issued by the FDA and FSIS used the labeling restriction with a supplementation as a marketing tool by adding “for your protection” or “for your safety” (US FDA, 2005). To reiterate the safety of lactoferrin, Taylor et al. (2004) determined a 2% lactoferrin solution as a antimicrobial spray for beef carcasses, which does not exceed 0.20 ml per kg of beef carcass, there is only an insignificant increase in lactoferrin. Taylor et al. (2004) deduced that National Beef Company could refuse the FDA and FSIS ruling of beef treated with lactoferrin. According to 21 CFR 101.100(a)(3)(ii), which designates, incidental additives in food at insignificant levels, used as processing aids could be exempt from labeling. Therefore, National Beef Company could appeal the labeling of lactoferrin as ruled by USDA FSIS (Taylor et al., 2004). National Beef Company estimated a typical consumer of beef products consumes 4.1 mg of lactoferrin per day. In addition, a heavy consumer is expected to ingest 9.1 mg of lactoferrin per day (all values for lactoferrin were tested in raw beef products) (US FDA, 2005). The amount of lactoferrin found in beef after treatment with Activin is minimal, compared to reports that teenagers 13 years to 19 years of age and those 20 years of age or older consume 75 mg per day and 50 mg per day, respectively, through the consumption of milk or milk-derived ingredients (US FDA, 2005).

Locke (2002) stated that lactoferrin is a natural antimicrobial that caters to criteria demanded by consumers and food processors. The following is the suggested criteria for preservatives and antimicrobials: (Naidu and Bidlack, 1998).

Non-toxic product that can be utilized on animals and humans

metabolized and excreted

water soluble, media of growth

heat stable to with stand thermal processes

active over a wide pH range

Lactoferrin, derived from bovine milk, is not only safe to consume, but meets all requirements set forth by Locke (2002).

Chemical and Physical Properties. Lactoferrin is a single, chained, bi-lobed, glycoprotein with a molecular weight of 80 kilodaltons (Masson et al., 1969; Brock, 2002; Farnaud and Evans 2003). Additionally, lactoferrin is a known member of the transferrin family of iron binding molecules that is present throughout the body (Masson et al., 1966). The two lobes of lactoferrin are very similar in structure and are known as the N-terminus and C-terminus. Each lobe is divided into 2 domains, which contain a single iron binding site (Anderson et al., 1987). Each lobe of lactoferrin has the ability to reversibly bind one iron (Fe^{3+}) molecule, accompanied with a synergistic anion with high affinity (K_a = 10^{20} L/mol) (Naidu and Bidlack, 1998). The natural anion binding molecule is either carbonate (CO_3^{2-}) or bicarbonate (HCO_3^-) (Brock, 2002, Farnaud and Evans, 2003). Grossman et al. (1992) observed a conformational change when an iron is bonded using x-ray solution scattering. Lactoferrin's change is described as a closing of the cleft where iron has been bound. Upon the release of the iron atom, the cleft opens. Lactoferrin deficient of iron is known as apo-

lactoferrin and that which has two atoms of iron bound to it is referred to as holo-lactoferrin (Naidu, 2001; Farnaud and Evans, 2003).

Lactoferrin's bacteriostatic properties are well documented and extend across a wide range of gram negative bacteria, including *E. coli* O157:H7, gram positive bacteria, viruses and fungi (Kirkpatrick et al., 1971; Arnold et al., 1977; Kalmar and Arnold, 1988; Yumauchi et al., 1993; Jones et al., 1994). This ability to inhibit bacterial growth in vitro was first attributed as its ability to sequester iron, an essential bacterial nutrient for metabolism (Chapple et al., 1988), from the environment (Groves, 1960; Arnold et al., 1977). Essentially lactoferrin starves the bacteria of this essential nutrient, causing a bacteriostatic effect (Naidu and Bidlack, 1998). This ability does work in most occasions, usually in vitro; yet some bacteria produce siderphores to release into the environment when stressed (Gray-Owen and Schrivers, 1996). Siderphores are produced by bacteria to aid in sequestering of iron from molecules which have a greater affinity (Gray-Owen and Schivers, 1996). Bacteria siderphores often have a higher binding affinity for iron than lactoferrin, and therefore acquire it readily from lactoferrin (Griffiths and Williams, 1999). Other species of bacteria do not require siderphores and are able to use previously sequestered iron straight from lactoferrin (Herrrington and Sparling, 1985). Unfortunately, in those situations lactoferrin is acting as a promicrobial molecule (Farnaud and Evans, 2003). Furthermore, concentration and form (apo- vs. holo-) of lactoferrin present can inhibit antimicrobial strength. For example, iron saturated lactoferrin (holo- lactoferrin) has reduced antimicrobial activity, when the same

concentration is compared to iron free lactoferrin (apo- lactoferrin) (Arnold et al., 1980, Yamauchi et al., 1993). Lactoferrin has some antimicrobial effect by sequestering iron in vitro; however, in vivo iron sequestering is highly debated (Brock, 2002). Brock (2002) explains that in vitro testing of lactoferrin's antimicrobial activity cannot mimic the complicated interactions that can happen in vivo.

In addition to lactoferrin's iron sequestering ability, it has demonstrated bactericidal properties revealed by interaction with the bacteria's surface (Arnold et al., 1977, 1982; Bortner et al., 1989). Lactoferrin has shown binding to active sites, and disruption or penetration of the cell membrane (Ellison and Geihl, 1981; Yumauchi et al., 1993). Lactoferrin specifically binds to the outer-membrane of gram-negative bacteria causing permeability or release of the lipopolysaccharide membrane (Ellison et al., 1988; Apllemink et al., 1994; Farnaud and Evans, 2003). Lactoferrin binds to the lipid A portion of the lipopolysaccharide membrane or outermembrane porins (Applemelk, 1994; Naidu et al., 1993). Unfortunately, changes in pH, Mg^{2+} concentrations, Ca^{2+} concentrations, as well as iron saturation decrease the efficacy of lactoferrin's damage to the outer-membrane of the bacteria (Botner et al., 1986, 1989; Kalmar and Arnold, 1988; Ellison et al., 1990; Farnaud and Evans, 2003). In addition, lactoferrin's antimicrobial ability to influence the bacteria has been shown to be decreased depending on the growth phase, the most susceptible is the early log phase. Lastly, some believe that bacteria grown from plates are completely resistant (Arnold et al., 1981; Bortner et al., 1989).

Lactoferrin can be hydrolyzed by pepsin resulting in a basic N-terminal end peptide, lactoferricin, which is more effective antimicrobial than lactoferrin (Saito et al., 1991; Bellamy, et al., 1992). Lactoferricin applies positive charges from within the peptide to interact with the negatively charged cell membrane (Nikaido and Vaara, 1985) resulting in a similar effect to the outer membrane as described for lactoferrin.

Table 2. Inhibitory Spectrum of Bovine Lactoferrin (LF) and Lactoferricin (Lfcin) against various bacteria (Naidu, 2000a; Locke, 2002).

Bacterial Species	**Form**	**Dose**	**Effect**	**Reference**
Aeromonas hydrophila	LF	0.1%	Adhesion-blockade (47%)	Paulsson et. al., 1993
Bacillus cereus	LFcin	6 µM	Cidal (4-log, 100%)	Hoek et. al., 1997
Bacillus circulans	LFcin	0.006%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Bacillus natto IFO3009	LFcin	0.002%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Bacillus stearothermophilus	LF	1:20	Stasis	Reiter & Oram 1967
Bacillus subtilis	LF	1:20	Stasis	Reiter & Oram 1967
Bacillus subtillis ATCC6633	LFcin	0.002%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Bifidobacterium longum	LF	0.1%	Agglutination	Tomita et. al., 1994
Corynebacterium diphtheriae	LFcin	0.018%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Corynebacterium ammaniagenes	LFcin	0.003%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Clostridium perfringens	LFcin	0.024%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Clostridium paraputrificum	LFcin	0.003%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Enterococcus faecalis	LFcin	0.06%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Escherichia coli E386	LF	0.1%	Stasis (24-h 100%)	Naidu et. al., 1993
Escherichia coli H10407	LF	0.1%	Adhesion-blockade (50%)	Paulsson et. al., 1993
Escherichia coli IID-861	LFcin	10 µM	Cidal (3-log reduction)	Bellamy et. al., 1992b
Escherichia coli CL99	LF	20 µM	LPS release, OM damage	Yamauchi et. al., 1993
Escherichia coli O157:H7	LF	2%	Cidal 90%	Ransom and Belk, 2003
Escherichia coli O157:H7	LFcin	15.6 mg	Cidal (99.9 %)	Jones et. al., 1994
Klebsiella pneumoniae	LFcin	10 µM	Cidal (3-log reduction)	Bellamy et. al., 1992b
Lactobacillus casei	LFcin	0.01%	Cidal (6-log, 100%)	Bellamy et. al., 1992b

Bacterial Species	Form	Dose	Effect	Reference
Listeria monocytogenes	LFcin	10 μM	Cidal (4-log reduction)	Bellamy et. al., 1992b
Listeria monocytogenes NCTC7073	LFcin	2 μM	Cidal (4-log, 100%)	Hoek et. al., 1997
Micrococcus luteus	LF	0.1%	Agglutination	Tomita et. al., 1994
Proteus vulgarus JCM1668T	LFcin	0.01%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Pseudomonas aeruginosa IFO3446	LFcin	10 μM	Cidal (4-log reduction)	Bellamy et. al., 1992b
Pseudomonas fluorescens	LFcin	8 μM	Cidal (4-log, 100%)	Hoek et. al., 1997
Salmonella abony	LF	0.8%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Salmonella Dublin	LF	0.2%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Salmonella enteritidis	LFcin	0.01%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Salmonella Hartford	LF	0.8%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Salmonella Kentucky	LF	0.2%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Salmonella panama	LF	0.1%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Salmonella pullorum	LF	0.2%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Salmonella rostock	LF	0.2%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Salmonella salford	LFcin	4 μM	Cidal (4-log, 100%)	Hoek et. al., 1997
Salmonella Montevideo	LF	20 μM	LPS release, OM damage	Yamauchi et. al., 1993
Salmonella Thompson	LF	0.1%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Salmonella typhimurium Rd	LF	0.5%	Stasis (64%)	Naidu et. al., 1993
Salmonella. Typhimurium R10	LF	0.1%	Adhesion-blockade (68%)	Paulsson et. al., 1993
Salmonella. Typhimurium SL696	LF	20 μM	LPS release, OM damage	Yamauchi et. al., 1993
Salmonella virchow	LF	0.8%	Stasis (24-h 100%)	Naidu & Arnold, 1994
Shigella flexeri	LF	0.1%	Adhesion-blockade (30%)	Paulsson et. al., 1993
Staphylococcus albus	LF	0.5%	Stasis	Masson et. al., 1966
Staphylococcus aureus	LF	0.1%	Adhesion-blockade (54%)	Paulsson et. al., 1993
Staphylococcus aureus JCM2151	LFcin	10 μM	Cidal (3-log reduction)	Bellamy et. al., 1992b
Staphylococcus epidermidis	LFcin	0.006%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Staphylococcus haemolyticus	LFcin	0.001%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Staphylococcus hominis	LFcin	0.003%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Streptococcus bovis	LFcin	0.006%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Streptococcus lactis	LFcin	0.003%	Cidal (6-log, 100%)	Bellamy et. al., 1992b
Streptococcus thermophilus	LFcin	0.003%	Cidal (6-log, 100%)	Bellamy et. al., 1992b

ACTIVIN™. Activin was developed by A. S. Naidu (Naidu, 2001). Activin is a mixture of ingredients developed to be sprayed electro-statically onto meat surfaces to reduce microbial numbers and growth. Activin is a mixture of immobilized lactoferrin, natural lactoferrin, an acid, a base and salt mixed into a deionized water.

Lactoferrin in the mixture must be derived from bovine milk, separated from whey. Immobilized lactoferrin is bond to a galactose rich polysaccharide (Naidu, 2001). The N-terminus region of the lactoferrin is bound to the substrate when iron concentrations are low. The native lactoferrin present in the mixture is non-substrate bound lactoferrin, which can be either in the apo- or holo- forms. The mixture is preferred to contain 1% immobilized lactoferrin and 1% natural lactoferrin (Naidu, 2001). The remaining buffer solution contains citric acid as the acid, sodium bicarbonate as the base, and sodium chloride as the salt. The optimum ratio of acid:base:salt is: 0.001M (acid): 0.01M (base): 0.1M (salt) (Naidu, 2001).

The lactoferrin and buffer solutions are then suspended in an aqueous solution using deionized water. The water must meet certain specifications as outlined by aLF Ventures and N-Terminus Laboratory (Pamona, CA). (See Appendix H)

According to Naidu (2002) and Naidu and Bidlack (1998), Activin results in a microbial blocking agent which interferes with adhesion/colonization, detaches live or dead organisms from surfaces, inhibits microbial growth/multiplication, and neutralizes the activity of endotoxins. Naidu (2002) and Naidu and Bidlack

(1998) claim that when Activin binds the outer-membrane of gram negative bacteria such as *E. coli* O157:H7, resulting in inhibition of cellular functions and deregulation of adhesion/fimbrial synthesis on the bacterial surface. This effect on fimbriae of *E. coli* was noticed 2 hours after exposure. Furthermore, Naidu (2002) hypothesized that Activin displaces bound live/dead microorganisms from their binding site on meat tissue causing bacterial detachment. Activin is designed to produce optimum apo- to holo- regulation of lactoferrin as well as reduce the instance of proteolysis. Efficacy of lactoferrin versus Activin has shown that Activin has greater antimicrobial effects on meat surfaces in 1% concentration when compared to lactoferrin in 1% concentration (Naidu, 2002). Therefore, Naidu (2002) reports that Activin is a microbial blocking agent that effectively prevents *E. coli* O157:H7 attaching to the surface, and inactivates it by binding to the cell membrane. Ransom and Belk (2003) found that Activin in combination with lactic acid could prevent or suppress growth in bologna and on adipose tissue (~ 1 log CFU/cm^2) after storage for 29 days.

Lactic Acid

The most used chemical decontaminants in spray wash systems found in commercial processing facilities are organic acids, usually lactic or acetic acids (Castillo et al., 2002). Lactic acid has been shown to effectively reduce pathogenic and spoilage bacteria on carcasses, sub-primals, retail cuts and trim for ground beef, as well as to have lasting effects on cuts in vacuum bags (Hamby et al., 1987; Dixon et al., 1987, 1991Anderson et al., 1989; Anderson et al., 1990; Dickson and Anderson, 1992; Ellebracht et al., 1999). However, Acuff

et al. (1987) found that lactic acid had very limited antimicrobial activity on meat surfaces. These inconsistencies of antimicrobial activity of lactic acid were clarified by Anderson and Marshall, (1990), who reported concentration and temperature deviations of lactic acid can reduce antimicrobial effects. In addition, method of acid application, length of application, time of application after removal of hide, type of tissue, use of a spray chill system and bacterial species being tested all have an effect on the efficacy of lactic acid (Sirugusa and Dickson, 1992; Dickson, 1991; Anderson et al., 1992).

The use of lactic acid is currently approved at concentrations of 1.5- 2.5% (USDA FSIS, 1996). The maximum effectiveness of lactic acid is achieved shortly following hide removal, while the carcass is still warm (Huffman, 2002). Nonetheless, studies have shown that 4% lactic acid is also effective in controlling pathogens on chilled beef carcasses (55°C) (Castillo et al., 2001). Lactic acid is considered to be most effective if applied at a temperature of 55° C or higher as a rinse. Rinses of varying concentrations (0.5% to 5%) resulted in reductions from 1 to 4 $\log_{10}$ CFU/cm^2, with variation due to factors previously stated (Greer et al., 1992). Currently, lactic acid rinses are suggested and used pre- and post-evisceration to maximize it effect on bacteria (Cutter and Siragusa, 1994; Dorsa et al., 1997, 1998; Delmore et al., 1998; Huffman, 2002).

Chemical and Physical Properties. Lactic Acid is naturally found in animal tissues, which increases during the conversion of muscle to meat (Baird Parker, 1980; Romans et al., 1994). Lactic acid ($CH_3CHOHCOOH$), a short chain organic acid, is highly soluble in water with a pKa of 3.1. Lactic acid is a stronger

decontaminate than acetic acid (CH_3COOH), being attributed to the longer chain length; Organic acid with longer chain lengths have stronger antimicrobial properties (Baird Parker, 1980).

Antimicrobial activity is attributed to the undissociated molecule of the organic acid (Ingram et al., 1956). The accumulation of the undissociated weak acids in the cytoplasm of the cell is due to the intracellular pH being higher than the pKa of the acid resulting in the dissociation of the acid releasing a proton and acidifying the microorganism's cytoplasm (Booth, 1985; Huffman, 2002). When organic acid is used in concentrations greater than 1%, Baird Parker (1980) reports they are very effective antimicrobials against a wide variety of microorganisms.

Hot Water Spray Washing

Exposure of animal tissues to hot water by various methods of application is effective in controlling many spoilage as well as pathogenic bacteria (Barkate et al., 1993; Smith, 1992; Davey and Smith, 1989; Smith and Graham, 1978; Patterson, 1969). USDA-FSIS (1996) acknowledged hot water washes, having temperatures in excess of 74°C, produced a sanitizing effect and are approved as a valid step in HACCP to control pathogens. Previous work demonstrates that hot water rinses reduce bacterial counts from 1-3 log CFU/cm^2 (Acuff et al., 1996; Gorman et al., 1995; Barkate et al., 1996; Kelly et al., 1981). Paterson (1969) first reported a reduction in bacterial numbers with the use of a hot water steam mixture (80-96° C) for 2 minutes delivering 18.9 liters of water to the samples surface. Smith and Graham (1978) found a reduction of two to three logs of *E.*

coli and *Salmonella* when beef and sheep surface tissue samples were immersed in hot water. Smith (1992) reported a reduction of pathogenic *E. coli* when inoculated on beef brisket tissue and treated with water (80°C) for 10 and 20 seconds. Gorman et al. (1995) and Kochevar et al. (1997) observed a reduction similar to that of Smith (1992) using a simulated spray-wash unit with water (74°C). In addition, this treatment showed an improvement in visual appearance, removing fecal and other contamination, required by the 'zero tolerance' policy in place by the USDA. Reagan et al. (1996) observed that trimming carcasses at line speed removed visual contamination, but hot water washing (74°-87.8° C, 11-18 seconds, 1310-2413 kPa) was better to remove microorganisms. The hot water wash was able to reduce bacterial numbers by injury and death due to elevated temperatures present in the study. However, consideration must be given that washing may potentially spread contamination from one area of a carcass to another (Barkate et al., 1993). Gorman et al. (1995) reported no potential spreading of microbial contamination using a pilot scale spray-washing unit. Despite this other problems have arose in the widespread application in commercial facilities such as amount of water needed, energy needed, spray nozzle problems, and condensation formation problems in and around the cabinets (Reagan et al., 1996). Also, problems with water loosing temperature and application flaws have increased the concern for use in the industry. Finally, the effectiveness of the hot water wash is dependent on temperature, exposure time, pressure, design of the cabinet and facilities.

High Pressure Water Washing

The use of high pressure spray-washing has been typically used to reduce visible contamination, microbial contamination and bone dust after splitting the carcass. A concern in the use of high pressures has been the possibility of physically driving the contamination into the muscle and adipose tissue (Gorman et al., 1995) and spreading contamination from one area of a carcass to another (Barkate et al., 1993). DeZuninga et al. (1991) recommends that the maximum pressure for use in a spray-washing cabinet to be 2070 kPa, based on their dye penetration model; the limit is suggested to decrease the possibility of physically driving bacteria into muscle and adipose tissue. Reagan et al. (1996) reported less *E. coli* O157:H7 positives were found on carcasses that were washed (28°-42° C, 410-2758 kPa, 18-39 seconds) compared to carcasses that have been trimmed or trimmed and then washed. Effectiveness of bacteria reduction is dependent on water pressure, angle of nozzles, time of exposure to treatment, and water temperature.

Other Harvest Microbial Interventions

Hide on Carcass Washing. Cargill Meat Solutions has recently spent millions of dollars on the instillation of a hide on carcass wash system to reduce initial bacterial numbers entering the facility (Allen, 2004). Reducing the number of bacteria from the major source of potential contamination, the hide, will reduce the microbial contamination of carcasses upon entering the hot box (Allen et al., 2004). Bosilevac et al. (2005) found that hides, when sampled before entering

and after exiting the cabinet, had lower aerobic plate counts and *Enterobacteriaceae* counts, being reduced by 2.1 and 3.4 log CFU per 100 cm^2, respectively. The prevalence of E. coli O157 on hides was reduced from 44% to 17% of hides when the cabinet was in use. Pre-evisceration carcass aerobic plate counts and Enterobacteriaceae counts were both reduced by 0.8 log CFU per 100 cm^2, and the prevalence of E. coli O157 was reduced from 17% to 2% when the cabinet was in use. The hide on carcass wash system uses a sodium hydroxide wash, followed by a chlorinated (1 ppm) water rinse and the use of a steam vacuum system to remove excess water from the pattern where the hide will be opened. The use of the additional steam vacuum increased the reduction of bacteria beyond carcass wash alone.

Chemical Dehairing. The dehairing process described and used in testing of the process consists of 3 steps considered to be bacteriostatic and/or bactericidal are: application of sodium sulfide, hydrogen peroxide and rinsing with lactic acid (Bowling and Clayton, 1992; Sofos and Smith 1998). Schnell et al. (1995) reported no reduction in aerobic plate counts. However, Catillo et al. (1998) and Graves-Delmore et al. (1997) reported reductions in pathogenic bacteria on the beef hides after being dehaired by the three chemical processes. Despite these findings, chemical dehairing is costly and slows chain speeds.

Steam Pasteurization According to Dr. Allen steam pasteurization is the most critical microbial intervention in Cargill Meat Solutions harvest process, stating “if it is down, we do not operate”. He goes on to comment that “all other interventions are just band aids in the slaughter process,” (Meat News, 2003).

Steam pasteurization is another means to obtain thermal destruction of bacteria on the surface of carcasses. The process begins with a drying process to remove any excess moisture from the carcass, followed by steam (≥83° C) treatment from 10-20 seconds, and followed by a cooling process. Reductions of bacteria are comparable to hot water rinse resulting in a 2-3 log CFU/cm^2 reduction (Gill et al., 1999). Steam pasteurization is more efficient than hot water and less likely to spread contamination, because of the "wrap around" effect of the steam. Currently, the larger beef packers in the United States and Oklahoma State University use steam pasteurization as one of their main critical control points.

Steam Vacuum The use of steam vacuuming of small areas of contamination is a widely accepted practice in the beef packing industry. Steam vacuuming uses steam and/or hot water to loosen soil and kill bacteria, followed by application of a vacuum to remove contaminants (Castillo et al., 1999; Dorsa et al., 1996; Kochevar et al., 1997; Sofos et al., 1999). Most steam vacuuming occurs after hide removal and follows the pattern where the hide is initially opened. Castillo et al. (1999) found that steam vacuuming reduced the number of indicator organisms 3 log cycles; unfortunately, vacuuming tended to spread contamination to adjacent sites. FSIS approved the use of steam vacuuming a contaminated area of up to 2.5 cm in diameter.

Multi-Hurdle Approach

Leistner (1995) describes the "hurdle approach' as sequential use of decontamination technologies. The multi-hurdle approach simply implies that if a

single application of a single decontamination treatment achieves a certain reduction of microbes, then the use of two, three or more different treatment technologies, will yield a synergistic effect when combined. Bacon et al. (2000) observed aerobic plate counts, *E. coli* counts and total coliforms gradually reduced through out the stages of slaughter process after decontamination steps were applied. The eight commercial facilities used a number interventions including steam vacuuming, pre-evisceration washes (water and organic acid), hot water, post-evisceration washes and steam pasteurization. Elder et al. (2000), also reported similar results where 43.4% of the lots evaluated were positive for *E. coli* O157:H7, however after all interventions were applied only 1.9% were positive. Despite the so called additive effect, Dr. Keith Belk in an interview stated “Multi-hurdle systems can become overwhelmed and could not handle the load of *E. coli* O157:H7” (Ishmeal, 2003). Hurdle technology has shown an additive effect, however the systems that we have in place have a thresh hold and cannot always decrease the levels to zero (Meat News, 2003). Cargill Meat Solutions has implemented a carcass wash system to their approach to help reduce the load to a number in which their hurdle approach can effectively handle (Allen, 2004). Furthermore, in the last few years research to eliminate or reduce pathogenic bacteria from cattle before entering the harvest floor has increased.

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CHAPTER III

EFFICACY OF ELECTROSTATICALLY SPRAYED ACTIVIN™ (ACTIVATED LACTOFERRIN), HOT WATER, HIGH PRESSURE WATER, AND LACTIC ACID INDIVIDUALLY AND USED IN A MULTI-HURDLE APPROACH TO REMOVE *ESCHERICHIA COLI* O157:H7 FROM BEEF TISSUES

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ABSTRACT

Validation of electrostatically sprayed Activin as a microbial intervention was investigated through 3 experiments: Experiment 1) Comparison of Activin (A), hot water rinse (HW), 2% lactic acid (LA) spray, Activin Buffer solution (B), high pressure water rinse 3s 15s (HP3) and high pressure water rinse 15 s (HP15) to reduce *E. coli* O157:H7 on inoculated (6 log CFU/cm^2) adipose tissue. Greater ($P< 0.05$) reduction of *E. coli O157:H7* was shown for adipose tissue samples treated with HW than HP3, B, HP15 and A. No differences ($P> 0.05$) were evident between treatments of LA, HP3, HP15, and A on *E. coli* O157:H7. Experiment 2) Evaluation of National Beef Packing Company's multi-hurdle intervention sequence with Activin (MH-A) and without Activin (MH-NA) on *E. coli* O157:H7 (6 log CFU/cm^2) on adipose and lean tissue. No difference ($P> 0.05$) in reduction of *E. coli* O157:H7 was found on samples treated with MH-A and MH-NA on lean or adipose tissue, 2.4-2.9 log CFU/cm^2. Experiment 3) Reduction of

total plate counts (TPC), Coliforms, Enterobacteriaceae (*ENT*), and lactic acid bacteria (LAB) on uninoculated lean and adipose tissue treated with MH-A and MH-NA and stored for 7 days (7° C). Adipose tissue treated with MH-A displayed reduced (P< 0.05) coliform counts following 7 d storage than adipose tissue treated with MH-NA. Lean samples treated with MH-A or MH-NA had significant (P< 0.05) higher reductions of TPC and LAB than adipose tissue treated with MH-A and MH-NA. No differences were observed for *ENT* for tissues or treatments. Greater reductions (P< 0.05) for LAB were observed on lean samples treated with MH-A than MH-NA following storage. In summary, Activin used either in a single treatment or a multi-hurdle approach was not significantly more effective than any other treatment against *E. coli O157:H7*. However, Activin did show an effect on coliforms and LAB following a 7 day storage period.
Key Words: Activin, lactoferrin, decontaminates, beef, *E. coli O157:H7*

INTRODUCTION

E. coli O157:H7 is the major food safety concern for the beef industry, and has cost the industry over $2.6 billion in the past 10 years (NCBA, 2005). Since 1982, there have been over one hundred outbreaks of *E. coli,* of which 52% were linked to beef (Elder et al., 2000, Arthur et al., 2004). In 2005, the FSIS reported a 43.3% decrease in *E. coli* O157:H7 positives in ground beef from 2003 to 2004 and an 80% decrease in positives since 2000 (NCBA, 2005c). This reduction is due to the industry's implementation of HACCP and carcass washes/sanitizing practices including steam pasteurization, hot water washes (>74° C), organic

acid rinses, steam/hot water vacuum, and high pressure water rinses (NCBA, 2005c)

In 2001, A. S. Naidu developed and patented a new organic microbial blocking agent, Activin. Activin's main ingredient, lactoferrin derived from bovine milk whey, is proven to be bacteriostatic and bactericidal against many gram-negative and gram positive bacteria (Farnaurd and Evans, 2003). Lactoferrin has the ability to sequester iron, attach to the bacterial cell's membrane causing damage and/or death, detach bacteria from the surface of beef tissue, and attach to the surface of beef tissue blocking bacteria attachment sites (Naidu and Bidlack, 1998). Locke (2002) observed MAP beef steaks treated with Activin suppressed total plate counts and extended desirable color by two days.

The objectives of this research was to compare Activin treatments to other proven interventions (i.e. hot water, high pressure, lactic acid) on the reduction of *E. coli* O157:H7 on adipose tissue, evaluate Activin's ability used in a multi-hurdle spray wash sanitizing sequence to enhance reduction of *E. coli* O157:H7 on lean and adipose tissues, and evaluate Activin's ability used in a multi-hurdle sequence to reduce natural bacterial loads on lean and adipose tissue following a 7d refrigerated storage period. The multi-hurdle sequence was National Beef Packing Company's. All times, temperatures, treatments, and chain speeds simulated actual commercial processing.

MATERIALS AND METHODS

Description of Model Spray Cabinets

Test Wash & Sanitizing Spray Cabinet. A model spray washing and sanitization cabinet was designed by Chad Co. (Olathe, KS), (See Figure 2). The cabinet had two chambers with the ability to spray three different liquids at any one time. The cabinet included a mechanized plate (20 cm x 20 cm) to suspend samples during treatment and was able to simulate chain speeds. Samples were suspended 30.5 cm from all spray nozzles. The first chamber was capable of supplying sanitizing chemical agents in a fine mist through four nozzles (H1/8VVSS80015; UniJet, Wheaton, Illinois). The nozzle pattern created a vertical spread of solution to cover the sample while in motion. The second chamber contained the remaining two spraying apparatus for application of spray treatments. A set stationary pattern of 2 nozzles (H1/8USS5020; UniJet) for low pressures created a vertical fan pattern that completely covered the sample plate area. These nozzles where placed one above the other. The second had 6 oscillating (64 revolutions per minute) nozzles (1/8MEG2510; UniJet) for higher pressures (758-2500 kPa) with horizontal spray patterns. The nozzles were stationed 3 sets of 2 on top of each other. Each nozzle during rotation sprayed water from the top of the cabinet to the bottom covering the sample plate. The cabinet was self contained and all runoff from various treatments was captured in a tank and sanitized with a predetermined amount of sodium hypochlorite to kill any pathogenic bacteria. During application of heavy spray treatments in chamber two, the cabinet was fully closed with doors on both ends to prevent the

spread of any pathogenic bacteria. For additional nozzle information see tables in Appendix F.

Electrostatic Spray System (ESS). A model electrostatic spray system and cabinet was designed to replicate National Beef Packing Company's system currently in use by Electrostatic Spraying Systems (Watkinsville, GA). The system was comprised of a plastic cabinet (Figure 8), a 2 dimensional aluminum carcass (238.7 cm x 72.66 cm) for sample suspension, a four nozzle electrostatic module assembly (using 3 of the nozzles) and an Alan-Bradley Control Module. The system was designed to allow 3 milliliters of solution to be dispersed per nozzle (n=3), at an air flow rate of 0.218 standard cubic meters per minute, with a droplet charge of 20 μ Amperes to 8 μ Amperes. The sample was suspended in the middle of the grounded aluminum carcass with hooks and faced directly at the nozzles approximately 91.5 cm from nozzle to sample.

Inoculum preparation

A six strain inoculum of *Escheria coli* O157:H7 was prepared from five viable strains (237AC1, 299AB3, 133AC1, 114AC1 and 55AC1) obtained from United States Department of Agriculture (USDA) Meat Animal Research Center (Clay Center, Nebraska). A sixth *E. coli* O157:H7 strain (ATCC 43895) was obtained from the American Type Culture Collection (Manassas, VA). Each strain was prepared in a tryptic soy broth (TSB), and grown overnight at 37° C. A portion of each culture was combined, vortexed, and diluted (1 x 10^8 cfu/ml) immediately prior to use. Following preparation of the six strain inoculum, it was placed into coolers with ice packs, and immediately transported to the abattoir of

Oklahoma State University Food and Agriculture Products Center (FAPC) (Stillwater, OK), a USDA inspected facility, for inoculation of samples.

Sanitizing treatments

The sanitizing treatments consisted of Activin, Activin's buffer, 2 % lactic acid, high pressure water and hot water. Activin and buffer solutions (aLF Ventures LLC, Pomona, CA; Naidu, 2001) were prepared by mixing de-ionized water meeting specifications set forth by aLF Ventures LLC, 24 h prior to use and held in closed containers at 7° C until use. Activin and buffer were applied at 7° C and each sample received 9 ml of spray (3 ml from 3 nozzles) from the ESS for all experiments. A 2% lactic acid solution (88 % stock solution, Birko Corporation, Henderson, CO) was diluted using purified bottled water 24 hrs prior to using the liquid. Lactic acid was held and applied at room temperature (24° C-28° C). To simulate chain speed application (270-300 carcasses per hour) at a distance of 15.24 cm per second, a flow rate of 0.416 liters per minute at a pressure of 137.9 kPa per nozzle. The high pressure water wash was applied as samples were stationary, using tap water at 32° C and a pressure of 758.4 kPa rinsing dispensing 7.04 liters per minute. Lastly, the hot water wash used a mixture of tap water and steam (93.3° C upon entering the cabinet) to achieve a meat surface temperature of 71° C, verified by a temperature recording decal (Wahl Instruments Inc., Asheville, NC). The hot water rinse dispensed 3.13 liters per minute at 34.47 kPa.

Sample Handling

Hot (25° C to 30° C) adipose tissue, removed from the area covering the deep pectoral (brisket), and/or hot lean tissue (25° C to 30° C), Cutaneous omo-brachialis (shoulder rose), were removed from carcasses following hide removal, but prior to application of any post-mortem antimicrobial washes from a commercial slaughtering facility (Creekstone Farms, Arkansas City, KS). The tissue samples were transported to FAPC using unsealed vacuum bags (Cryovac, Duncan, SC) in a ice chest to maintain temperature (25° C to 30° C), until inoculation (< 3 hrs). Each sample was placed horizontally on chemically sterilized metal trays, aseptically cut with a sterile knife blade into approximately 15 cm x 10 cm rectangular portion, four 25 cm^2 areas were marked with sterile stainless steel templates (5 cm x 5 cm, 25 cm^2) using edible ink, and inoculation was accomplished by evenly dispensing 0.25 mL over the 25 cm^2 area, to accomplish an inoculation of 1 x 10^6 CFU/cm^2. The inoculum was then carefully spread not to allow run off outside of the marked area. Following inoculation each sample was assigned a treatment and held at room temperature (25° C to 28° C) for 30 min to allow for attachment of bacteria. Two 25 cm^2 areas were aseptically removed for pre-treatment (control) enumeration using a sterile knife and forceps. The remaining two 25 cm^2 areas were then spray-washed with specified treatments under specific conditions. Following specified treatments, the remaining two 25 cm^2 samples were aseptically removed for post-treatment enumeration using a sterile knife and forceps. All samples were aerobically

packaged in a Whirl-Pak bag, sealed and placed on ice for overnight shipment to Food Safety Net Services (San Antonio, TX).

Description of Experiments.

Validation Experiment. *A v*alidation attachment experiment was carried out to determine the effect length of time between hide removal and sample inoculation has on attachment of *E. coli* O157:H7. Adipose tissue was removed from beef carcasses (as described earlier) at the FAPC abattoir, placed in bags to simulate transport, and carried to the FAPC pathogen lab (room 307). Samples (n=25) were then inoculated after 60, 90, 120 150, 180 min as described below. After inoculation, there was an attachment period of 30 min. Samples (25 cm^2) were excised as described and placed in a Whirl-Pak bag, with 25 ml of buffered peptone water. Samples were pummeled using a Seward Laboratory Stomacher/Blender 400 (Seward Company, United Kingdom) at low speed for 5 s to remove loosely attached cells. Samples were removed, placed in sterile Whirl-Pak bag, and enumerated at FAPC pathogen lab using the same procedure as below.

Experiment 1. This experiment evaluated all treatments using adipose tissue.

Treatment 1: Activin (A) application by electrostatic spray, wait for 30 min followed by a high pressure water rinse for 15s.

Treatment 2: Activin buffer (B) application by electrostatic spray, wait for 30 min followed by a high pressure water rinse for 15s.

Treatment 3: Lactic Acid rinse (LA) for 15.24cm/s , wait for 30 min followed by a high pressure water rinse for 15s.

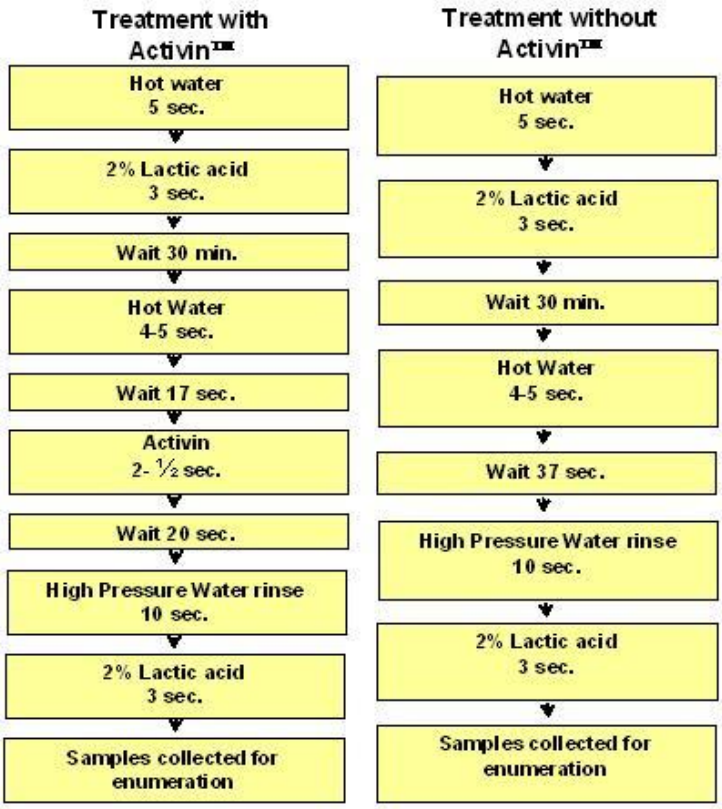
Treatment 4: Hot water rinse (HW) for 3 s, wait for 30 min followed by a high pressure water rinse for 15s.

Treatment 5: High pressure water rinse (HP3) for 3 s, wait for 30 min followed by a high pressure water rinse for 15 s.

Treatment 6: Single high pressure water (HP15) rinse for 15 s.

Experiment 2 (National Beef Company Multi-Hurdle Sequence). Experiment 2 evaluated the National Beef Packing Company's multi-hurdle sequence with Activin (MH-A) and without Activin (MH-NA) on lean and adipose tissue. Information of the National Beef Company multi-hurdle sequence is overviewed in Figure 4.

Figure 4: National Beef Company intervention sequence with Activin (MH-A) and without Activin (MH-NA).



Experiment 3 (Shelf-life). The objective was to evaluate differences of natural microbial loads (uninoculated) on adipose and lean tissue treated with MH-A and MH-NA after 7 days stored at 7° C. All the samples were removed aseptically as described above and placed in a Whirl- pak bag and stored (7°C) for 7 days and shipped to Food Safety Net Services for enumeration of total plate counts, coliform counts, *Enterobacteriaceae*, and Lactic Acid Bacterial counts.

Microbial Enumeration

E. coli O157:H7. 25 ml of buffered peptone water to the bags containing the 25 cm^2 samples. The bags were pummeled using a Seward Laboratory Stomacher/Blender 400 (Seward Company, United Kingdom) for 1 minute at 230 RPM, diluted when necessary and 1 ml of diluent was plated in duplicate onto ntRainbow plates. The plates were incubated (37° C) for 24 hrs and counted. This method allowed for a limit of detection down to 1 cfu/ cm^2. (AOAC, 2000)

Total Plate Count. Food Safety Net Services followed standard plating methodology as outlined by FDA's Bacteriological Analytical Method (US-FDA, 2005). Samples were diluted with peptone in a sterile stomacher bag and stomached for 1 minute. The homogenate was then spiral plated (0.25 ml per plate in quadruplet) onto tryptic soy agar. Plates were incubated at 25° C for 48 hrs, counted and reported in CFU per cm^2.

Coliform Count. Coliforms were enumerated using 3M Petrifilm Coliform Count Plates (St. Paul, MN) (3M, 2005a). Samples were diluted with peptone in a sterile stomacher bag and stomached for 1 minute. The homogenate was then

plated in quadruplet. The films then were incubated at 32° C for 24 hrs, counted and reported in CFU per cm^2.

Enterobacteriaceae. Enterobacteriaceae were enumerated using 3M Petrifilm Enterobacteriaceae Count Plates (St. Paul, MN) (3M, 2005b). Samples were diluted with peptone in a sterile stomacher bag and stomached for 1 minute. The homogenate was plated in quadruplet. The films then were incubated at 35° C for 24 hrs, counted and reported in CFU per cm^2.

Lactic Acid Bacteria. Food Safety Net Services used a standard method for enumeration of lactic acid bacteria from Compendium for the Microbiological Examination of the Foods (1992)

Data Analysis

Data in the form of colony forming units per cm^2 were analyzed using Proc Mixed method (PROC mixed Version 8, SAS Institute, Cary, NC) to analyze attachment of *E. coli* O157:H7 over time on adipose tissue (Validation Experiment), compare reductions of *E. coli* O157:H7 by the first treatment (Experiment 1), reduction of *E. coli* O157:H7 due to different sequential spray treatments (Experiment 2) and the reduction of bacteria detected at the end of the storage period (Experiment 3). Reductions for each treatment were determined by first averaging amount of bacteria in CFU/cm^2 on the controls for each individual sample (n=2), minus the treated samples (n=2). A predetermined significance level of $P< 0.05$ was used. Mean separation was completed using Least Significance Difference.

RESULTS

Validation Experiment (E. coli 0157:H7 Attachment). The amount of tightly bound bacteria on fat samples decreased as time between hide removal and inoculation increased ($P < 0.05$) (See Figure 5). Greatest attachment, 5.24 log CFU/cm^2, occurred at only 60 min following hide removal, having significantly ($P < 0.05$) greater attachment of *E. coli* O157:H7 on adipose tissue, than inoculated 120, 150 and 180 min following hide removal. Furthermore, samples inoculated 90 min following hide removal were significantly different ($P < 0.05$) than those inoculated 180 min following hide removal, having attachment of *E. coli* O157:H7 of 5.07 log CFU/cm^2 and 4.79 CFU/cm^2, respectively. There was no difference ($P > 0.05$) in attachment of *E. coli* O157:H7 on samples inoculated 90 min following hide removal to samples inoculated 120 and 150 min following hide removal. All samples inoculated at 120, 150 and 180 min, displayed no difference ($P > 0.05$) in attachment of *E. coli* O157:H7 on adipose tissue.

Experiment 1. All spray-washing/rinsing treatments effectively reduced the amount of *E. coli* O157:H7 on adipose tissue between 1.6-2.8 log CFU/cm^2 (Table 3 & Figure 6). The treatment-by-day interaction was not significant ($P > 0.05$) of the single treatments. However, significant main effect differences ($P < 0.05$) were observed for the reduction of *E. coli O157:H7* across the two days of sampling, with the samples that were subjected to treatments on d 1 having higher ($P < 0.05$) reductions of *E. coli O157:H7* than that of samples being subjected to treatments on d 2 of sampling, 2.6 log CFU/cm^2 vs. 2.1 log CFU/cm^2 respectively. All samples subjected to the six treatments on d 1 responded

similarly, having no significant difference (P> 0.05). However, d 2 of sampling revealed differences (P< 0.05) in reductions of *E. coli* O157:H7 across means of the six treatments. Samples subjected to HW had the highest average reduction of *E. coli O157:H7*, 2.8 logs CFU/cm^2, being significantly greater (P< 0.05) than samples subjected to treatments of HP15, A, HP3 and B. No reduction differences (P> 0.05) of *E. coli* O157:H7 were observed between samples receiving HW and LA treatments. Furthermore, LA treatments displayed larger reductions (P< 0.05) of *E. coli O157:H7* than samples treated with B. Samples treated with A, B, HP3 and HP15 displayed no differences (P> 0.05) in reduction of *E. coli* O157:H7 on adipose tissue.

Experiment 2 (National Beef Multi-Hurdle Sequences). Both intervention sequences (MH-A, MH-NA) were effective in reducing *E. coli* O157 from both adipose and lean tissues, 2.6-2.8 log CFU/cm^2. No differences (P> 0.05) were observed for the tissue-by-treatment interaction. Additionally, neither treatment nor tissue main effects were found be significant (P> 0.05) as well. (Figure 7)

Experiment 3 (Shelf-life). The uninoculated/unwashed controls on both lean and adipose tissue reached total plate counts (TPC) in excess of 6 logs CFU/cm^2, following a 7 d storage period (7° C). As for TPC, there was no (P> 0.05) treatment-by- tissue interaction, nor was there a difference (P> 0.05) in suppression of growth due to treatment of both adipose and lean samples MH-A or MH-NA. (See Figure 8) However, both sequential spray treatments applied to lean tissue displayed a much greater (P> 0.05) reduction, 2.7 log CFU/cm^2, on

TPCs than did the treatments applied to adipose tissue, having a suppression of growth of 0.37 CFU/cm^2.

There was no (P> 0.05) treatment-by-tissue sample interaction on reduction of coliforms. Furthermore, there was no (P> 0.05) difference for the main effect of tissue type. Nonetheless, uninoculated adipose samples treated with MH-A significantly (P< 0.05) suppressed the growth of coliforms, when compared to adipose tissue treated with MH-NA following the storage period (See Figure 9).

There was no differences (P> 0.05) observed in the reduction of *Enterobacteriaceae* on tissue sample or treatment type. There was a slight trend (P=0.15) showing both lean and adipose tissue samples treated with MH-A suppressed *Enterobacteriaceae* counts over both lean and adipose tissue samples treated with MH-NA, having a reduction of 2.12 CFU/cm^2 and 1.16 CFU/cm^2 over control samples, respectively (Figure 10).

No treatment-by-tissue interaction was observed (P> 0.05) for the reduction of lactic acid bacteria. However, lean samples treated with MH-A and MH-NA had significantly (P< 0.05) decreased growth of lactic acid bacteria following storage, when compared to adipose samples treated with MH-A and MH-NA following storage. In addition, lean samples treated with MH-A had an extremely higher (P< 0.05) reduction of lactic acid bacteria, than lean samples treated with MH-NA. Lastly, there was no difference (P> 0.05) in the suppression of lactic acid bacteria due to treatment on adipose tissue following a storage period. (See Figure 11).

DISCUSSION

The validation experiment proved there was differences ($P < 0.05$) between 60 min following hide removal and 180 min following hide removal to inoculation for the attachment of *E. coli* O157:H7; however, sample collection (25 min) and transportation (75 min) would cause inoculation of samples to be after 90 min post-hide removal. Statistical analysis revealed that 120 min, 150min and 180 min post-hide removal inoculation times were not different ($P > 0.05$) in attachment of *E. coli* O157:H7. Thus, the point in which we would stop inoculating would be 180 min. In addition, to reduce the variation from the sample that was inoculated at the beginning until the last sample, treatments where randomly pre-determined to reduce the variation in the number of organisms that would attach. This difference might be due to the noticeable change in appearance of adipose tissue as time after hide removal increased. The adipose tissue appeared dry and plastic like, which was thought to be caused by the loss of moisture. The inoculum seemed to take longer to absorb onto adipose tissue as length of post-hide removal to inoculum increased. According to Firstenberg-Eden (1981), attachment of bacteria happens in a two stage process, a loose reversible stage and an irreversible stage. If the inoculum took longer to absorb, the bacteria did not reach the surface at the same time, and the second stage of attachment of some bacteria may either not have occurred or be weak enough that the quick (5 s) pummeling released these bacteria. Background bacteria present on the adipose tissue may have also

played a small role in the number of *E. coli* O157:H7 attached as time increased from hide removal.

On the first day of Experiment 1, an unexplained amount of condensation and iron was found in the filtered high pressure air line used for A and B applications. The problem was eliminated by installing a drier/filter into the line prior to the air entering the electrostatic spray system. This was accomplished before any testing was done on the second day. There is an unexplained significant ($P< 0.05$) main effect, that occurred in which the first day not only had greater reductions ($P< 0.05$), but data shown in table 7 revealed there is a much a higher and consistent number of bacteria found on the first day's control samples, 5.2-6.1 log CFU/cm^2, than the controls on the second day, 4.4-5.3 log CFU/cm^2. However, the treated samples show similar levels on d 1 and d 2, having 2.4-3.0 log CFU/cm^2 and 1.6 -2.8 log CFU/cm^2, respectively. Bacterial attachment to animal tissues is complex (Firstenberg-Eden, 1981). The attachment of bacteria on the control samples could be affected by inoculum concentration (Butler et al., 1979; Notermans and Kampelmacher, 1975), temperature of the medium used for attachment (Butler et al., 1979), structure and morphology (Butler et al., 1979), the individual inoculating, or individual enumerating the samples. In Experiment 2 (National Beef Company sequence), bacterial attachment differences between adipose tissue and lean tissue was not statistically significant , which is in agreement with findings by Cabedo et al. (1997) and Dickson and Frank (1993). The reductions between adipose tissue and lean tissue in Experiment 2 were not significantly different ($P> 0.05$).

However, Dickson (1998) suggested a stronger attachment occurs on muscle tissue than adipose tissue when washed with NaOH and KOH. Dickson and Frank (1993) reported an unexplained high variability in irreversible attachment of cells attached to adipose tissue. Thus our findings demonstrate there is no difference in attachment or strength of irreversible attachment of *E. coli* O157:H7 to adipose or lean tissues for experiment 2.

The results obtained in experiment 1 for HW for overall reduction of *E. coli* O157:H7 having 2.8-3.0 log CFU/cm^2, is similar to the reductions reported by Gorman et al. (1995). Working with an inoculation fecal cocktail containing *E. coli,* they found that when the first spray washing treatment involved used hot water (74° C), reductions in counts achieved were in excess of 3.0 log CFU/cm^2. Additional treatments did not further reduce *E. coli* O157:H7. The authors suggested chemical interventions would not be necessary if hot water (74° C; pressures 276, 689, or 2068 kPa) was the intervention chosen for carcass decontamination (Gorman et al., 1995).

LA was as effective as HP15 rinse alone at reducing *E. coli* O157:H7 adipose tissue ($P<0.05$). Thus, LA could possibly take longer than the time allotted to affect *E. coli* O157:H7. LA has been shown to reduce bacteria from 3.7 to 4.7 log CFU/cm^2 (Hardin et al., 1995). LA bactericidal activity is attributed to the undissociated molecule of the acid (Ingram et al., 1956). This causes a build up of acid in the cytoplasm of the cell and ends in acidifying the cell's cytoplasm causing death (Booth, 1985; Huffman, 2002). Gorman et al. (1995) reported when sanitizing agents were followed by plain water spray-washing, the

effectiveness of the sanitizers was lowered. They suggested that loss of activity was possibly due to the physical removal or dilution of the sanitizing agents. The authors stressed the importance of sequence of interventions including chemical sanitizers. Furthermore, LA effectiveness as a final carcass rinse has shown to be reduced, due to the current practice of spray-chilling carcasses, causing a dilution of the acid (Pipek et al., 2005). Dorsa et al. (1998) determined that 2% lactic acid spray could effectively suppress the growth of spoilage bacteria, *Salmonella typhimrium* and *E. coli* O157:H7 stored for 21 days at 4° C. LA in this study was used at ambient temperature (24°-28° C), research has shown that elevating LA temperature to 55° C increases effectiveness and reduction of spoilage and pathogenic bacteria (Pipek et al., 2005). Stopforth et al. (2003) discovered *E. coli* O157:H7 formed biofilms and remained detectable (>1.3 log CFU/cm2) on stainless steel surfaces, when surfaces were expose to organic acid runoff. A growing concern is that forms of *E. coli* O157:H7 have adapted to acidic concentrations and may arise as a major problem in the future.

A and MH-A treatments both effectively reduced *E. coli* O157:H7 to levels of 2.0-2.8 log CFU/cm^2. However, A displayed no difference (P> 0.05) in the reduction of *E. coli O157:H7,* from HP15, LA, HP3. In addition, MH-A displayed no difference (P> 0.05) in the reduction of *E. coli* O157:H7*,* from MH-NA. These findings, were the first to use Activin that was ESS and used in a multi-hurdle approach to combat *E. coli* O157:H7, and were not consistent with findings of in vitro research, where *E. coli* O157:H7 was reduced by 3 log CFU/cm^2 (Jones et al. 1992). Ransom and Belk (2003) found that Activin suppressed growth of *E.*

coli O157:H7 and *Listeria monocytogenes* in sliced bologna by 0.7-1.3 log and 6.2 log, respectively. They also found treatments to beef cuts after inoculation of *E. coli* O157:H7 and *Listeria monocytogenes*, and stored for 29 d in a vacuum bag, showed Activin suppressed growth by 3.3 and 4.7 log, respectively. Activin used in combination with 2% LA was effective in suppressing growth of *E. coli* O157:H7 (1.3 log), *Listeria monocytogenes* (0.8 log) at 2 days of storage at 12° C (Ransom and Belk, 2003). Locke (2002) observed that application of Activin suppressed growth and total plate count numbers during storage. In the present study, treatments containing Activin in Experiment 3 revealed significant ($P< 0.05$) suppression of lactic acid bacteria on lean samples treated with MH-A, a slight trend ($P=0.15$) of suppression of *Enterobacteriaceae* when samples where treated with MH-A and a significant difference ($P< 0.05$) of suppression of coliform counts on adipose tissue treated with MH-A compared to MH-NA treated samples. All the findings from the present study, Locke (2002) and Ransom and Belk (2003) suggests the possibility Activin may need more time than allotted (20 seconds and 30 min) by the sequence before rinsing in this study to aid in the suppression or reduction of microbial growth. This could be supported by Naidu (2002) were *E. coli* O157:H7 lost its fimbriae after 2 hrs of exposure to lactoferrin. Lactoferrin in Activin has never been tested (only used in a plant setting) in an electrostatically spray system, and the molecule could possible undergo a conformation change when charged in the system. Lactoferrin isolated from milk becomes highly susceptible to molecular changes resulting from pH, heat, proteolysis, ionic imbalance, any of which can greatly decrease its effectiveness

as an antimicrobial (Sebranek, 2003). Charging the protein during ESS possibly effects the conformation, iron binding properties, effect its ability to bind to the cell surface or binding ability to the meat surface. Lastly, treatments of hot water or organic acid that precedes and/or follows the Activin spray treatment could possibly affect its antimicrobial effectiveness.

High pressure washes are typically used to physically remove decontamination. HP3 and HP15 both effectively reduced *E. coli* O157:H7 from adipose tissue, and were no more effective at reducing *E. coli* O157:H7 on adipose tissue ($P > 0.05$) than A, B, and LA treatments. A concern in the use of high pressures has been the possibility of physically driving contamination into the muscle and adipose tissues (Groman et al., 1995), and spreading contamination from one area of a carcass to another (Barkate et al. 1993). DeZuniga et al. (1991) through the use of dye penetration models, recommends the maximum pressure used for a spray washing system be 2070 kPa. Reagen et al. (1996) observed less *E. coli* O157:H7 positives were found on carcasses that were washed (410-2758 kPa, 18-39 seconds) compared to carcasses that were trimmed or trimmed and washed. The effectiveness of high pressure washes is dependent upon pressure, angle of nozzles, time of exposure and temperature of water.

Leistner (1995) describes the “hurdle approach” as sequential use of decontamination steps. The sequences if used properly could achieve a synergistic effect. Many studies have tried to quantify this number. However, Gorman et al., (1995), as stated earlier, indicated the use of chemicals before or

after washes reduced the effectiveness of washes. Both MH-A and MH-NA sequences effectively reduced *E. coli* O157:H7, total plate counts, lactic acid bacteria, *Enterobacteriaceae*, and coliform counts in both of the last experiments. Elder et al. (2000) reported that in lots sampled before and after multiple sequence processes in a plant the *E. coli* O157:H7 was reduced from 43.4% of lots positive to 1.9%. Bacon et al. (2000) evaluated multiple-sequential interventions at eight commercial beef plants and found that *E. coli* was reduced from a range of 2.6–5.3 log CFU/ 100 cm^2. In most cases multiple spray washes are more effective than knife trimming on carcasses (Hardin et al., 1995; Dorsa et al., 1996; Reagan et al., 1996). On an interesting note, all treatments in experiment 1 had reductions of *E. coli O157:H7* on adipose Tissue from 1.6 – 3.0 log CFU/cm^2 and the National Beef multi-hurdle intervention sequence had 2.6-2.8 log CFU/cm^2 reduction on adipose tissue.

CONCLUSIONS

All treatments were effective in reducing *E. coli* O157:H7. All treatments containing Activin did not show an additive reduction against *E. coli* O157:H7 when compare to treatments that did not use Activin. However, multi-hurdle sequences that contain Activin had greater suppression of Coliforms and Lactic acid bacteria over 7 d of storage. Recommendations of trying different methods of application for Activin, and allowing longer exposure times to work on spoilage and pathogenic bacteria on carcasses as well as on packaged products should be further explored.

Figure 2: Chad Company (Olathe, Ks) Test Wash and Sanitizing Spray Cabinet.

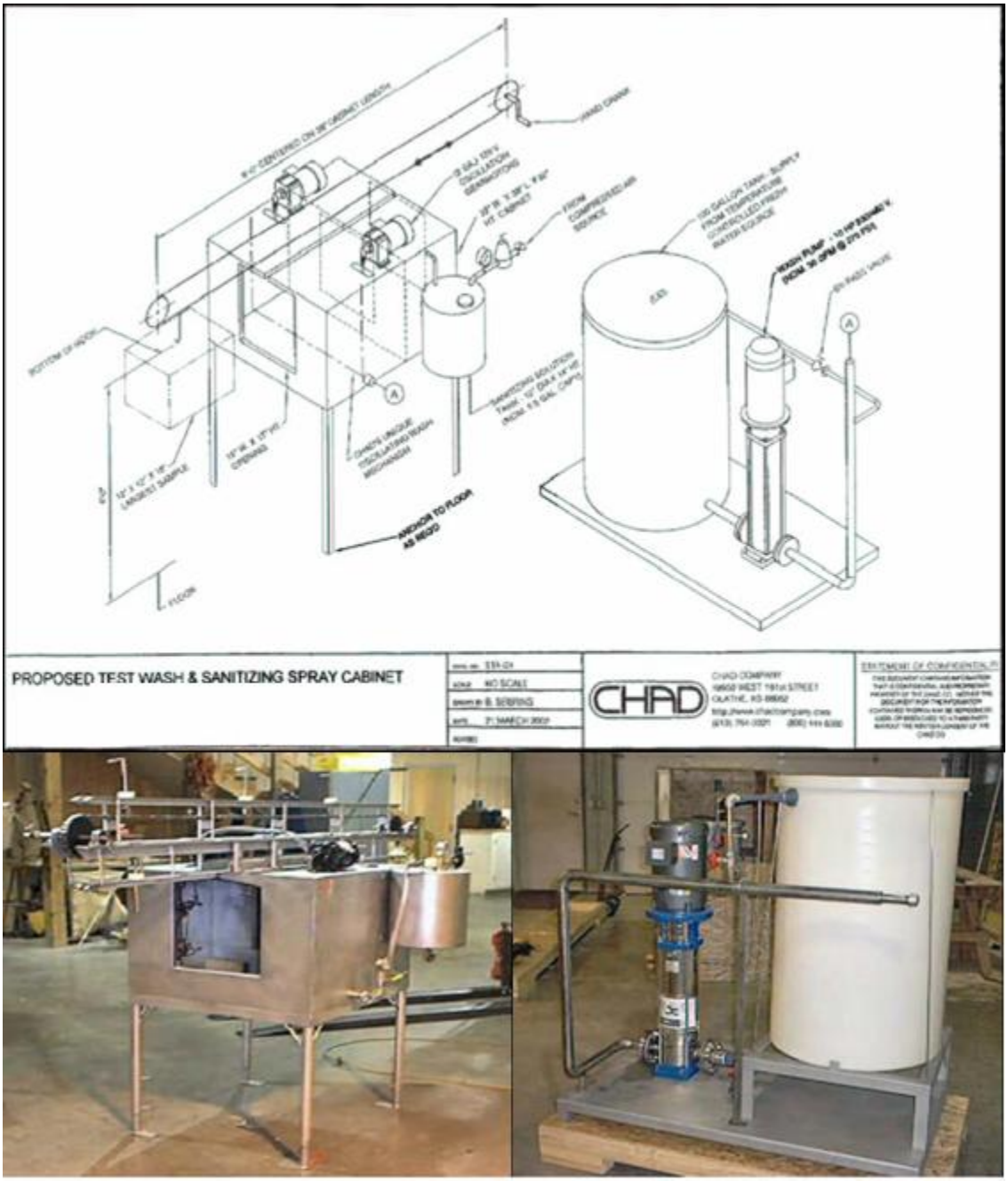


Figure 3: Electrostatic Spray System Test Cabinet, aluminum carcass and spray tower.

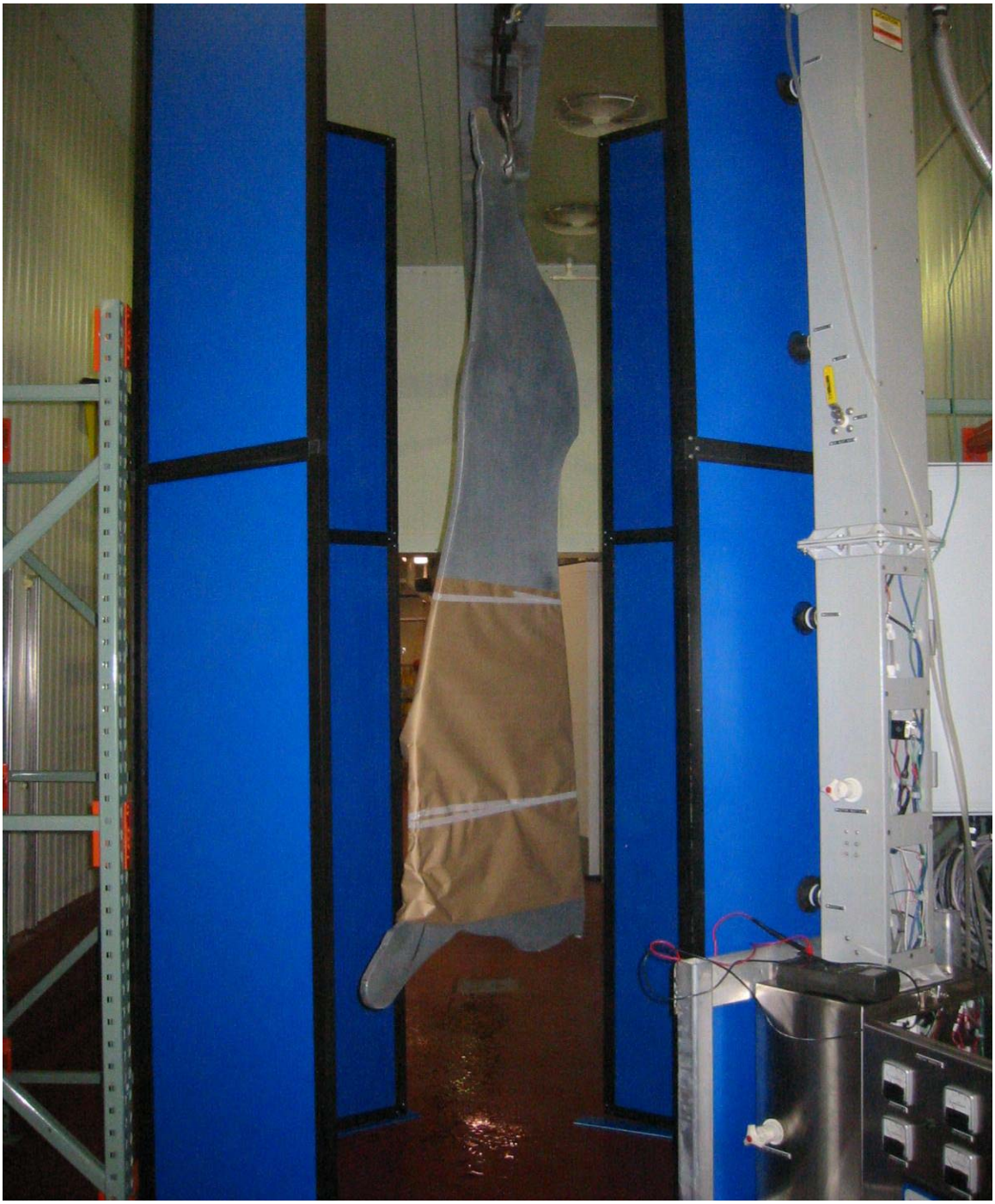
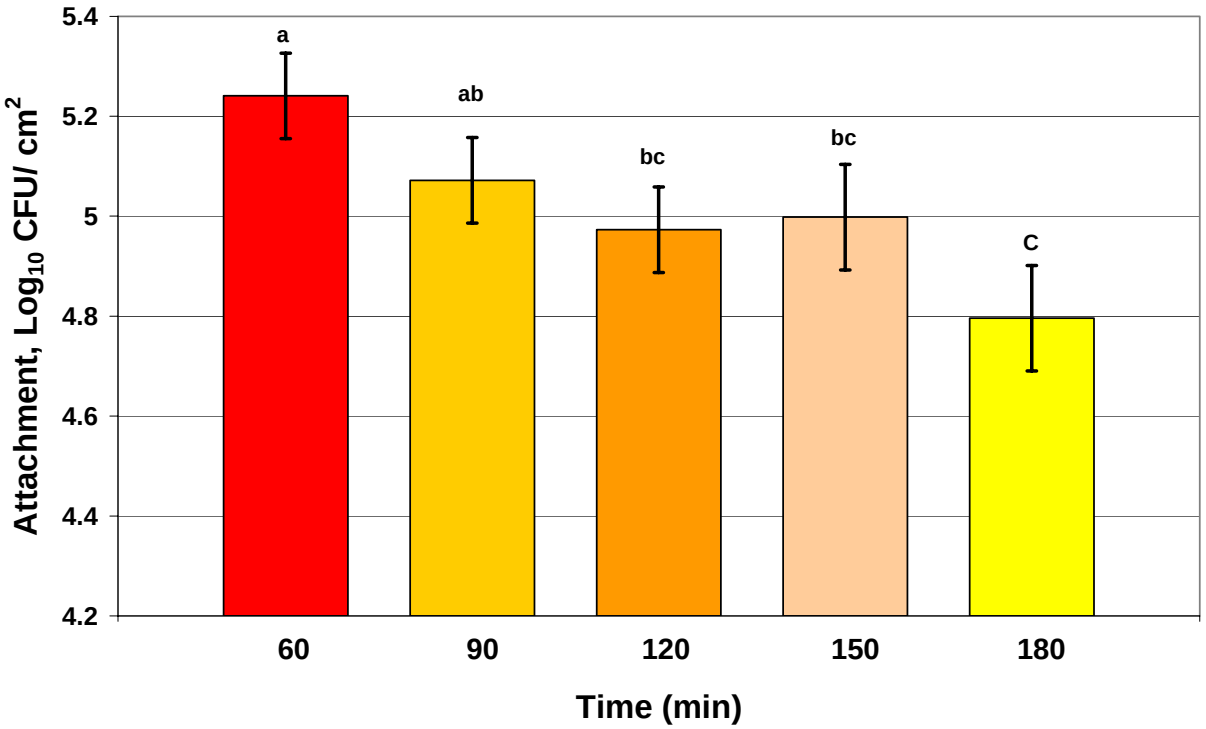
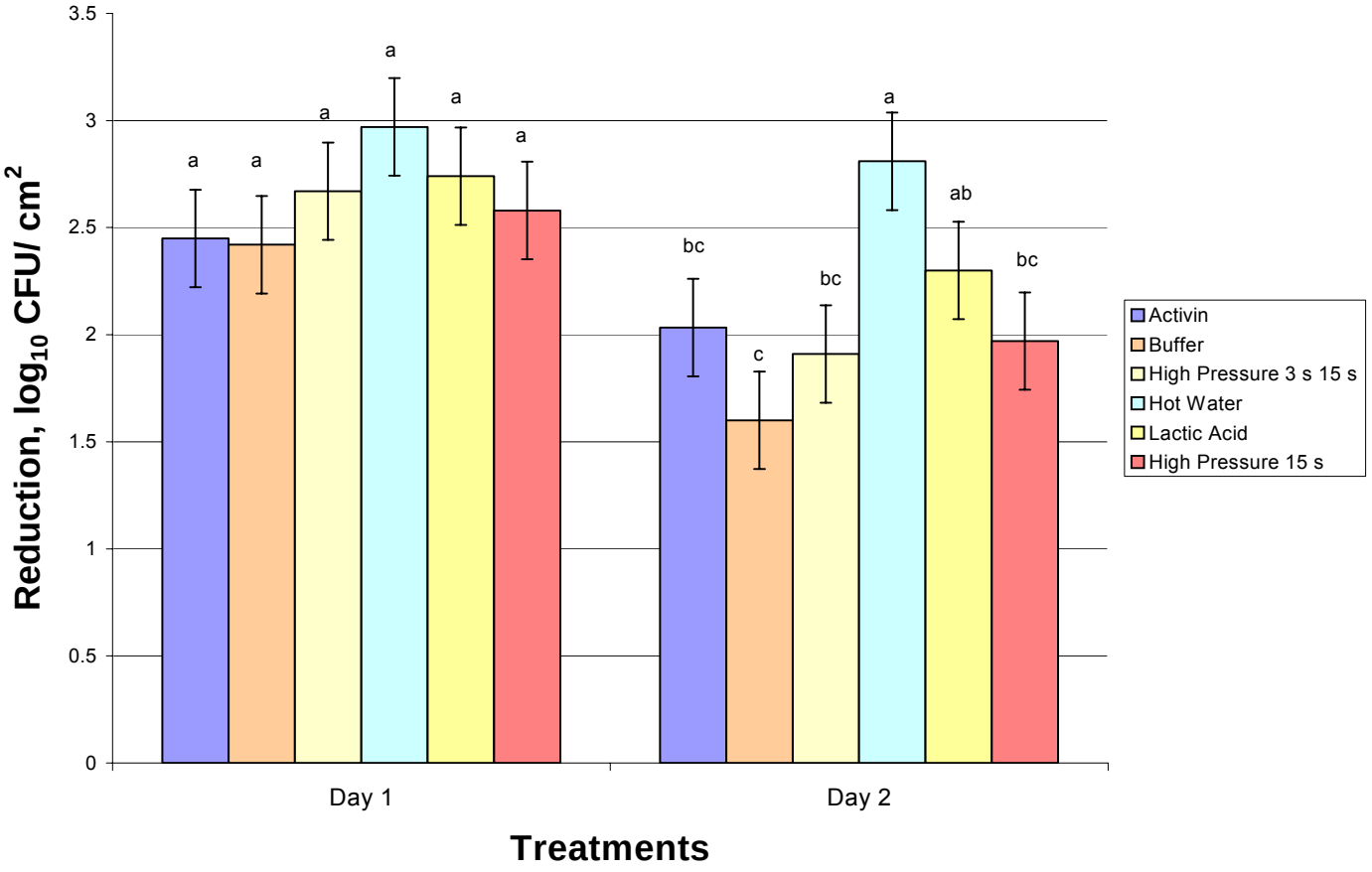


Figure 5- Mean E. coli O157:H7 counts (Log_{10} CFU/cm^2) attached to beef adipose tissue samples inoculated at 1 x 10^6 CFU/cm^2 60, 90, 120, 150, and 180 minutes following hide removal.



[a, b, c] Mean values with different superscripts are significantly different (P< 0.05).

Figure 6 – Mean E. coli O157:H7 counts (Log_{10} CFU/cm^2) as affected by spraying/rinsing treatments applied to beef adipose tissue samples inoculated at 1 x 10^6 CFU/cm^2.



a, b, c Mean values with different superscripts within a day are significantly different (P< 0.05).

Table 3 – Mean E. coli O157:H7 counts (Log_{10} CFU/cm^2) as affected by spraying/rinsing treatments applied to beef adipose tissue samples inoculated at 1 x 10^6 CFU/cm^2.

Treatment	Day 1			Day 2		
	Before Treatment	After Treatment	Reduction[e] (sem)[d]	Before Treatment	After Treatment	Reduction[e] (sem)[d]
Buffer	5.6	3.2	**2.4[a]** (0.02)	4.8	3.2	**1.6[c]** (0.08)
Activin	5.7	3.3	**2.4[a]** (0.11)	5.3	3.3	**2.0[bc]** (0.32)
Hot Water	5.6	2.6	**3.0[a]** (0.32)	4.6	1.8	**2.8[a]** (0.11)
High Pressure 3 s 15 s	5.2	2.6	**2.6[a]** (0.17)	4.4	2.5	**1.9[bc]** (0.33)
High Pressure 15 s	6.1	3.5	**2.6[a]** (0.04)	4.8	2.8	**2.0[bc]** (0.16)
Lactic Acid	5.6	2.9	**2.7[a]** (0.26)	4.8	2.5	**2.3[ab]** (0.13)
Total	5.6	3.0	2.6	4.8	2.7	2.1

[a, b, c] Mean values with different superscripts within a column are significantly different (P< 0.05)

[d] Standard Error of the least squares mean

[e] Least Squares Means

Figure 7- Mean E. coli O157:H7 counts (Log_{10} CFU/cm^2) as affected by Multi-Hurdle Sequences with Activin (MH-A) and without Activin (MH-NA) applied to beef adipose and lean tissue samples inoculated at 1 x 10^6 CFU/cm^2.

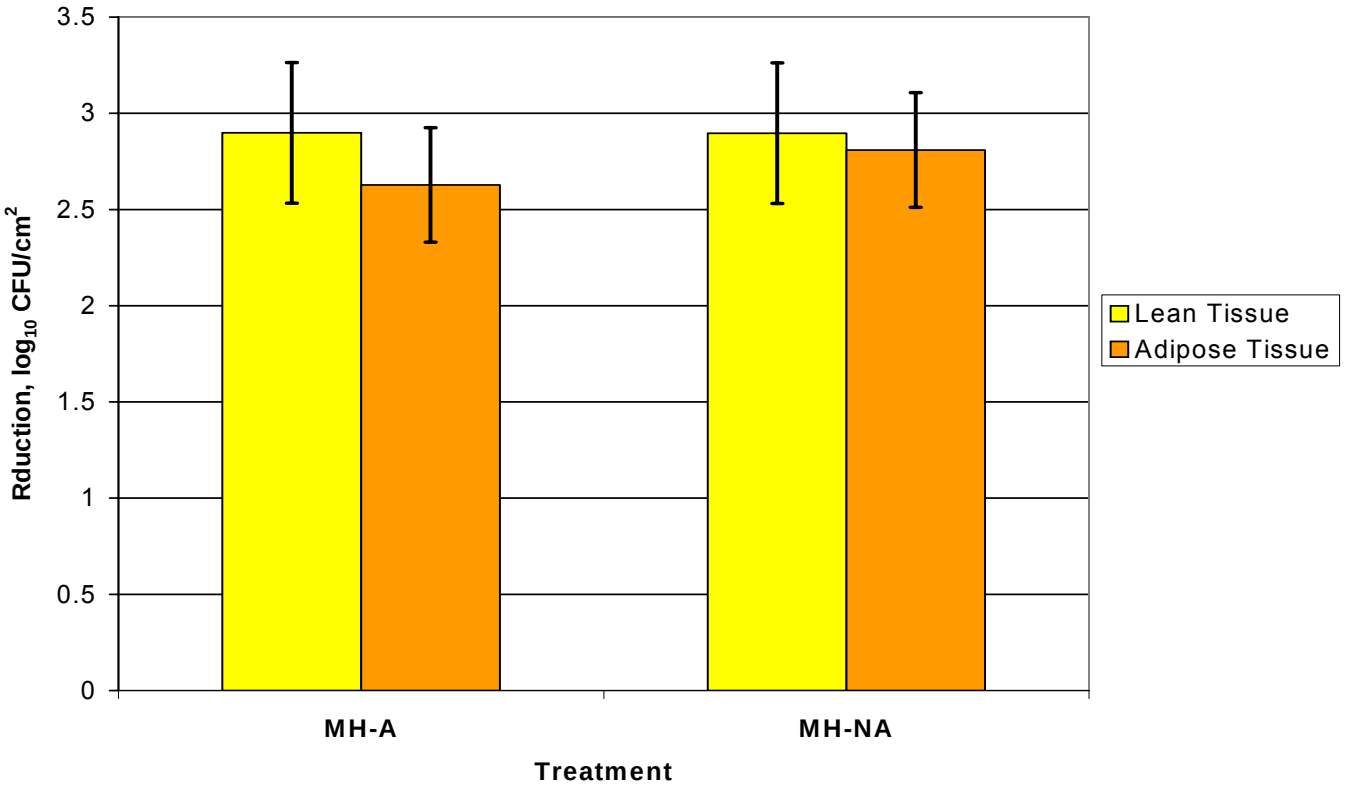
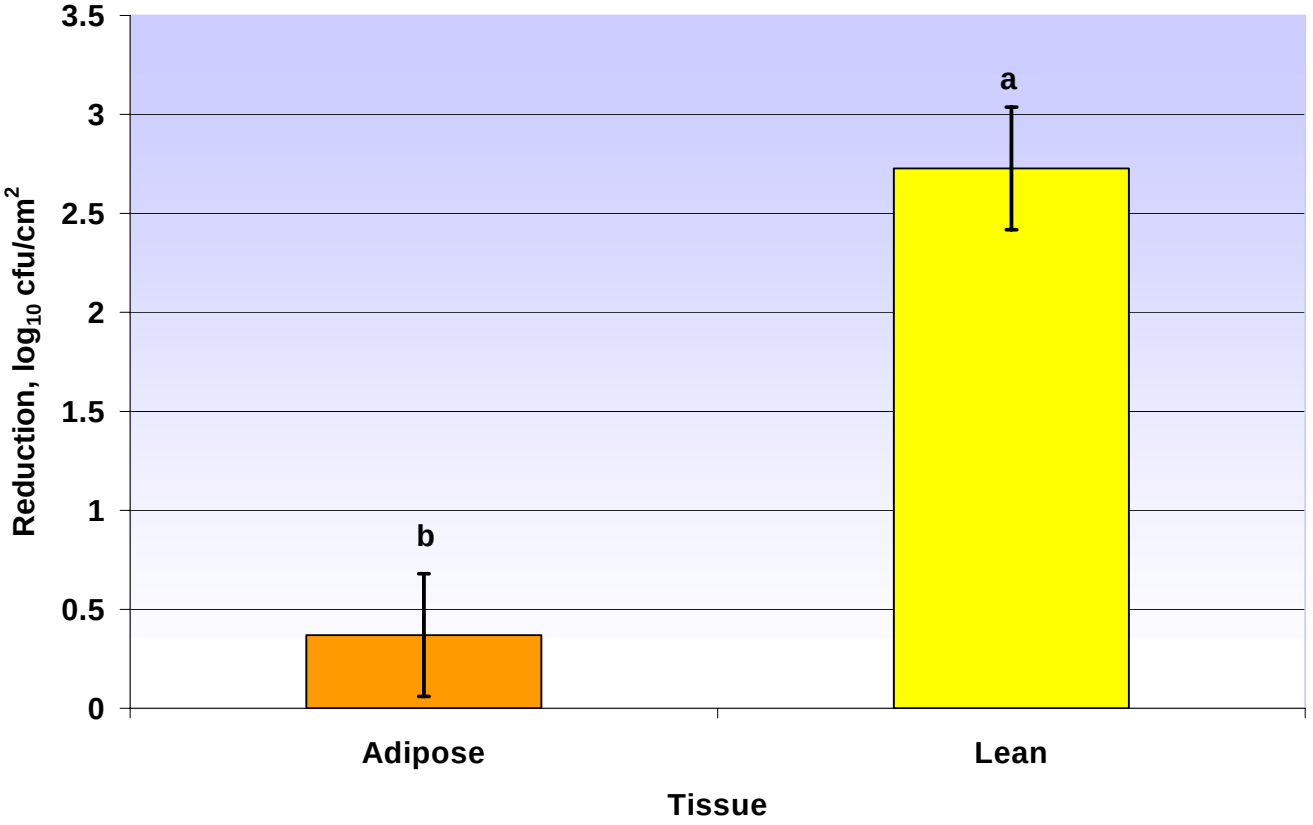
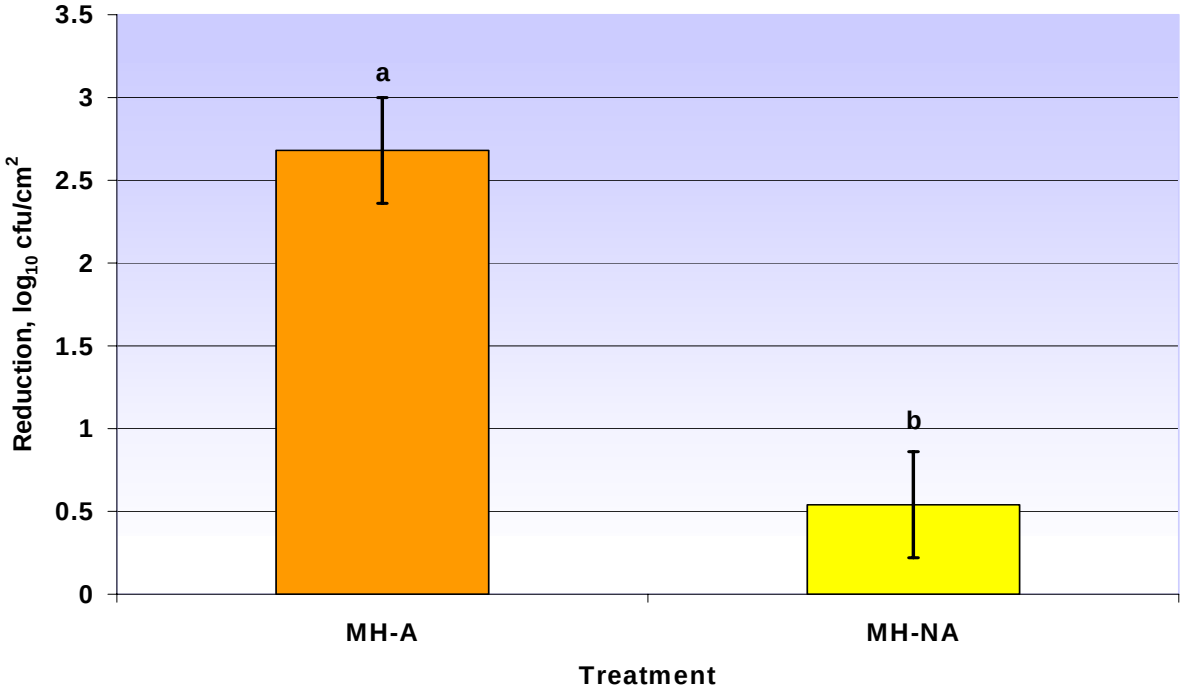


Figure 8- Mean Reduction of Total Plate Counts as affected by Multi-Hurdle Sequences with Activin (MH-A) and without Activin (MH-NA) on lean and adipose tissues combined following 7 d refrigerated (7°C) storage.



[a, b,]Mean values with different superscripts are significantly different (P< 0.05).

Figure 9- Mean Reduction of Colifrom Counts as affected by Multi-Hurdle Sequences with Activin (MH-A) and without Activin (MH-NA) on adipose tissue following 7 d refrigerated (7°C) storage.



[a, b] Mean values with different superscripts are significantly different (P< 0.05).

Figure 10– Mean Reduction of *Enterobacteriaceae* as affected by Multi-Hurdle Sequences with Activin (MH-A) and without Activin (MH-NA) on lean and adipose tissues combined following 7 d refrigerated (7°C) storage.

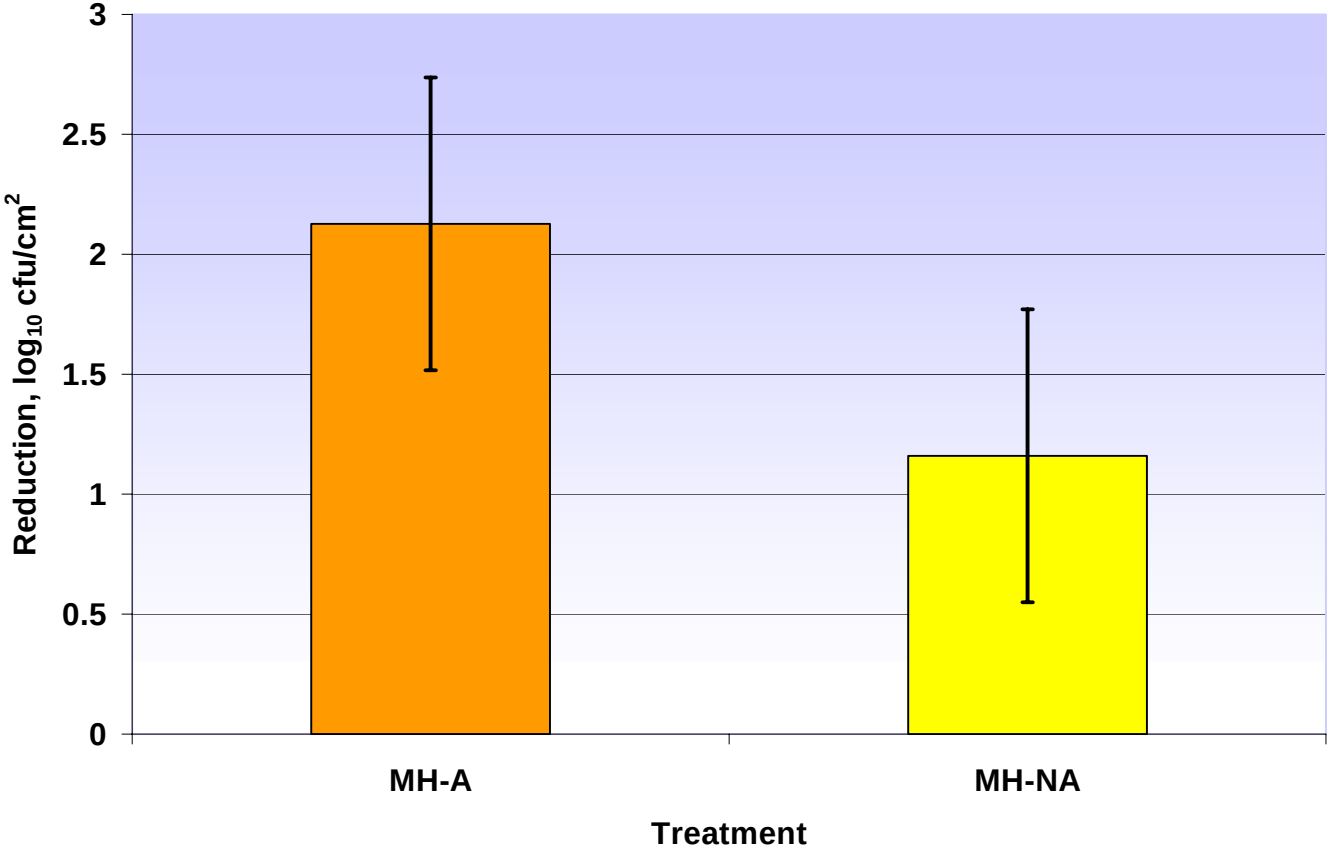
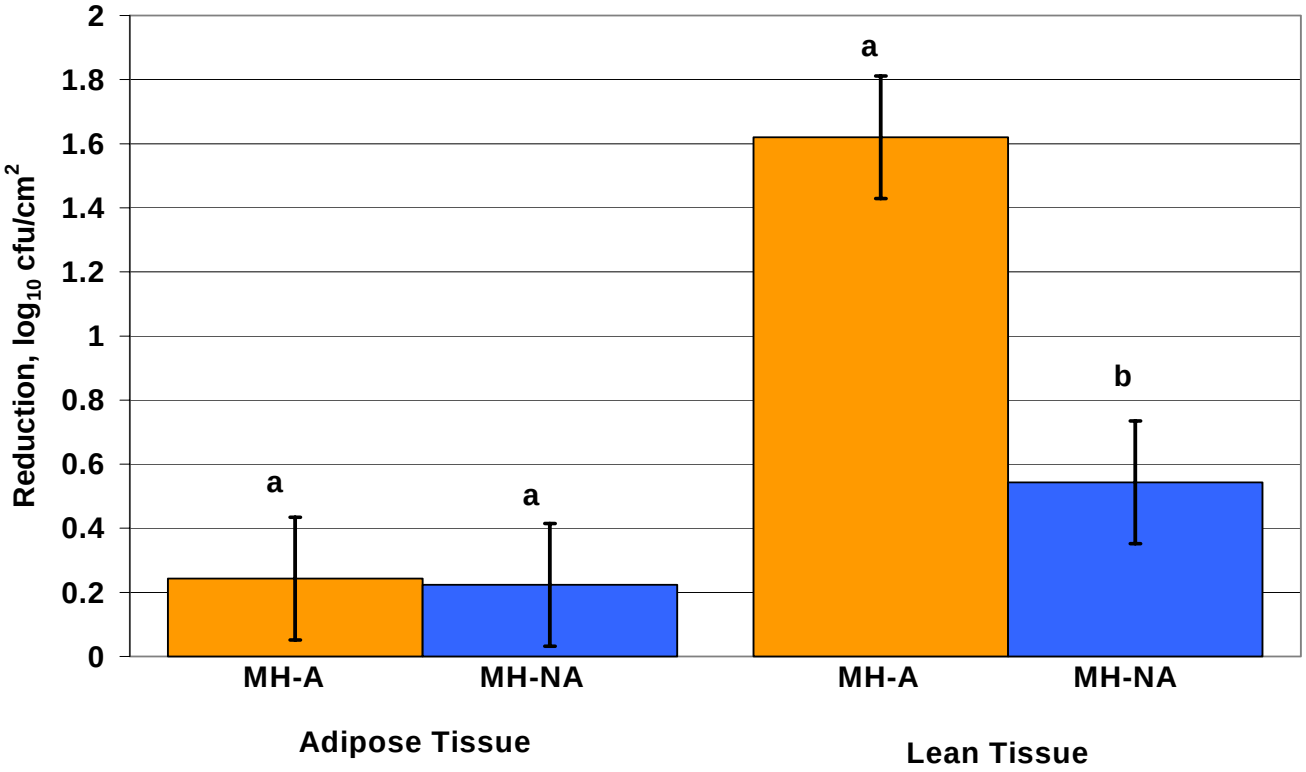


Figure 11- Mean Reduction of Lactic Acid Bacteria as affected by Multi-Hurdle Sequences with Activin (MH-A) and without Activin (MH-NA) on lean and adipose tissues combined following 7 d refrigerated (7°C) storage.



[a, b, c] Mean values in the same tissue type with different superscripts within a column are significantly different (P< 0.05).

Table 4 – Mean Reduction of Lactic Acid Bacteria as affected by Multi-Hurdle Sequences with Activin (MH-A) and without Activin (MH-NA) on lean and adipose tissues combined following 7 d refrigerated (7°C) storage.

Treatment	Adipose			Lean			
	Before Treatment	After Treatment	Reduction[e] (sem)[d]	Before Treatment	After Treatment	Reduction[e] (sem)[d]	Total Reduction
MH-NA	5.51	5.74	**-0.22[a]** (0.13)	5.90	5.36	**0.54[b]** (0.14)	**0.16[b]** (0.14)
MH-A	5.33	5.08	**0.24[a]** (0.24)	5.71	4.09	**1.62[a]** (0.24)	**0.93[a]** (0.24)
Total	5.4	5.4	**0.0[b]** (0.17)	5.8	4.7	**1.1[a]** (0.19)	

[a, b, c] Mean values with different superscripts within a column are significantly different (P< 0.05)

[d] Standard Error of the least squares mean

[e] Least Squares Means

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Appendix

Appendix A

United States Department of Agriculture
Food Safety and Inspection Service
Field Operations
Springdale District Office
4700 South Thompson, B-201
Springdale, AR 72764

September 21, 2004

Mr. Peter Muriana, Associate Professor
Oklahoma State University
Food & Agricultural Products Res. & Technology Center
Department of Animal Science
109 FAPC
Stillwater, Okla. 74078-6055

Dear Mr. Muriana:

The Springdale District Office received the documents pertaining to the Activin research project. This office contacted the New Technology Division of the Office of Policy, Program and Employee Development in Washington, DC for discussion and guidance. FSIS reviews new technologies that companies employ to ensure that their use is consistent with Agency regulations and will not adversely affect product safety, inspection procedures, or the safety of FSIS inspectors.

It is our understanding that since the beef short plates inoculated with the cocktail of E.Coli 0157:H7 will be discarded in plastic-bag-lined containers for incineration, an in-depth, pre-use review by the New Technology Division would not be necessary at this point in time.

In conducting this project, the following steps should be followed:

1) Measures taken to assure that applicable regulations and rules are followed.
2) This process does not affect product safety.
3) It is acceptable to the local inspection team.
4) The establishment follows its SSOP.

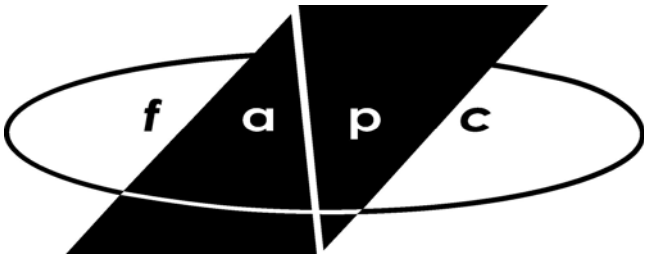
If you have further questions, please contact this office at 479/751-8412.

Sincerely,

Marcia Endersby, DVM
District Manager

c: Dr. Kevin Ehlers, FLS (via electronic mail)
Mr. Freddie Sizemore, IIC (via electronic mail)

Appendix B



HAZARDS ANALYSIS
AND
CRITICAL CONTROL POINTS

ACTIVIN IN-PLANT BEEF CARCASS VALIDATION

PROCESS DESCRIPTION

PROCESS: *Activin In-Plant Beef Carcass Validation*

1. COMMON NAME ?	The Activin Project
2. HOW IS IT TO BE USED ?	A project designed to test the antimicrobial efficacy of Activin against *E. Coli 0157:H7.*
3. TYPES OF PACKAGES USED ?	Plastic bags (including bio-hazard labeled bags), whirl-pack bags, and plastic drums will be used for sample storage and/or residual product storage and disposal.
4. LENGTH OF SHELF LIFE, AT WHAT TEMPERATURE ?	Not applicable
5. WHERE WILL THE PRODUCT BE SOLD ?	All residual products and applicable packaging and containers will be condemned and removed by a medical waste removal company.
6. LABELING INSTRUCTIONS ?	After research is complete, all products and/or samples will be labeled as either “Biohazard” or “Inedible/Condemned”.
7. IS SPECIAL DISTRIBUTION CONTROL NEEDED ?	Yes, according to the subsequent outline of this plan and the written sanitation standard operating procedures for the project.

PRODUCTS, SUPPLIES and SPECIAL CONSIDERATIONS

PROCESS: *Activin In-Plant Beef Carcass Validation*

MEAT MODEL – Beef short plates to represent freshly slaughtered beef carcasses.

CARCASS MODEL – A fabricated aluminum one-dimensional profile of a life-sized beef carcass.

SLAUGHTER FLOOR MODEL – Room 217 of the Food & Ag. Products Center.

TREATMENT MODEL – A stainless steel "spray-cabinet" constructed specifically for this research, which will be set in place to represent normal carcass flow through an industry-type spray cabinet.

INOCULUMS – Strains of *E. Coli* 0157:H7, described in the document titled *Activin In-Plant Carcass Validation* (Muriana).

TREATMENT – Described in the document titled *Activin In-Plant Carcass Validation* (Muriana).

CONTAINMENT – Plastic sheeting will be installed with adhesive tape above and around the spray cabinet to contain any aerosolization of inoculum or treatments. Furthermore, any liquid run-off of inoculums or treatments liquids will be collected in a catch-pan and treated to destroy the pathogen, before it is released into the environment.

PERSONAL PROTECTIVE EQUIPMENT (PPE) – Described in SSOPs and Microbial Pathogen SOPs.

HAZARD IDENTIFICATION / PREVENTITIVE MEASURES

PROCESS *Activity: In-Plant Beef Carcass Validation*

PROCESS STEP	HAZARD (B=Biological)	PREVENTIVE **MEASURES**
Receive Research Supplies	No hazards identified	
Store Research Supplies	No hazards identified	
Discard Used Research Supplies	B-Supplies may be contaminated with the pathogen in use	B-Sterilize or sanitize contaminated supplies and place them in properly identified containers for treatment and disposal
Receive Beef Short Plates	No hazards identified	
Stage Beef Short Plates	No hazards identified	
Inoculate Beef Short Plates	B-The environment may become contaminated with the pathogen in use	B-Proper control of inoculums according to good research practices. Also, collection of run-off in a "catch-pan"
Treat Beef Short Plates	B-The environment may become contaminated with the pathogen in use due to liquid run-off	B-Collection of run-off in a "catch-pan"
Treat Catch Pan	B-Inadequate treatment of catch pan	B-Sanitizing of catch pan materials
Excise Samples From Beef Short Plates	B-Equipment used for excisions may become contaminated with the pathogen in use	B-Good research practices for work with pathogens (Microbial SOPs).
Package Excised Samples	B-Samples in the package will have the pathogen present	B-Proper labeling of packages
Store Excised Samples	B-Samples in the package will have the pathogen present	B-Proper labeling of packages
Distribute Excised Samples	B-Proper labeling of packages	B-Proper labeling of packages

HAZARD IDENTIFICATION / PREVENTITIVE MEASURES

PROCESS STEP	HAZARD (B=Biological)	PREVENTIVE **MEASURES**
Discard Beef Short Plates in Containers	B-Product in the container may have the pathogen present	B-Proper labeling of the containers
Clean/Sanitize Beef Short Plates	B-Beef short plates may not be effectively treated	B-Follow procedures for cleaning/sanitizing the product
Seal Containers	B-Product in the container may have the pathogen present	B-Proper sealing of the container lids
Distribute Containers to Storage	B-The containers may have the pathogen present on the outside of the container	B-Sterilize/sanitize outside of containers before distribution
Distribute Containers to Waste Removal Company	B-Product in the container may have the pathogen present	B-Proper labeling of the containers
Receive Pathogen (*E.Coli* 0157:H7)	B-*E. Coli* 0157:H7 is a pathogen known to cause human illness.	B-Proper labeling and handling according to good research practices (Microbial SOPs)
Store Pathogen	B-*E. Coli* 0157:H7 is a pathogen known to cause human illness.	B-Proper labeling and handling according to good research practices (Microbial SOPs)
Prepare Pathogen for Inoculation	B-*E. Coli* 0157:H7 is a pathogen known to cause human illness.	B-Proper labeling and handling according to good research practices (Microbial SOPs)
Destroy Residual Pathogen	B-Survival of the pathogen	B-Proper handling and destruction according to good research practices (Microbial SOPs)
Discard the Destroyed Pathogen	B-Survival of the pathogen	B-Proper handling and destruction according to good research practices (Microbial SOPs)

CCP DETERMINATION (1 OF 4)

PROCESS: *Activin In-Plant Beef Carcass Validation*

PROCESS STEP	HAZARD (S) BIOLOGICAL – B CHEMICAL – C PHYSICAL – P	Q1. DO PREVENTITIVE MEASURES EXIST FOR THE IDENTIFIED HAZARDS? *If no = not a CCP- Identify how and where This hazard will be controlled *If yes = move to the next question	Q2. DOES THIS PREVENTIVE MEASURE ELIMINATE OR REDUCE THE LIKELY OCCURRENCE OF A HAZARD(S) TO AN ACCEPTABLE LEVEL? *If no = move to the next question *If yes = CCP	Q3. COULD CONTAMINATION WITH IDENTIFIED HAZARDD(S) OCCUR IN EXCESS OF ACCEPTABLE LEVELS OR COULD THESE INCREASE TO UNACCEPTABLE LEVELS? *If no = not a CCP *If yes = move to the next question	Q4. WILL A SUBSEQUENT STEP ELIMINATE THE HAZARD(S) OR REDUCE THE LIKELY OCCURRENCE TO AN ACCEPTABLE LEVEL? *If no = CCP If yes = not a CCP	# CCP
Receive Research Supplies	No hazards identified					
Store Research Supplies	No hazards identified					
Discard Used Research Supplies	B-Supplies may be contaminated with the pathogen in use	No – Supplies are certain to be contaminated and will be handled according to SOPs for microbiological research				
Receive Beef Short Plates	No hazards identified					
Stage Beef Short Plates	No hazards identified					
Inoculate Beef Short Plates	B-The environment may become contaminated with the pathogen in use	Yes – Control of inoculum run-off into a catch pan	No – While the inoculum is collected in a catch pan, it is still hazardous	Yes	Yes – treatment of the materials in the catch pan before released to the environment	
Treat Beef Short Plates	B-The environment may become contaminated with the pathogen in use due to liquid run-off	Yes – Control and collection of the treatment and run-off associated with the step	No – While the treatment liquids and inoculum are collected in a catch pan, it is still hazardous	Yes	Yes – treatment of the materials in the catch pan before released to the environment	
Treat Catch Pan	B – Inadequate destruction of pathogens in the catch pan	Yes – proper concentration and type of sanitizer/sterilizer used	Yes			1

CCP DETERMINATION (2 OF 4)

PROCESS: *Activin In-Plant Beef Carcass Validation*

PROCESS STEP	HAZARD (S) BIOLOGICAL – B CHEMICAL – C PHYSICAL – P	Q1. DO PREVENTITIVE MEASURES EXIST FOR THE IDENTIFIED HAZARDS? *If no = not a CCP- Identify how and where This hazard will be controlled *If yes = move to the next question	Q2. DOES THIS PREVENTIVE MEASURE ELIMINATE OR REDUCE THE LIKELY OCCURRENCE OF A HAZARD(S) TO AN ACCEPTABLE LEVEL? *If no = move to the next question *If yes = CCP	Q3. COULD CONTAMINATION WITH IDENTIFIED HAZARDD(S) OCCUR IN EXCESS OF ACCEPTABLE LEVELS OR COULD THESE INCREASE TO UNACCEPTABLE LEVELS? *If no = not a CCP *If yes = move to the next question	Q4. WILL A SUBSEQUENT STEP ELIMINATE THE HAZARD(S) OR REDUCE THE LIKELY OCCURRENCE TO AN ACCEPTABLE LEVEL? *If no = CCP If yes = not a CCP	# CCP
Excise Samples From Beef Short Plates	B-Equipment used for excisions may become contaminated with the pathogen in use	No – Equipment used is certain to be contaminated and will be handled according to SOPs for microbiological research				
Package Excised Samples	B-Samples in the package will have the pathogen present	No – The samples are certain to have the pathogen present, and will be handled according to SOPs for microbiological research				
Store Excised Samples	B-Samples in the package will have the pathogen present	No – The samples are certain to have the pathogen present, and will be handled and labeled according to SOPs for microbiological research				
Distribute Excised Samples	B-Samples in the package will have the pathogen present	No – The samples are certain to have the pathogen present, and will be handled and labeled according to SOPs for microbiological research				

CCP DETERMINATION (3 OF 4)

PROCESS: *Activin In-Plant Beef Carcass Validation*

PROCESS STEP	**HAZARD (S)** **BIOLOGICAL – B** **CHEMICAL – C** **PHYSICAL – P**	**Q1. DO PREVENTITIVE MEASURES EXIST FOR THE IDENTIFIED HAZARDS?** ***If no = not a CCP- Identify how and where This hazard will be controlled** ***If yes = move to the next question**	**Q2. DOES THIS PREVENTIVE MEASURE ELIMINATE OR REDUCE THE LIKELY OCCURRENCE OF A HAZARD(S) TO AN ACCEPTABLE LEVEL?** ***If no = move to the next question** ***If yes = CCP**	**Q3. COULD CONTAMINATION WITH IDENTIFIED HAZARDD(S) OCCUR IN EXCESS OF ACCEPTABLE LEVELS OR COULD THESE INCREASE TO UNACCEPTABLE LEVELS?** ***If no = not a CCP** ***If yes = move to the next question**	**Q4. WILL A SUBSEQUENT STEP ELIMINATE THE HAZARD(S) OR REDUCE THE LIKELY OCCURRENCE TO AN ACCEPTABLE LEVEL?** ***If no = CCP** **If yes = not a CCP**	**# CCP**
Sanitize Beef Short Plates	B-Beef short plates may not be effectively treated	No-The sanitization of the beef short plates is a precautionary measure to help reduce the presence of the pathogen. The plates will still be labeled and handled as if they have live pathogens present on the surface				
Discard Beef Short Plates in Containers	B-Product in the container may have the pathogen present	No – The plates are certain to have the pathogen present, and will be handled and labeled as “condemned”, “inedible”, and “biohazard”.				

Seal Containers	B-Product in the container may have the pathogen present	No – The containers are certain to have the pathogen present, and will be handled and labeled as “condemned”, “inedible”, and “biohazard”.				
Distribute Containers to Storage	B-The containers may have the pathogen present on the outside of the container	Yes – Sanitizing of the outside of the containers after they are sealed	Yes			2

CCP DETERMINATION (4 OF 4)

PROCESS: *Activin In-Plant Beef Carcass Validation*

PROCESS STEP	HAZARD (S) BIOLOGICAL – B CHEMICAL – C PHYSICAL – P	Q1. DO PREVENTITIVE MEASURES EXIST FOR THE IDENTIFIED HAZARDS? *If no = not a CCP- Identify how and where This hazard will be controlled *If yes = move to the next question	Q2. DOES THIS PREVENTIVE MEASURE ELIMINATE OR REDUCE THE LIKELY OCCURRENCE OF A HAZARD(S) TO AN ACCEPTABLE LEVEL? *If no = move to the next question *If yes = CCP	Q3. COULD CONTAMINATION WITH IDENTIFIED HAZARDD(S) OCCUR IN EXCESS OF ACCEPTABLE LEVELS OR COULD THESE INCREASE TO UNACCEPTABLE LEVELS? *If no = not a CCP *If yes = move to the next question	Q4. WILL A SUBSEQUENT STEP ELIMINATE THE HAZARD(S) OR REDUCE THE LIKELY OCCURRENCE TO AN ACCEPTABLE LEVEL? *If no = CCP If yes = not a CCP	# CCP
Distribute Containers to Waste Removal Company	B-Product in the container may have the pathogen present	No – The containers are certain to have the pathogen present, and will be handled and labeled as “condemned”, “inedible”, and “biohazard”.				
Receive Pathogen (*E.Coli* 0157:H7)	B-*E. Coli* 0157:H7 is a pathogen known to cause human illness.	No – Pathogens will be labeled and handled according to SOPs for microbiological research				
Store Pathogen	B-*E. Coli* 0157:H7 is a pathogen known to cause human illness.	No – Pathogens will be labeled and handled according to SOPs for microbiological research				
Prepare Pathogen for Inoculation	B-*E. Coli* 0157:H7 is a pathogen known to cause human illness.	No – Pathogens will be labeled and handled according to SOPs for microbiological research				

Destroy Residual Pathogen	B-Survival of the pathogen	Yes – Proper destruction methods (autoclave)	Yes			3
Discard the Destroyed Pathogen						

CRITICAL LIMITS, MONITORING AND CORRECTIVE ACTIONS			
PROCESS: *Activin In-Plant Beef Carcass Validation*			
PROCESS STEP / CCP	**CRITICAL LIMITS**	**MONITORING PROCEDURES (WHO/WHAT/WHEN/HOW)**	**CORRECTIVE ACTIONS**
Treatment of the catch pan and materials collected by the additioin of chlorine bleach. **CCP 1**	1000 ppm free chlorine or ammonium chloride in the run-off after the addition of either chlorine bleach or ammonium chloride	1. Who – person assigned to the task by the MPM. 2. What – measure the amount of free chlorine in the liquid run-off after the addition of chlorine bleach. 3. When – before every time the contents of the catch pan are released to the environment (floor drain). 4. How – by using Quantofix or similar brand test sticks for chlorine quantitative determinations.	1. Identify the cause of the problem. 2. Bring the problem under control. 3. Take action to prevent re-occurrence. 4. Retain product until corrected.
Distribute containers to storage – complete and proper sanitizing (by means of flooding with electric pump) before distribution **CCP 2**	1000 ppm of chlorine or ammonium chloride sanitizer	1. Who – person assigned to the task by the MPM. 2. What – record the amount of concentrated sanitizer and water used to formulate the sanitizer. 3. When – each occurrence when sanitizer is formulated. 4. How – by observing the measurement and addition of each ingredient and recording the observations.	1. Identify the cause of the problem. 2. Bring the problem under control. 3. Take action to prevent re-occurrence. 4. Retain product until corrected.

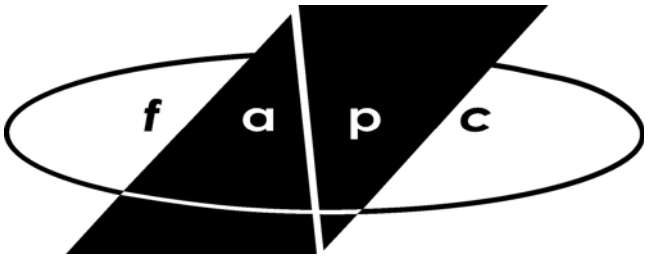
CRITICAL LIMITS, MONITORING AND CORRECTIVE ACTIONS			
PROCESS: *Activin In-Plant Beef Carcass Validation*			
PROCESS STEP / CCP	**CRITICAL LIMITS**	**MONITORING PROCEDURES (WHO/WHAT/WHEN/HOW)**	**CORRECTIVE ACTIONS**
Submit and destroy residual pathogens by use of autoclave **CCP 3**	Autoclave containers of residual pathogens at ≥121°C, at 15 psi, and for 30 minutes or longer	1. Who – FAPC pathogens laboratory personnel. 2. What – Autoclave the product and print the results. 3. When – each occurrence when pathogens remain after the project. 4. How – by following autoclaving SOPs.	1. Identify the cause of the problem 2. Bring the problem under control 3. Take action to prevent re-occurrence 4. Retain product until corrected

RECORD KEEPING AND VERIFICATION

PROCESS: *Activin In-Plant Beef Carcass Validation*

PROCESS STEP / CCP	RECORDS	VERIFICATION PROCEDURES✦
Treatment of the catch pan and materials collected – proper sanitizing concentration **CCP 1**	Sanitizer test sticks (strips) – to be attached to test stick form with proper identification	1. Direct observation of monitoring procedures – once per day 2. Verification of sanitizer concentration with test sticks against a know concentration – once per day 3. Review monitoring records – once per day
Distribute containers to storage – proper sanitizing before distribution **CCP 2**	Sanitizer concentration formulation sheet for CCP2	1. Direct observation of monitoring procedures – once per day 2. Verification of sanitizer concentration with test sticks against a know concentration – once per day 3. Review monitoring records – once per day
Destroy residual pathogens **CCP 3**	Autoclave record form and autoclave print-out	Use of autoclave tape.

Appendix C



SPECIAL SANITATION STANDARD OPERATING PROCEDURES

FOR THE

ACTIVIN IN-PLANT BEEF CARCASS VALIDATION PROJECT

Establishment 526, Stillwater, OK is a part of the Oklahoma Food and Agricultural Products Research and Technology Center (FAPC). It is a very small slaughter and processing establishment for live cattle, hogs and sheep, and processes wholesale & retail cuts, ground meats and cured and ready to eat meat products.

Management structure is:

Director, FAPC:.....Dr. J. Roy Escoubas
Meat Processing Manager (MPM):.....Jacob Nelson
Meat Processing Coordinator (MPC):.....Russell Nabors
Pilot Plant Manager (PPM):.....David Moe

The FAPC is participating in special research activities for testing the efficacy of an anti-microbial treatment against *E. Coli* 0157:H7. This research will partially be conducted on the slaughter floor of the establishment. Because the eventual and subsequent slaughter and processing of animals will occur in the establishment when the research is concluded, special precautions and procedures are defined in this document to ensure proper sanitary conditions and to prevent cross-contamination of other areas in the establishment. Procedures for conducting the research and control of the pathogen (*E.*

Coli 0157:H7) are outlined in a HACCP plan written specifically for the research project.

I. PRE-OPERATIONAL PROCEDURES

Objective 1: Rooms 217, 228, 233, 235 and the inedible corridor will be used during the research, and will be segregated from the remainder of the plant.

Objective 2: All equipment, detergents and sanitizers used during active research and for final cleaning of the area will be prepared and placed in room 217 before the daily research begins.

Task(s) performed by: Employees of the meat pilot plant, or others as directed by the MPM.

A. Post signs indicating entry restrictions.

1. Post “No Entry” signs on the following nine (9) doors using signs provided and yellow caution tape. Signs and tape will be stored in room 207 in a box labeled “Activin Project”. (See attached floor plan).

   a. Entry into 217 from 217A.
   b. Entry into 216 from corridor near 215.
   c. Entry into 216 from North exterior entrance.
   d. Entry into 235 from corridor near 234.
   e. Entry into inedible corridor from corridor near 234.
   f. Entry into inedible corridor from 231.
   g. Entry into inedible corridor from 230B.
   h. Entry into 217 from 229.
   i. Entry into 228 from corridor near 227.

B. Seal doorways

1. Use plastic sheeting (e.g. – Visqueen or similar brand/style) and a strong adhesive tape (e.g. – 3M Brand gray ‘Duck Tape’), to cover and seal the doorways at the following eight (8) locations and descriptions. Plastic sheeting and tape will be stored in room 207 in boxes labeled “Activin Project”. (See attached floor plan for details).

   a. North side of doorway between 217 and 217A.

b. West side of doorway between 216 and the corridor near 215.
c. North side of doorway between 235 and the corridor near 234.
d. East side of doorway entry to the inedible corridor near 234.
e. East entry into 231. *Note – disconnect electrical power to door opener before sealing door.*
f. East entry into 230B.
g. East entry into 229.
h. East side of entry into corridor from 228.

C. Prepare sanitation materials.

1. Cleaning materials – place the following in room 217.

a. Foaming machine with 25-foot air hose.
b. One (1) gallon of normally used detergent (Liquik 5 or 20).
c. One (1) gallon of chlorine bleach (5.25% sodium hypochlorite).
d. Two (2) scrub brushes (red bristles).
e. Five (5) green scour pads.
f. Two (2) floor scrub brushes (red handle).
g. Two (2) containers labeled “Trash” with liners installed.
h. Paper towel rolls in dispensers provided.
i. Hand soap in dispensers provided.

2. Sanitizing materials – place the following in room 217.

a. Sanitizing canister with 50-foot air hose.
b. Hand-held siphon gun.
c. Electric pump with pick-up and discharge hose.
d. One (1) empty plastic 1-gallon container (for preparing sanitizer).
e. One (1) empty 55-gallon plastic drum.
f. Two (2) plastic graduated cylinders (10ml maximum and 50ml maximum).
g. One (1) squirt-style pump to fit 1-gallon container.
h. Four (4) gallons of quaternary ammonia.
i. Two (2) additional gallons of chlorine bleach (5.25% sodium hypochlorite).

j. Test strip kits for chlorine and quaternary ammonia.
k. Two (2) rubber foot baths (see II., A., 1., a.).
l. Two (2) hand-dip containers (see II., A., 1., a.).

II. OPERATIONAL PROCEDURES

Objective: Personal hygiene and personnel traffic flow will be controlled in a manner to prevent contamination of other areas in the establishment.

Task(s) performed by: Research participants and employees of the establishment, as assigned by the project manager or MPM.

A. While research is actively conducted:

1. Research participants and employees providing assistance will clean and/or sanitize their hands, arms, gloves, outer garments, boots, etc., or change any garments as often as necessary during research operations to maintain sanitary conditions. Special attention must be given for those employees that must leave the rooms or establishment for retrieving products or other needed supplies.

    a. Rubber foot bath mats and the hand dip containers shall contain a 1000 ppm solution of ammonium chloride sanitizer, to be changed as often as necessary if they become soiled.

2. Research participants will only enter and leave room 217 through the main East entrance, or through the inedible corridor through rooms 233 and 235.

III. POST OPERATIONAL PROCEDURES

Objective 1: At the conclusion of research activities for each day, rooms 217, 228, 233, 235, the inedible corridor, and all equipment used for the research will be cleaned, sanitized and restored to its original condition.

Objective 2: At the conclusion of cleaning and sanitizing procedures, rooms 217, 228, 233, 235, the inedible corridor, and all equipment used for the research will be tested for the presence of *E. Coli* 0157:H7.

Task(s) performed by: Employees of the establishment as directed by the MPM.

A. Dress appropriately.

1. Employees should wear.

   a. Typical slaughter coveralls.
   b. Rubber boots.
   c. Nitrile aprons.
   d. Nitrile gloves.
   e. Other attire normally required by existing SOPs and GMPs.
   f. Other attire required by microbiological SOPs.

B. Perform dry clean-up.

1. Collect all trash items and place in appropriate containers.
2. Collect any residual meat items from floor, equipment, etc. and place in appropriate containers.
3. Seal containers with lids and store in room 233.

C. Perform wet clean-up.

2. Rinse all equipment used and soiled areas with hot water (>180^{o}F).
3. Prepare foaming machine according to normal procedure.
4. Apply foam to all equipment used (including water hoses, doors, sinks, etc.) and other soiled areas per normal procedure.
5. Scrub soiled areas with brushes and green pads per normal procedure.

6. Rinse all areas with hot water (>180^{o}F).

   a. Inspect and re-clean if necessary.
   b. Rinse all areas again with hot water (>180^{o}F) if necessary.

7. Empty foaming machine.
8. Rinse foaming machine, brushes, air hoses, water hoses, and green scour pads with hot water (>180^{o}F), and leave exposed for subsequent sanitizing procedures.

D. Sanitize

1. Prepare 50-gallon batches (as many as needed to perform the task) of chlorine sanitizer with a concentration of 1000 ppm sodium hypochlorite.
2. Using the electric pump, flood entire area (equipment, floors, walls, doors, etc.) with chlorine sanitizer.

   a. Allow areas to remain wet for 15 min. minimum.

3. Rinse entire area with hot water (>180^{o}F).
4. Prepare 2 gallons of 1000 ppm ammonium chloride sanitizer.

   a. Use siphon gun to sanitize cleaning equipment (foaming machine, brushes, green pads, air hoses, etc, paper towel dispensers and hand soap dispensers, employee garments (rubber aprons, rubber boots).

5. Prepare sanitizing canister with 200 ppm ammonium chloride sanitizer, and fog room 217.

E. Perform microbial sampling.

1. Collect samples from the environment and test for the presence of *E. Coli* 0157:H7, according to the procedures outlined by the Activin Environmental Sampling Program (to be drafted and performed by a third-party auditor [Food ProTech, Stillwater, OK]).
2. Record results of the microbial tests on form “Activin Environmental Sampling Results”.

a. No processing shall occur in the rooms used until test results demonstrate the absence of *E. Coli* 0157:H7 from the environment.

F. Restore rooms.

1. Remove plastic sheeting and tape, and discard in waste receptacles.
2. Remove “No Entry” signs and store
3. Remove cleaning equipment to storage

IV. MONITORING AND RECORD KEEPING

Objective: Procedures will be monitored and the results recorded.

A. The MPM or other assigned personnel will perform daily inspection (when applicable) during and after post-operational cleaning and sanitizing. The assigned person will use this document as a “check-list” to perform the inspections. The results of the inspection will be recorded on form “Activin M.L.1”.

1. If the MPM or other assigned personnel determine that the procedures are not followed, or equipment and rooms are out of compliance, corrective and preventive actions will be performed. The actions performed will be recorded on form “Activin M.L.1”.

B. According to (III) (E) (1) & (2) of this document, the third-party auditor will record and submit to the establishment the findings of the environmental microbial sampling plan. Establishment management will maintain these reports.

V. SANITIZER PREPARATIONS

Objective: To prepare the proper concentrations of sanitizer to meet the requirements of this document.

Task(s) performed by: Establishment employees as directed by the MPM.

	Bi-Quat (5.0% / 5.0%)	Chlorine Bleach (5.25%)	Water
Chlorine, 1000 ppm	--	3.6 liters or (1.0 gallons)	50 gallons
Ammonium chloride, 1000 ppm	38.0 ml	--	1 gallon
Ammonium chloride, 200 ppm	7.5 ml	--	1 gallon

ACTIVIN M.L.1 – SSOP INSPECTION REPORT

NAME _____ DATE

1. Were all items described in the SSOP in place and executed correctly?

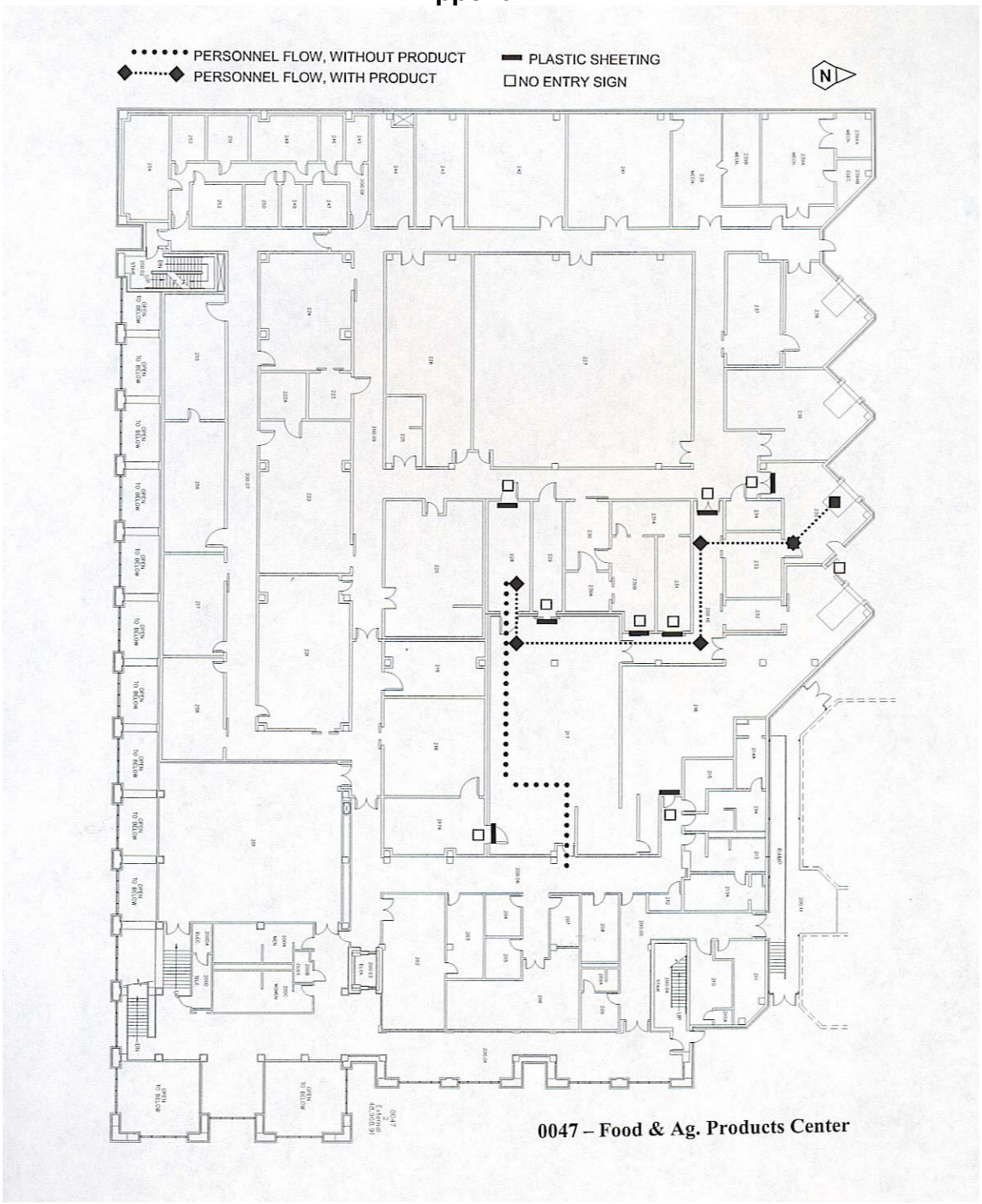
   Yes

   No

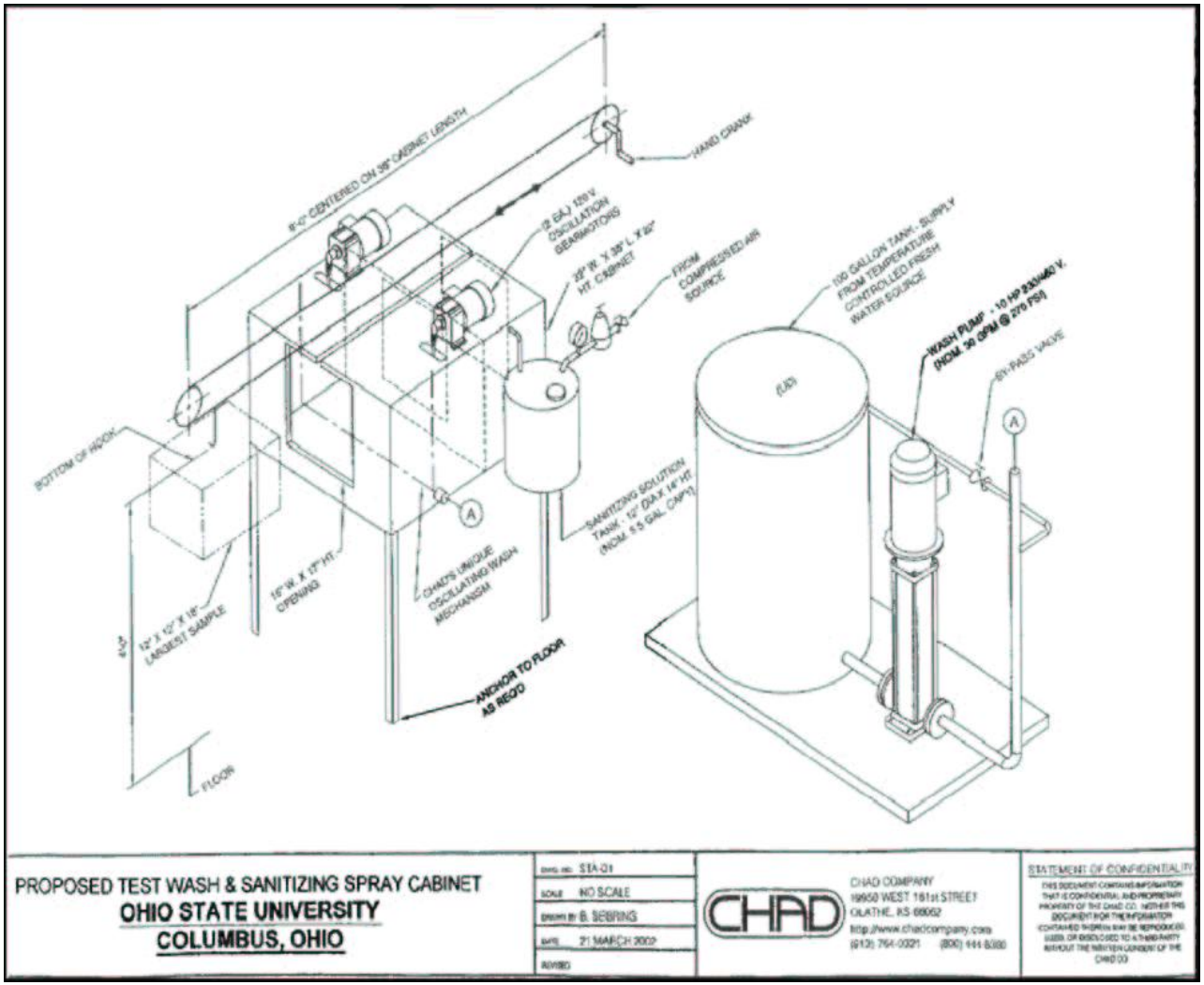
2. If No, cite the section of this document in violation (Example: I. C. 2. d.)

3. Describe below the corrective actions performed.

Appendix D


PERSONNEL FLOW, WITHOUT PRODUCT
PERSONNEL FLOW, WITH PRODUCT
PLASTIC SHEETING
NO ENTRY SIGN
N
0047 – Food & Ag. Products Center

Appendix E


8'-0" CENTERED ON 36" CABINET LENGTH
HAND CRANK
(2 EA.) 115 V. OSCILLATION GEARMOTORS
28" W. X 36" L. X 60" HT. CABINET
FROM COMPRESSED AIR SOURCE
100 GALLON TANK - SUPPLY FROM TEMPERATURE CONTROLLED FRESH WATER SOURCE
WASH PUMP - 10 HP 230/460 V. (NOM. 50 GPM @ 270 PSI)
BY-PASS VALVE
BOTTOM OF HOOK
12" X 12" X 18" LARGEST SAMPLE
15" W. X 13" HT. OPENING
CHAD'S UNIQUE OSCILLATING WASH MECHANISM
SANITIZING SOLUTION TANK - 12" DIA X 14" HT (NOM. 5.5 GAL. CAPY.)
ANCHOR TO FLOOR AS REQ'D
FLOOR
PROPOSED TEST WASH & SANITIZING SPRAY CABINET
OHIO STATE UNIVERSITY
COLUMBUS, OHIO
DWG. NO. STA-01
SCALE NO SCALE
DRAWN BY B. SEBRING
DATE 21 MARCH 2002
REVISED
CHAD
CHAD COMPANY
16950 WEST 161st STREET
OLATHE, KS 66062
http://www.chadcompany.com
(913) 764-0321 (800) 444-8388
STATEMENT OF CONFIDENTIALITY

Appendix F

CHAD

19950 W. 161st Street
Olathe, Kansas 66062
(800) 444-8360
Phone: (913) 764-0321
Fax: (913) 764-0779

DATE: November 18, 2004
TO: Jake Nelson
COMPANY: Oklahoma State Univ.
FAX NUMBER: 405-744-6313

FAX NUMBER: (913) 764-0779
NUMBER OF PAGES TO FOLLOW: 4
LOCATION: Stillwater, OK
PHONE NUMBER: 405-744-6329

Jake,

Attached are four sheets that give the "performance data" for the nozzles that are used on the Test Spray Cabinet.

1. The nozzles in the 1st station, "oscillating carcass wash", are "1/8MEG2510". As you can see in the table, the flow is 0.71 GPM @ 20 psi per "each nozzle". The total flow is determined by the "**spray pressure capacity**" multiplied by the "**number**" of nozzles in that station, (e.g., .71 GPM x 6 nozzles = 4.26 GPM). If you spray at 25 psi, then you would have to interpolate the capacity from the table.

2. The nozzles in the 2nd station, "hot water pasteurization", are "H1/8USS5020". As you can see in the table, the flow is 1.40 GPM @ 20 psi per "each nozzle.

3. The nozzles in the 3rd station, "sanitizing", are "H1/8VVSS80015". As you can see in the table, the flow is 0.11 GPM @ 20 psi per "each nozzle".

I think Mike Gangel left some 1/8MEG2508 nozzles when he was there. If you put these nozzles in the 1st station, then the flow would be 0.56 GPM @ 20 psi per "each nozzle".

Hopefully, this gives you the information that you requested.

Please call if you have any comments or questions.

Good luck with your project.

Regards,

Ron

Ron Witt
Sales Engineer

UniJet® Spray Nozzles • Standard Spray
Small Capacity

1ST STATION
"2510" NOZZLE
(CARCASS WASH — OSCILLATING)

FLAT SPRAY NOZZLES

PERFORMANCE DATA

Spray Angle at 40 psi	Capacity Size	Equiv. Orifice Dia.	Capacity (gallons per minute)											Spray Angle			
			5 psi	10 psi	20 psi	30 psi	40 psi	60 psi	80 psi	100 psi	200 psi	300 psi	500 psi	20 psi	40 psi	80 psi	200 psi
25°	10	.079"	—	.50	.71	.86	1.0	1.2	1.4	1.6	2.2	2.7	3.5	18°	25°	31°	37°
	13	.090"	—	.65	.92	1.1	1.3	1.6	1.8	2.1	2.9	3.6	4.6	18°	25°	31°	37°
	15	.097"	—	.75	1.1	1.3	1.5	1.8	2.1	2.4	3.4	4.1	5.3	18°	25°	31°	37°
	20	.112"	—	1.0	1.4	1.7	2.0	2.5	2.8	3.2	4.5	5.5	7.1	19°	25°	31°	37°
	30	.133"	1.1	1.5	2.1	2.6	3.0	3.7	4.2	4.7	6.7	8.2	10.6	20°	25°	30°	36°
	40	.153"	1.4	2.0	2.8	3.5	4.0	4.9	5.7	6.3	8.9	11.0	14.2	21°	25°	29°	35°
	50	.172"	1.8	2.5	3.5	4.3	5.0	6.1	7.1	7.9	11.2	13.7	17.7	21°	25°	29°	35°
	60	.188"	2.1	3.0	4.2	5.2	6.0	7.3	8.5	9.5	13.4	16.4	21	22°	25°	29°	35°
	70	.203"	2.5	3.5	4.9	6.1	7.0	8.6	9.9	11.1	15.7	19.2	25	22°	25°	29°	35°
15°	0017	.011"	—	—	—	.015	.017	.021	.024	.027	.038	.047	.06	—	15°	30°	37°
	0025	.013"	—	—	—	.022	.025	.031	.035	.040	.06	.07	.09	—	15°	28°	34°
	0033	.015"	—	—	—	.029	.033	.040	.047	.052	.07	.09	.12	—	15°	27°	32°
	0050	.018"	—	—	—	.043	.050	.06	.07	.08	.11	.14	.18	—	15°	26°	30°
	0067	.021"	—	—	—	.06	.067	.08	.09	.11	.15	.18	.24	—	15°	25°	29°
	01	.026"	—	—	—	.09	.10	.12	.14	.16	.22	.27	.35	—	15°	24°	28°
	015	.032"	—	—	—	.13	.15	.18	.21	.24	.34	.41	.53	—	15°	23°	27°
	02	.035"	—	—	.14	.17	.20	.25	.28	.32	.45	.55	.71	6°	15°	22°	27°
	03	.043"	—	—	.21	.26	.30	.37	.42	.47	.67	.82	1.1	6°	15°	22°	27°
	04	.050"	—	—	.28	.35	.40	.49	.57	.63	.89	1.1	1.4	7°	15°	21°	26°
	05	.056"	—	—	.35	.43	.50	.61	.71	.79	1.1	1.4	1.8	7°	15°	21°	26°
	055	.059"	—	—	.39	.48	.55	.67	.78	.87	1.2	1.5	1.9	7°	15°	21°	26°
	06	.061"	—	—	.42	.52	.60	.73	.85	.95	1.3	1.6	2.1	8°	15°	21°	26°
	07	.066"	—	—	.49	.61	.70	.86	.99	1.1	1.6	1.9	2.5	8°	15°	21°	26°
	08	.071"	—	—	.56	.69	.80	.98	1.1	1.3	1.8	2.2	2.8	9°	15°	20°	25°
	09	.075"	—	—	.64	.78	.90	1.1	1.3	1.4	2.0	2.5	3.2	9°	15°	20°	25°
	10	.079"	—	—	.71	.86	1.0	1.2	1.4	1.6	2.2	2.7	3.5	10°	15°	19°	24°
	11	.083"	—	.55	.78	.95	1.1	1.4	1.6	1.7	2.5	3.0	3.9	10°	15°	19°	24°
	12	.087"	—	.60	.85	1.0	1.2	1.5	1.7	1.9	2.7	3.3	4.2	10°	15°	19°	24°
	15	.097"	—	.75	1.1	1.3	1.5	1.8	2.1	2.4	3.4	4.1	5.3	10°	15°	19°	24°
	20	.112"	—	1.0	1.4	1.7	2.0	2.5	2.8	3.2	4.5	5.5	7.1	10°	15°	19°	23°
	30	.133"	—	1.5	2.1	2.6	3.0	3.7	4.2	4.7	6.7	8.2	10.6	10°	15°	19°	21°
	40	.153"	—	2.0	2.8	3.5	4.0	4.9	5.7	6.3	8.9	11.0	14.2	10°	15°	18°	21°
	50	.172"	—	2.5	3.5	4.3	5.0	6.1	7.1	7.9	11.2	13.7	17.7	11°	15°	18°	21°
	60	.188"	—	3.0	4.2	5.2	6.0	7.3	8.5	9.5	13.4	16.4	21	11°	15°	18°	21°
	70	.203"	—	3.5	4.9	6.1	7.0	8.6	9.9	11.1	15.7	19.2	25	11°	15°	18°	21°

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Small Capacity

2 ND STATION

"5010" NOZZLE

(HOT WATER PASTUERIZATION)

PERFORMANCE DATA

Spray Angle at 40 psi	Capacity Size	Equiv. Orifice Dia.	Capacity (gallons per minute)											Spray Angle			
			5 psi	10 psi	20 psi	30 psi	40 psi	60 psi	80 psi	100 psi	200 psi	300 psi	500 psi	20 psi	40 psi	80 psi	200 psi
	0033	.015"	—	—	.023	.029	.033	.040	.047	.052	.07	.09	.12	30°	50°	62°	68°
	0050	.018"	—	—	.035	.043	.050	.06	.07	.08	.11	.14	.18	32°	50°	60°	66°
	0067	.021"	—	—	.05	.06	.067	.08	.09	.11	.15	.18	.24	35°	50°	60°	66°
	01	.026"	—	.05	.07	.09	.10	.12	.14	.16	.22	.27	.35	37°	50°	59°	65°
	015	.032"	—	.07	.11	.13	.15	.18	.21	.24	.34	.41	.53	38°	50°	58°	64°
	02	.035"	—	.10	.14	.17	.20	.25	.28	.32	.45	.55	.71	39°	50°	57°	63°
	025	.039"	.09	.13	.18	.22	.25	.31	.35	.40	.56	.68	.88	40°	50°	57°	63°
	03	.043"	.11	.15	.21	.26	.30	.37	.42	.47	.67	.82	1.1	40°	50°	56°	62°
	035	.047"	.12	.18	.25	.30	.35	.43	.49	.55	.78	.96	1.2	40°	50°	56°	61°
	04	.050"	.14	.20	.28	.35	.40	.49	.57	.63	.89	1.1	1.4	42°	50°	56°	61°
	05	.056"	.18	.25	.35	.43	.50	.61	.71	.79	1.1	1.4	1.8	44°	50°	56°	61°
	06	.061"	.21	.30	.42	.52	.60	.73	.85	.95	1.3	1.6	2.1	45°	50°	56°	60°
(50°)	07	.066"	.25	.35	.49	.61	.70	.86	.99	1.1	1.6	1.9	2.5	45°	50°	56°	60°
	075	.068"	.27	.38	.53	.65	.75	.92	1.1	1.2	1.7	2.1	2.7	45°	50°	55°	60°
	08	.071"	.28	.40	.56	.69	.80	.98	1.1	1.3	1.8	2.2	2.8	45°	50°	55°	60°
	09	.075"	.32	.45	.64	.78	.90	1.1	1.3	1.4	2.0	2.5	3.2	45°	50°	55°	59°
	10	.079"	.35	.50	.71	.86	1.0	1.2	1.4	1.6	2.2	2.7	3.5	45°	50°	55°	59°
	13	.090"	.46	.65	.92	1.1	1.3	1.6	1.8	2.1	2.9	3.6	4.6	45°	50°	55°	59°
	15	.097"	.53	.75	1.1	1.3	1.5	1.8	2.1	2.4	3.4	4.1	5.3	45°	50°	55°	59°
	(20)	.112"	(.71)	1.0	(1.4)	1.7	2.0	2.5	2.8	3.2	4.5	5.5	7.1	45°	50°	55°	59°
	30	.133"	1.1	1.5	2.1	2.6	3.0	3.7	4.2	4.7	6.7	8.2	10.6	45°	50°	55°	59°
	40	.153"	1.4	2.0	2.8	3.5	4.0	4.9	5.7	6.3	8.9	11.0	14.2	46°	50°	54°	59°
	50	.172"	1.8	2.5	3.5	4.3	5.0	6.1	7.1	7.9	11.2	13.7	17.7	46°	50°	54°	59°
	60	.188"	2.1	3.0	4.2	5.2	6.0	7.3	8.5	9.5	13.4	16.4	21	46°	50°	54°	59°
	70	.203"	2.5	3.5	4.9	6.1	7.0	8.6	9.9	11.1	15.7	19.2	25	46°	50°	54°	59°
	0017	.011"	—	—	.012	.015	.017	.021	.024	.027	.038	.047	.06	21°	40°	54°	61°
	0025	.013"	—	—	.018	.022	.025	.031	.035	.04	.06	.07	.09	22°	40°	53°	60°
	0033	.015"	—	—	.023	.029	.033	.040	.047	.057	.07	.09	.12	22°	40°	53°	60°
	0050	.018"	—	—	.035	.043	.050	.06	.07	.08	.11	.14	.18	22°	40°	53°	60°
40°	0067	.021"	—	—	.05	.06	.067	.08	.09	.11	.15	.18	.24	24°	40°	53°	60°
	01	.026"	—	—	.07	.09	.10	.12	.14	.16	.22	.27	.35	26°	40°	52°	59°
	015	.032"	—	—	.11	.13	.15	.18	.21	.24	.34	.41	.53	27°	40°	52°	59°
	02	.035"	—	.10	.14	.17	.20	.25	.28	.32	.45	.55	.71	29°	40°	51°	58°
	025	.039"	—	.13	.18	.22	.25	.31	.35	.40	.56	.68	.88	29°	40°	51°	58°
	03	.043"	—	.15	.21	.26	.30	.37	.42	.47	.67	.82	1.1	30°	40°	50°	57°

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1ST STATION

"OPTIONAL" "2508" NOZZLE

PERFORMANCE DATA

("CARCASS WASH - OSCILLATING"

Spray Angle at 40 psi	Capacity Size	Equiv. Orifice Dia.	Capacity (gallons per minute)											Spray Angle			
			5 psi	10 psi	20 psi	30 psi	40 psi	60 psi	80 psi	100 psi	200 psi	300 psi	500 psi	20 psi	40 psi	80 psi	200 psi
40°	04	.050"	—	.20	.28	.35	.40	.49	.57	.63	.89	1.1	1.4	30°	40°	50°	56°
	05	.056"	—	.25	.35	.43	.50	.61	.71	.79	1.1	1.4	1.8	31°	40°	49°	55°
	055	.059"	—	.28	.39	.48	.55	.67	.78	.87	1.2	1.5	1.9	31°	40°	49°	55°
	06	.061"	—	.30	.42	.52	.60	.73	.85	.95	1.3	1.6	2.1	31°	40°	49°	55°
	07	.066"	.25	.35	.49	.61	.70	.86	.99	1.1	1.6	1.9	2.5	31°	40°	49°	55°
	08	.071"	.28	.40	.56	.69	.80	.98	1.1	1.3	1.8	2.2	2.8	31°	40°	47°	53°
	09	.075"	.32	.45	.64	.78	.90	1.1	1.3	1.4	2.0	2.5	3.2	32°	40°	45°	48°
	10	.079"	.35	.50	.71	.86	1.0	1.2	1.4	1.6	2.2	2.7	3.5	32°	40°	45°	48°
	11	.083"	.39	.55	.78	.95	1.1	1.4	1.6	1.7	2.5	3.0	3.9	32°	40°	45°	48°
	12	.087"	.42	.60	.85	1.0	1.2	1.5	1.7	1.9	2.7	3.3	4.2	32°	40°	45°	48°
	13	.090"	.46	.65	.92	1.1	1.3	1.6	1.8	2.1	2.9	3.6	4.6	32°	40°	45°	48°
	15	.097"	.53	.75	1.1	1.3	1.5	1.8	2.1	2.4	3.4	4.1	5.3	32°	40°	45°	48°
	20	.112"	.71	1.0	1.4	1.7	2.0	2.5	2.8	3.2	4.5	5.5	7.1	32°	40°	45°	48°
	25	.121"	.88	1.3	1.8	2.2	2.5	3.1	3.5	4.0	5.6	6.9	8.8	32°	40°	45°	48°
	30	.133"	1.1	1.5	2.1	2.6	3.0	3.7	4.2	4.7	6.7	8.2	10.6	33°	40°	45°	48°
	40	.153"	1.4	2.0	2.8	3.5	4.0	4.9	5.7	6.3	8.9	11.0	14.2	34°	40°	45°	48°
	50	.172"	1.8	2.5	3.5	4.3	5.0	6.1	7.1	7.9	11.2	13.7	17.7	35°	40°	45°	48°
	60	.188"	2.1	3.0	4.2	5.2	6.0	7.3	8.5	9.5	13.4	16.4	21	35°	40°	45°	48°
	70	.203"	2.5	3.5	4.9	6.1	7.0	8.6	9.9	11.1	15.7	19.2	25	35°	40°	45°	48°
25°	0017	.011"	—	—	—	.015	.017	.021	.024	.027	.038	.047	.06	—	25°	35°	47°
	0025	.013"	—	—	—	.022	.025	.031	.035	.040	.06	.07	.09	—	25°	35°	45°
	0033	.015"	—	—	—	.029	.033	.040	.047	.052	.07	.09	.12	—	25°	34°	44°
	0050	.018"	—	—	—	.043	.050	.06	.07	.08	.11	.14	.18	—	25°	34°	43°
	0067	.021"	—	—	—	.06	.067	.08	.09	.11	.15	.18	.24	—	25°	34°	42°
	01	.026"	—	—	.07	.09	.10	.12	.14	.16	.22	.27	.35	14°	25°	34°	42°
	015	.032"	—	—	.11	.13	.15	.18	.21	.24	.34	.41	.53	15°	25°	34°	41°
	02	.035"	—	—	.14	.17	.20	.25	.28	.32	.45	.55	.71	15°	25°	33°	40°
	03	.043"	—	—	.21	.26	.30	.37	.42	.47	.67	.82	1.1	15°	25°	33°	40°
	04	.050"	—	.20	.28	.35	.40	.49	.57	.63	.89	1.1	1.4	16°	25°	32°	39°
	05	.056"	—	.25	.35	.43	.50	.61	.71	.79	1.1	1.4	1.8	16°	25°	32°	39°
	055	.059"	—	.28	.39	.48	.55	.67	.78	.87	1.2	1.5	1.9	16°	25°	32°	39°
	06	.061"	—	.30	.42	.52	.60	.73	.85	.95	1.3	1.6	2.1	17°	25°	31°	38°
	07	.066"	—	.35	.49	.61	.70	.86	.99	1.1	1.6	1.9	2.5	17°	25°	31°	38°
	08	.071"	—	.40	.56	.69	.80	.98	1.1	1.3	1.8	2.2	2.8	17°	25°	31°	36°
	09	.075"	—	.45	.64	.78	.90	1.1	1.3	1.4	2.0	2.5	3.2	17°	25°	31°	38°

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TOTAL P.05

VeeJet® Spray Nozzles • Standard Spray
Small Capacity

3RD STATION
" 80015 NOZZLE
(SANITIZING)

PERFORMANCE DATA

Spray Angle at 40 psi	Nozzle Type/ Inlet Connection												Capacity Size	Equiv. Orifice Dia.	Capacity (gallons per minute)										Spray Angle			
	H-VV		H-VVL		H-U				H-DT		H-DU				5 psi	10 psi	20 psi	30 psi	40 psi	60 psi	80 psi	100 psi	200 psi	300 psi	500 psi	20 psi	40 psi	80 psi	200 psi
	1/8	1/4	1/8	1/4	1/8	1/4	3/8	1/2	1/8	1/4	1/8	1/4																
	•	•	•	•					•	•			01	.026"	—	.05	.07	.09	.10	.12	.14	.16	.22	.27	.35	68°	80°	89°	92°
		•	•	•	•					•			(015)	.032"	—	.07	.11	.13	.15	.18	.21	.24	.34	.41	.53	68°	80°	89°	92°
	•	•	•	•					•	•			02	.035"	.07	.10	.14	.17	.20	.25	.28	.32	.45	.55	.71	69°	80°	88°	91°
	•	•	•	•					•	•			03	.043"	.11	.15	.21	.26	.30	.37	.42	.47	.67	.82	1.1	70°	80°	87°	90°
	•	•	•	•					•	•			04	.050"	.14	.20	.28	.35	.40	.49	.57	.63	.89	1.1	1.4	71°	80°	86°	89°
	•	•	•	•					•	•			05	.056"	.18	.25	.35	.43	.50	.61	.71	.79	1.1	1.4	1.8	71°	80°	86°	89°
	•	•	•	•					•	•			06	.061"	.21	.30	.42	.52	.60	.73	.85	.95	1.3	1.6	2.1	72°	80°	85°	88°
	•								•	•			07	.066"	.25	.35	.49	.61	.70	.86	.99	1.1	1.6	1.9	2.5	72°	80°	85°	88°
	•	•	•	•					•	•			08	.071"	.28	.40	.56	.69	.80	.98	1.1	1.3	1.8	2.2	2.8	72°	80°	84°	87°
		•		•					•	•			09	.075"	.32	.45	.64	.78	.90	1.1	1.3	1.4	2.0	1.5	3.2	73°	80°	84°	87°
(80°)					•	•	•	•			•	•	10	.079"	.35	.50	.71	.86	1.0	1.2	1.4	1.6	2.2	2.7	3.5	73°	80°	84°	87°
					•	•		•			•	•	15	.094"	.53	.75	1.1	1.3	1.5	1.8	2.1	2.4	3.4	4.1	5.3	74°	80°	83°	86°
					•	•	•	•			•	•	20	.109"	.71	1.0	1.4	1.7	2.0	2.5	2.8	3.2	4.5	5.5	7.1	74°	80°	83°	86°
					•	•	•	•			•	•	30	.133"	1.1	1.5	2.1	2.6	3.0	3.7	4.2	4.7	6.7	8.2	10.6	74°	80°	83°	86°
					•	•	•	•			•	•	40	.153"	1.4	2.0	2.8	3.5	4.0	4.9	5.7	6.3	8.9	11.0	14.2	74°	80°	83°	86°
						•	•	•				•	50	.172"	1.8	2.5	3.5	4.3	5.0	6.1	7.1	7.9	11.2	13.7	17.7	74°	80°	83°	85°
						•	•	•				•	60	.188"	2.1	3.0	4.2	5.2	6.0	7.3	8.5	9.5	13.4	16.4	21	75°	80°	83°	85°
						•	•	•				•	70	.203"	2.5	3.5	4.9	6.1	7.0	8.6	9.9	11.1	15.7	19.2	25	75°	80°	83°	86°
							•	•					100	.243"	3.5	5.0	7.1	8.6	10.0	12.2	14.1	15.8	22	27	35	75°	80°	83°	86°
							•	•					150	.297"	5.3	7.5	10.6	13.0	15.0	18.4	21	24	34	41	53	73°	80°	84°	86°
								•					200	.343"	7.1	10.0	14.1	17.3	20	25	28	32	44	55	71	74°	80°	82°	85°
	•	•	•	•					•				0077	.023"	—	.039	.055	.067	.077	.09	.11	.12	.17	.21	.27	53°	73°	86°	92°
	•	•	•	•									0154	.032"	.05	.08	.11	.13	.15	.19	.22	.24	.34	.42	.54	55°	73°	84°	88°
73°		•		•									0231	.038"	.08	.12	.16	.20	.23	.28	.33	.37	.52	.63	.82	56°	73°	83°	87°
	•	•	•	•									0308	.044"	.11	.15	.22	.27	.31	.38	.44	.49	.69	.84	1.1	58°	73°	82°	86°
		•		•									0462	.054"	.16	.23	.33	.40	.46	.57	.65	.73	1.0	1.3	1.6	60°	73°	80°	84°
	•		•										0770	.069"	.27	.38	.54	.67	.77	.94	1.1	1.2	1.7	2.1	2.7	64°	73°	77°	82°
	•		•										0017	.011"	—	—	.012	.015	.017	.021	.024	.027	.038	.047	.06	44°	65°	77°	86°
	•		•										0033	.015"	—	—	.023	.029	.033	.040	.047	.052	.07	.09	.12	47°	65°	76°	83°
	•	•	•	•					•				0067	.021"	—	.03	.05	.06	.067	.08	.09	.11	.15	.18	.24	50°	65°	75°	81°
65°	•	•	•	•					•	•			01	.026"	—	.05	.07	.09	.10	.12	.14	.16	.22	.27	.35	51°	65°	74°	80°
	•	•	•	•									015	.032"	—	.07	.11	.13	.15	.18	.21	.24	.34	.41	.53	51°	65°	74°	80°
	•	•	•	•					•	•			02	.035"	.07	.10	.14	.17	.20	.25	.28	.32	.45	.55	.71	52°	65°	73°	79°

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CHAIN SPD HD/HR	HVS/HR	SPG	IPH	/60	IPM	/60	IPS	X5	IN. TRAVEL/ 5 SEC.
40	80	48"	3840		64		1.07		5.35
50	100	48"	4800		80		1.33		6.65
60	120	48"	5760		96		1.6		8.00
70	140	48"	6720		112		1.87		9.35
80	160	48"	7680		128		2.13		10.65
90	180	48"	8640		144		2.4		12.00
100	200	48"	9600		160		2.67		13.35
110	220	48"	10560		176		2.93		14.65
120	240	48"	11520		192		3.2		16.0
130	260	48"	12480		208		3.47		17.35
140	280	48"	13440		224		3.73		18.65
150	300	48"	14400		240		4.0		20.00
160	320	48"	15360		256		4.27		21.35
170	340	48"	16320		272		4.53		22.65
180	360	48"	17280		288		4.8		24.00
190	380	48"	18240		304		5.07		25.35
200	400	48"	19200		320		5.33		26.65
210	420	48"	20160		336		5.6		28.0
220	440	48"	21120		352		5.87		29.35
230	460	48"	22080		368		6.13		30.65
240	480	48"	23040		384		6.4		32.0
250	500	48"	24000		400		6.67		33.35
260	520	48"	24960		416		6.93		34.65
270	540	48"	25920		432		7.20		36.0
280	560	48"	26880		448		7.47		37.35
290	580	48"	27840		464		7.73		38.65
300	600	48"	28800		480		8.0		40.0
310	620	48"	29760		496		8.27		41.35
320	640	48"	30720		512		8.53		42.65
330	660	48"	31680		528		8.8		44.0
340	680	48"	32640		544		9.07		45.35
350	700	48"	33600		560		9.33		46.65
360	720	48"	34560		576		9.6		48.0
370	740	48"	35520		592		9.87		49.35
380	760	48"	36480		608		10.13		50.65
390	780	48"	37440		624		10.4		52.0
400	800	48"	38400		640		10.67		53.35
410	820	48"	39360		656		10.93		54.65
420	840	48"	40320		672		11.2		56.0
425	850	48"	40800		680		11.33		56.65
450							12.0		

sec/exp.

—600 hogs/hr

—800 hd/hr
—840 hogs/hr

9.1

—1100 hogs/hr
7.5 sec

7
1200 hogs/hr

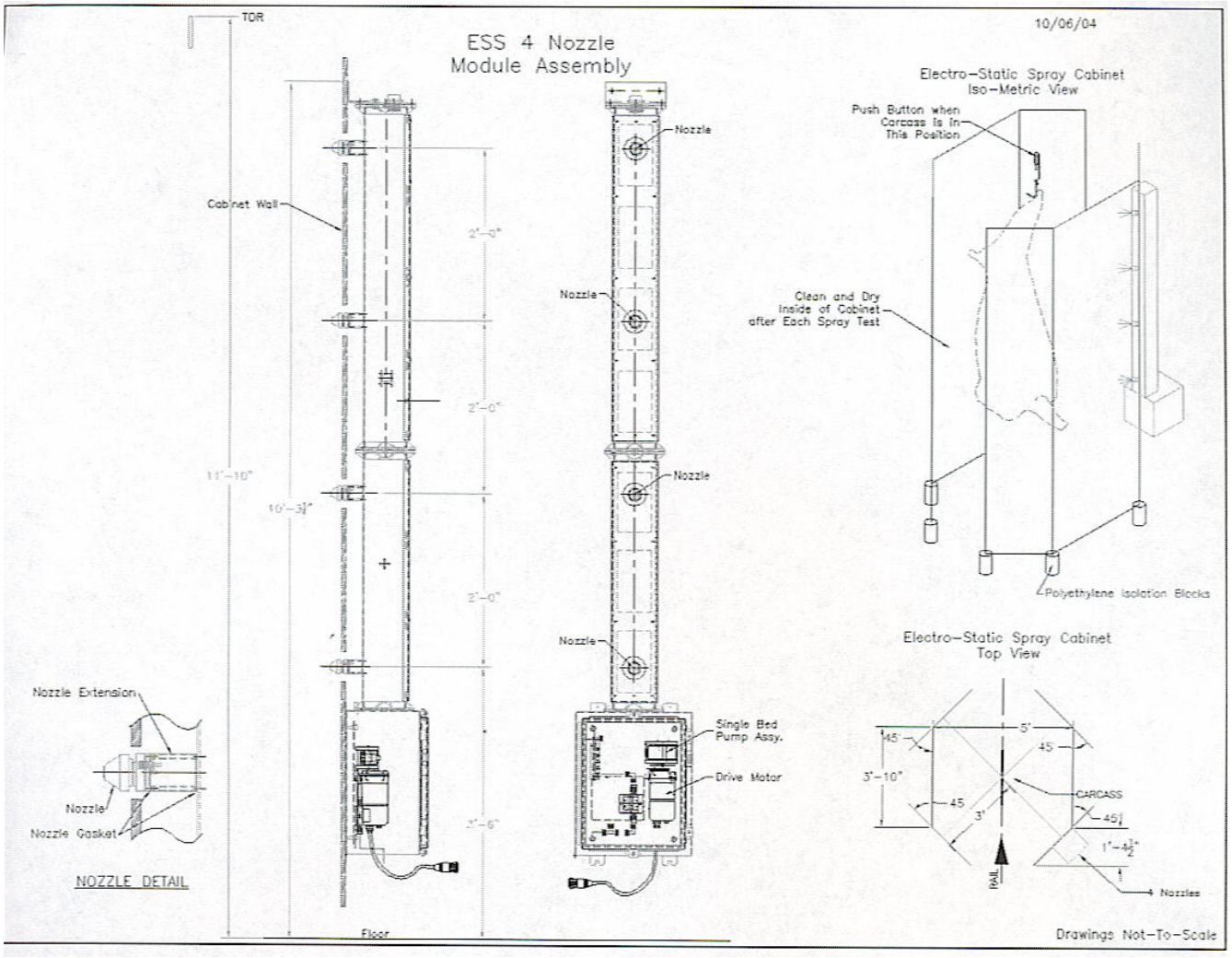
5

ANGLE/OSC.	ACTUAL COVERAGE @ C RAIL(INCHES)	USE FOR COVERAGE (INCHES)
20°	4.827	4.75
25°	6.070	6.000
30°	7.340	7.250
35°	8.630	8.750
40°	9.960	10.000
45°	11.340	11.250
50°	12.765	12.750
55°	14.250	14.250
60°	15.804	15.750
65°	17.440	17.500
70°	19.168	19.250
75°	21.005	21.000
80°	22.970	23.000
85°	25.084	25.000
90°	27.374	27.375

NOTE:

1. FACE OF NOZZLE TO CENTER OF RAIL IS ≈ 12"
2. ¢ of shaft to ¢ of [illegible]
   90° — 2 5/16
   70°
   60° — 1 5/8

Appendix G


ESS 4 Nozzle
Module Assembly
10/06/04
TOR
Cabinet Wall
Nozzle
Nozzle
Nozzle
Nozzle
2'-0"
2'-0"
2'-0"
2'-6"
11'-10"
10'-3"
Single Bed
Pump Assy.
Drive Motor
Floor
Nozzle Extension
Nozzle
Nozzle Gasket
NOZZLE DETAIL
Electro-Static Spray Cabinet
Iso-Metric View
Push Button when
Carcass Is In
This Position
Clean and Dry
Inside of Cabinet
after Each Spray Test
Polyethylene Isolation Blocks
Electro-Static Spray Cabinet
Top View
5"
45°
45°
3'-10"
45°
3"
CARCASS
45°
1"-4½"
PAIL
Nozzles
Drawings Not-To-Scale

Appendix H

Metals' specifications of water for proper activation of lactoferrin (ALF Ventures, 2005).

Metal	**Spec. (ppm)**	**Metal**	**Spec. (ppm)**
Aluminum	<0.1	Magnesium	<1
Antimony	<0.1	Manganese	<0.01
Arsenic	<0.1	Mercury	<0.005
Barium	<0.1	Molybdenum	<0.02
Beryllium	<0.01	Nickel	<0.1
Cadmium	<0.01	Potassium	<0.5
Calcium	<5	Selenium	<0.1
Chromium	<0.01	Silver	<0.01
Cobalt	<0.04	Sodium	<10
Copper	<0.1	Thallium	<0.1
Iron	<0.1	Vanadium	<0.1
Lead	<0.05	Zinc	<0.3

Physical specifications of water for proper activation of lactoferrin (ALF Ventures, 2005).

Physical	**Spec. (ppm)**
pH	5.0 - 6.7
Hardness	<6

Inorganic Specifications of water for proper activation of lactoferrin (ALF Ventures, 2005).

Inorganic	Spec. (ppm)
Total Cyanide	<0.1
Chloride	<1
Fluoride	<0.1
Nitrogen, Nitrite	<0.5
Ortho- phosphate	<0.1
Phosphate	<0.1
Sulfate	<1

Microbiological Specifications of water proper activation of lactoferrin (ALF Ventures, 2005).

Media	Spec. (CFU/100 mL)
Total Plate Counts	< 1
Coliform Counts	< 0.1
E. coli	0

Vita

Adam Wayne Tittor

Candidate for the Degree of

Master of Science

Thesis: EFFICACY OF ELECTROSTATICALLY SPRAYED ACTIVIN (ACTIVIATED LACTOFERRIN), HOT WATER, HIGH PRESSURE WATER AND LATIC ACID INDIVIDUALLY AND USED IN A MULTI-HURDLE APPROACH TO REMOVE *ESCERICHIA COLI* O157:H7 FROM BEEF TISSUE

Major Field Animal Science

Biographical

Personal Data: Born in Fort Worth, Texas On February 2, 1981, the son of Paul & Scotty Tittor and Bonnie Musser.

Education: Graduated Salutatorian from Paradise High School, Paradise, Texas in May, 1999; received Bachelor of Science degree in Animal Science from Texas Tech University, Lubbock Texas in 2003; Completed the requirements for the Master of Science degree with a Major in Animal Science specializing in Meat Science in July 2005.

Experience: Raised in Paradise, Texas; raised and showed cattle, swine and horses; employed by Texas Tech University, Department of Animal Science Meat Laboratory as an undergraduate 2000-2003; judged on the 2001 Texas Tech University Intercollegiate Meats Judging Team; IBP (Tyson) Quality Control Intern 2001, Excel (Cargill Meats Solutions) Production intern 2002, and Oklahoma State University as a graduate research assistant and 2005 Spring Meats Coach.

Professional Memberships:
American Meat Science Association (Student Board Directors)
American Society of Animal Science

Name: Adam Wayne Tittor Date of Degree: July, 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFICACY OF ELECTROSTATICALLY SPRAYED ACTIVIN (ACTIVIATED LACTOFERRIN), HOT WATER, HIGH PRESSURE WATER AND LATIC ACID INDIVIDUALLY AND USED IN A MULTI-HURDLE APPROACH TO REMOVE *ESCERICHIA COLI* O157:H7 FROM BEEF TISSUE

Pages in Study: 118 Candidate for the Degree of Master of Science

Major Field: Animal Science

Scope of Study: The purpose of this study was to: determine 1) the efficacy of electrostatically sprayed Activin (A) against *Escherichia coli* O157:H7 inoculated on adipose tissue, compared to treatments of hot water rinse (HW), high pressure spray (HP3, HP15), Activin's buffer (B) and lactic acid spray (LA); 2) if Activin has a synergistic effect when applied in a multi-sequence spray (MH-A) wash used by National Beef Packing Company on the reduction of *Escherichia coli* O157:H7 inoculated on beef lean and adipose tissue compared to the sequence without Activin (MH-NA); 3) MH-A and MH-NA's effects on total plate counts (TPC), coliform counts (CC), *Enterobacteriaceae (ENT)* and lactic acid bacteria (LAB) when applied to uninoculated lean and adipose tissue, and stored for 7 days at 7° C.

Findings and Conclusions:

Greater ($P < 0.05$) reduction of *E. coli* O157:H7 was shown for adipose tissue samples treated with HW than HP3, B, HP15 and A. No differences ($P > 0.05$) were evident between treatments of LA, HP3, HP15, and A on *E. coli* O157:H7. No difference ($P > 0.05$) in reduction of *E. coli* O157:H7 was found on samples treated with MH-A and MH-NA on lean or adipose tissue, 2.4-2.9 log CFU/cm^2. Adipose tissue treated with MH-A displayed reduced ($P < 0.05$) coliform counts following a 7 d storage period compared to adipose tissue treated with MH-NA. Lean samples treated with MH-A displayed reduced ($P < 0.05$) LAB compared to MH-NA treated lean tissue following a 7 d storage period. Lean samples treated with MH-A had greater ($P < 0.05$) reductions than samples treated with MH-NA for TPC and LAB after 7 d. No differences ($P > 0.05$) were observed for *ENT* on lean and adipose tissue. These findings suggest using electrostatically sprayed Activin as a single treatment or a multi-hurdle intervention sequence is not effective to reduce *E. coli* O157:H7 on hot adipose or lean tissue.

ADVISER'S Approval:_____