Translocation of Orally Inoculated *Salmonella* Following Mild Immunosuppression via Dexamethasone Infusions in Dairy Steers and the Presence of the *Salmonella* in Atypical Anatomical Locations.

by

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Abstract

Salmonella is a foodborne pathogen of increasing concern in the U.S. as foodborne and legislative action against foods containing this pathogen is being developed. *Salmonella* is the leading bacteria associated with foodborne illness in the U.S. (CDC, 2018). The objective of this study was to determine if immunosuppression via daily dexamethasone (DEX) infusion altered *Salmonella* Typhimurium (SAL) translocation from the GI tract to atypical locations. Weaned Holstein steer calves (n = 20; BW = 102 ± 2.7kg) received DEX (n = 10; 0.5mg/kg BW) or saline (CON; n = 10; 0.5mg/kg BW) for 4 d (from d -1 to d 2) prior to oral inoculation of SAL (3.4 x 10^6 CFU/animal) via milk replacer on d 0. Fecal swabs for SAL shedding were obtained daily and samples were confirmed positive beginning 24 h post inoculation (d 1) to harvest (d 5). Dexamethasone administration was achieved via indwelling jugular catheters fitted simultaneously with rectal temperature (RT) recording devices on d −2 relative to inoculation and placed in individual pens in an environmentally controlled facility. Whole blood was collected at -24, -12, and 0 h; and 8-h intervals from 8 to 120 h for hematology. Upon harvest (d 5), the ileum, cecal content, lymph nodes (ileocecal, mandibular, popliteal, and prescapular), and synovial (stifle, coxofemoral and shoulder) swabs were collected for the isolation of the inoculated strain of SAL. Following inoculation, 100% of DEX calves shed the experimental strain of SAL for 5 d, 90% of CON calves shed from d 1 to 3, and 100% of CON calves shed from d 4 to 5. There was a time x treatment ($P < 0.01$) for RT such that the DEX treatment resulted in a decreased RT on d -1 to 2. There were increased WBC and neutrophils in DEX steers ($P < 0.0001$), and lymphocytes increased following DEX administration ($P = 0.02$ at 24 and 48h, respectively. Greater ($P < 0.01$) concentrations of SAL were quantified from the cecum of DEX calves (3.86 ± 0.37 log CFU) than CON (1.37 ± 0.37 log CFU); There was no difference in SAL concentrations between DEX and CON calves in ileal tissue ($P = 0.07$), nor ileocecal ($P = 0.57$), mandibular ($P =0.12$), popliteal ($P = 0.99$), nor prescapular ($P = 0.83$) lymph nodes. *Salmonella* was isolated from the stifle joint of one calf in the CON group; however, SAL was not isolated from any other joint fluids sampled. Of the stifle samples collected, 3.3% were positive for SAL indicating the opportunity of contaminating the meat during hind quarter fabrication. These data may be interpreted as either a mild immunosuppression due to multiple

DEX exposures, or a combination of both mild immunosuppression and slight return of immune function when juxtaposing DEX with *Salmonella* inoculation. While more research is needed to elucidate the interactions of immunosuppression and pathogen migration patterns, these data confirm that orally inoculated SAL can translocate from the G.I. and be harbored in atypical locations representing a food safety risk.

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List of Abbreviations

ARS	Agricultural Research Service
BGA	Brilliant Green Agar
BW	Body Weight
CBC	Complete Blood Count
CDC	Center of Disease Control
CFU	Colony Forming Units
CON	Control
CTL	Cytotoxic T-cell
DEX	Dexamethasone
FDA	Food and Drug Administration
Fig.	Figure
FSIS	Food Safety Inspection Services
GC	Glucocorticoid
GI	Gastrointestinal
HPA	Hypothalamic Pituitary Adrenal
H_2O_2	Hydrogen Peroxide
H_2S	Hydrogen Sulfide
IACUC	Institutional Animal Care and Use Committee
IFN-γ	Interferon-Gamma
IL-6	Interlukin-6
IM	Inner Membrane

LB	Luria-Bertani Agar
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MHC	Major Histocompatibility Complex
O_2^{-}	Superoxide Anion
ODPHP	Office of Disease Prevention and Health Promotion
OM	Outer Membrane
PAMP	Pathogen-Associated Molecular Pattern
PBS	Peptone Buffered Saline
pH	Potential Hydrogen
ROS	Reactive Oxygen Species
RT	Rectal Temperature
RV	Rappaport Vassiliadis Broth
SAL	Naladixic Acid Resistant *Salmonella* Typhimurium
SAS	Statistical Analysis System
SCV	*Salmonella* Containing Vacuole
$S_4O_6^{2-}$	Tetrathionate
$S_2O_3^{2-}$	Thiosulfate
SPI-1	*Salmonella* Pathogenicity Island-1
SPI-2	*Salmonella* Pathogenicity Island-2
S. Enteritidis	*Salmonella Enteritidis*
S. Newport	*Salmonella Newport*
S. Typhimurium	*Salmonella* Typhimurium

TLR	Toll like Receptor
TNF-α	Tumor Necrosis Factor-Alpha
TS	Transport Swab with Modified Liquid Stuarts
TSI	Triple Sugar Iron Slant
TT	Tetrarhionate Broth
USDA	United States Department of Agriculture
APHIS	Animal and Plant Health Inspection Service
WBC	White Blood Cell
XLT	Xylose Lysine Tergitol-4
°C	Degrees Celsius

Chapter 1. Literature Review

1.1 *Salmonella* Introduction

Salmonella is a foodborne pathogen of increasing concern in the U.S. as foodborne nd legislative action against foods containing this pathogen are in continuous discussion (ODPHP, 2010). *Salmonella* in beef cattle may be harbored within peripheral tissues that are not reached by topical pathogen interventions within the harvest facility; however, the mechanisms by which enteric *Salmonella* translocate to these atypical peripheral tissue locations are not well understood (Gragg et al., 2013). Therefore, the objective of this study was to determine if immunosuppression via daily dexamethasone (DEX) infusion altered *Salmonella* translocation from the gastrointestinal (GI) tract. To understand the translocation of *Salmonella* into the suspected tissues, the overall structure and survival methods of *Salmonella* in the microbiota in the GI of a beef animal must be examined.

1.2 *Salmonella* Structure

1.2.1. "Simple" Structure

Unlike many other organisms, bacteria is faced with hostile environments from dry external climates to the acidity of the stomach content, even making its way to the inside of blood cells (Nieto et al., 2016). Through these diverse environments, bacterium have evolved highly sophisticated and complex surface components to provide protection for this "simple" organism. *Salmonella* is a gram-negative bacterium, which contains a cell envelope consisting of three primary layers as well as flagella for transport (Sterzenbach et al., 2013). These three layers include a peptidoglycan layer, inner membrane (IM), and an outer membrane (OM) (Sterzenbach et al., 2013).

1.2.2. The Inner Membrane

Bacteria, like *Salmonella*, lack intracellular organelles, therefore, many functions such as energy synthesis, protein secretion, and biosynthesis of structural components are performed in the inner phospholipid bilayer membrane (Silhavy et al., 2010; Sterzenbach et al., 2013). These components include phospholipid, peptidoglycan molecules, and lipopolysaccharide (LPS) units for the growth and division (Sterzenbach et al., 2013).

1.2.3. The Periplasm and Peptidoglycan

Between the IM and OM lies a viscous cellular cavity packed densely with soluble proteins first termed the periplasm (Mullineax et al. 2006; Silhavy et al., 2010; Sterzenbach et al., 2013). The periplasm has an array of functions which include degradative potentially harmful enzymes (such as those from the immune system and antibiotics), nutrient transport, and transport of molecules for envelope biogenesis (Silhavy et al., 2010). The peptidoglycan, a rigid exoskeleton made up of cross-linked peptide side chains, is essential for the integrity of bacteria cell walls (Vollmer et al., 2008). Without the peptidoglycan layer's inhibition during biosynthesis, the cell will die from lysosomes and other mutations (Vollmer et al., 2008). Along with the survival of the cell, peptidoglycan is used as a scaffold, anchoring the OM membrane with lipoproteins called LPP (Braun, 1975; Silhavy et al., 2010). The LPP is a covalent attachment from the lipids of the peptidoglycan layers to the small proteins embedded in the OM (Vollmer et al., 2008; Silhavy et al., 2010).

1.2.4. The Outer Membrane

The outer most layer of the Gram-negative cell is the OM, a distinguishing feature of Gram-negative bacteria whereas, Gram-positive bacteria lack this lipid bilayer membrane (Silhavy et al., 2010). This bilayer is made up of small amounts of phospholipids but mainly glycolipids like LPS, which surrounds the peptidoglycan layer, shielding the periplasm from the external environment (Silhavy et al., 2010; Sterzenbach et al., 2013). This critical function is due to the selectively permeable barrier for specific small molecule transport through β sheet channel-forming proteins referred to as "porins" that allow the influx of nutrients and the excretion of waste products (Nikaido, 2003; Silhavy et al., 2010).

1.2.5. The Lipopolysaccharide Structure

The LPS is found in the outer leaflet of the OM though they are synthesized on the inner leaflet of the IM (Silhavy et al., 2010). Consisting of three regions, the LPS has a hydrophobic domain known as lipid A, the non-repeating core oligosaccharide, and a distal O side-chain repeating oligosaccharide (Raetz and Whitfield 2002; Silhavy et al., 2010; Sterzenbach et al., 2013). Lipid A is the structural anchor of LPS to the remaining OM; however, it has extremely potent biological effects (Raetz and Whitfield 2002; Sterzenbach et al., 2013). Throughout the

evolution of eukaryotes, lipid A has been present in the cell envelope of Gram-negative bacteria, therefore leading to the development of an infectious marker by the host immune system responsible for the endotoxin shock, pyrogenicity, activation of complement, coagulation and haemodynamic changes upon identification (Raetz and Whitfield 2002; Sterzenbach et al., 2013). Though adult cattle have adapted to *Salmonella* infections, the human innate immune system is sensitive to this molecule leading to a sure indicator of salmonellosis (Silhavy et al., 2010).

The core oligosaccharide is structurally conserved among related bacteria and serves as a site of attachment for the enterobacterial common antigen or the variable O side chains commonly referred to as O-antigens (Raetz and Whitfield, 2002). This structural feature is important for the outer membrane integrity and resilience (Raetz and Whitfield, 2002). Though enterobacterial common antigens are not well understood, recently Bridges et al (2015), theorized the function to be associated with the role in bile salt resistance which is a common trait of *Salmonella*.

O-antigens are the distal articulation of the LPS layer and are hydrophilic in nature, reaching out to the microenvironment (Sterzenbach et al., 2013). In *Salmonella*, O-antigens are important for the function of bacterial surface adherence, environmental persistence, and multicellular behavior (Barak et al., 2007; Crawford et al., 2008). The O-antigen component is also of importance in evading the host's immune response. The lymphocyte killing mechanism identifies the pathogen and releases membrane attack complex (MAC), a four protein binding complement component to destroy the bacteria (Muller-Eberhard, 1986). In this defense mechanism, hydrophobic sites of MAC allow the complex to insert itself into the lipid bilayer of the pathogenic membrane causing lysis of the bacteria (Muller-Eberhard, 1986). The O-antigen chains however aids in serum resistance due to the increased distance of the MAC to the cell surface, which prevents insert of larger channels into the bacterial OM (Bravo et al., 2008; Islam et al., 2014). Due to unique configuration, the specific O-antigen is used to define bacteria serology (Raetz and Whitfield, 2002; Silhavy et al., 2010; Sterzenbach et al., 2013). The system that is used to differentiate the serovars of *Salmonella* is based on the structure of the O-antigen paired with the specificity of the H (flagellar) antigen as well (Sterzenbach et al., 2013).

1.2.6. The Flagella

Though *Salmonella* uses flagella for transport, the flagellar proteins found in these motile structures are referred to as “H-antigens” which, like O antigens, are used for serotyping (Herrera-León et al, 2004; McQuiston et al., 2004; Sterzenbach et al., 2013). Unique among Enterobacteriaceae, *Salmonella* commonly has two distinct H-antigen, phase 1 and phase 2 flagellar proteins that have the ability to change phases to avoid detection from the host’s defense mechanisms (Herrera-León et al, 2004; McQuiston et al., 2004). *Salmonella* code these H antigens by one of two genes, fliC and fljB, however, only one H-antigen phases can be expressed by a single cell (McQuiston et al., 2004). The unique antigenic configurations of *Salmonella* are derived from the antigenic properties of their LPS sugar repeat units (O-antigens) and their flagellar structural protein subunits (H-antigens) which leads to the specific serotype of the bacteria.

1.3. *Salmonella* in the Gastrointestinal Tract

1.3.1. *Salmonella* Life Cycle

For *Salmonella*, the infectious life cycle exists in different habitats. The bacteria passes from the dry external environment, through the acidity of the stomach, the lumen of the gut, and the extracellular space of host tissues; therefore the protective mechanism must be highly developed for survival. After a *Salmonella* cell has been ingested, there are several infective mechanisms that mediate the invasion of the host’s intestinal epithelium, elicit inflammatory response which subsequently changes the intestinal epithelium, and evade detection and elimination that is essential for pathogenesis (LaRock et al., 2015).

1.3.2. *Salmonella* and the Bovine Microbiota

The intestinal environment of a healthy bovine is close to anaerobic with the normal ruminal microflora readily consuming what traces of oxygen exist (Thiennimitr et al., 2011). The microflora in the gut is used for the breakdown of nutrients through fermentation which generates cytotoxic compounds (Thiennimitr et al., 2011; Li et al., 2012). Hydrogen sulfide (H_2S) produced as a by-product of fermentation and can be harmful to the intestinal tissue;

however, the intestinal epithelium enzymatically detoxifies H_2S to thiosulfate ($S_2O_3{}^{2-}$) (Thiennimitr et al., 2011).

During gastroenteritis, large numbers neutrophils transmigrate into the intestinal lumen through the adhesion molecule, L-selectin, expressed on the surface of white blood cells (Burton et al 1995). The neutrophils transmigrate into the intestinal lumen, giving rise to characteristic of scours associated with pathogenic infection by damaging the lumen wall (Santos et al., 2001). Neutrophils harbor a diverse array of antimicrobial functions, including antimicrobial granules, phagocytic abilities, production of reactive oxygen species (ROS), and neutrophil extracellular trap (Sekirov et al., 2010). The neutrophil blood cell release ROS in an attempt to kill bacteria through oxidative burst of the bacteria cell (Thiennimitr et al., 2011). Though this is an immune function to terminate pathogenic cells, the host intestinal microbiota has been shown to be more susceptible to these antimicrobial mechanisms than the invading bacteria such as *Salmonella* Typhimurium (*S. Typhimurium*) (Sekirov et al., 2010). A secondary function of releasing ROS is the oxidation of thiosulfate ($S_2O_3{}^{2-}$) to tetrathionate ($S_4O_6{}^{2-}$) which *Salmonella* can utilize (Hensel et al., 1999). *Salmonella* employs tetrathionate as a terminal electron acceptor used for cellular respiration (Hensel et al., 1999). This mechanism enables *Salmonella* to use respiration to produce energy in the nutritionally competitive conditions of the gut (Winter et al., 2010). In the harsh environment of the gut, *Salmonella* can use the inflammation response of the host to outcompete microbes (Winter et al., 2010).

With *Salmonella* thriving in the gut, the customary diarrhea enhances the transmission rate of bacteria to the next host by fecal shedding of the organism via the fecal-oral route (Bäumler et al., 2011). Winter et al., (2010) suggests that the inactivation of genes required to utilize tetrathionate respiration, will in turn eliminate the ability of *Salmonella* to outgrow the microbiota during gastroenteritis. This data indicates that tetrathionate respiration is one of the main mechanisms enhancing outgrowth of *Salmonella* in an inflamed gut. This host response provides a new respiratory electron acceptor in the gut, enabling the pathogen to outgrow the competing microbiota in the lumen.

1.3.3. *Salmonella* resistance to pH Stress

S. Typhimurium possesses an adaptive response to acid that increases survival during exposure to extremely low pH values in the digestive tract (Park et al., 1996). Starting with its

ingestion of the pathogen, the microbe must endure severe acid in the bovine stomach, volatile fatty acids in the intestine, and mild to moderate acid in the phagosomes and phagolysosomes of epithelial cells and macrophages (Park et al., 1996). With these hurdles, *S. Typhimurium* have evolved to include a variety of acid survival mechanisms designed to counteract acid stress with mediated acid tolerance responses (Park et al., 1996). Inducible amino acid decarboxylases neutralize external pH around the bacteria cell rather than subjecting to acid stress (Foster, 1993; Foster and Hall, 1992; Lee et al., 1995). By the decarboxylation of an available amino acid, the highly alkaline amine can be utilized to pump out of the cell into the external surroundings (Foster, 1993; Foster and Hall, 1992; Lee et al., 1995). Though this seems to be an effective buffer against acidic conditions, high concentrations of bacteria are needed, which is an unlikely factor in the ingestion of *Salmonella* (Park et al., 1996). Another acid tolerance responses has been found to play a role in the survival of pathogenic cells exposed to extreme acidity. Instead of the decarboxylation of amino acids to buffer the external pH, a lysine decarboxylase was utilized to maintain the pH homeostasis of the *S. Typhimurium* cell internally (Park et al., 1996).

1.3.4. *Salmonella* transmission through the Epithelium

After surviving the acidity of the gastric portion of the GI tract in the rumen, the bacterium enters the small intestine, and binds to epithelial cells of the intestinal wall (Holt, 2000). Bile is an important antimicrobial component of the human digestive system in which cholesterol is enzymatically converted to bile salts by the liver (Urdaneta and Casadesús, 2017). Once produced, bile is sent to the small intestine by bile ducts to control the structure and function of the intestinal microbiome (Urdaneta and Casadesús, 2017). If the pathogen is compromised by bile salts, both DNA damage and membrane damage is induced via ROS easing replication and eventually cell death (Merritt and Donaldson, 2009). One mechanism against this destruction the use of efflux pumps to remove bile salts from the cell, thus preventing potential damage to the DNA so that the cell can repair effected membrane (Merritt and Donaldson, 2009). Another mechanism employs genetic repair of mismatched gene to suppress the sensitivity to bile if DNA damage is sustained (Urdaneta and Casadesús, 2017).

Upon ingestion, *S. Typhimurium* colonizes the terminal end of the ileum and colon, eliciting symptoms of gastroenteritis in the bovine species within 24 hours post inoculation

(Thiennimitr et al., 2011). Once bound, T3SS-1 mediates irreversible adhesion secreting effector proteins which elicit the uptake of *Salmonella* (Keestra-Gounder et al., 2015; Que et al., 2013). After traversing the epithelium, the second secretion of T3SS-2 aids in the survival of the pathogen in phagocytic immune cells such as macrophages, monocytes, and neutrophils (Que et al., 2013). Pathogen-associated molecular pattern (PAMP) are immune markers recognized by Toll-like receptors (TLR) causes pro-inflammatory responses in the host cell (Sterzenbach et al., 2013). The pathogen persists within an O-antigenic *Salmonella* containing vacuole (SCV) preventing the detection and fusion of lysosomes (Nieto et al., 2016). Specific genes mediate the development (*Salmonella* pathogenicity island 1 [SPI-1]) and maintain the integrity (*Salmonella* pathogenicity island 2 [SPI-2]) of the SCV where the pathogen can proliferate (Thiennimitr et al., 2011; Figueira and Holden, 2012; Que et al., 2013; Hurley et al., 2014). Effector proteins are translocated from the SCV via SPI-2, to capture nutrients needed for replication (Hurley et al., 2014). The SCV enhances this resistance to the innate immune system by diminishing surface expression of PAMP (Marshall and Gunn, 2015).

1.4. Bovine Immunity to *Salmonella*

In a review by Carroll and Forsberg (2007), the immune system can by separated into the innate immunity and adaptive immunity. The first line of defense is that of the innate immune system, in which nonspecific defense mechanisms are elicited immediately following pathogenic exposure. Characterized as the first line of defense, physical barriers such as saliva, mucosal secretion, stomach acid, and bile salts, even those of engulfing blood cells are categorized as innate immunity. With this initial defense, the innate system of a mature animal commonly provides adequate time for the adaptive immune system to launch a specialized attack against the invader.

1.4.1. The Innate Immune System

Once the pathogen infects the body, the innate immune system engulfs the invader with phagocytic cells (i.e. neutrophils, monocytes, macrophages, and dendritic cells) and attempts to eliminate the pathogen (Carroll and Forsberg, 2007). In contrast to the adaptive immunity, the phagocytic cells do not recognize the antigen of the pathogen, however, they do use TLR for recognizing the immune marker PAMP (Carroll and Forsberg, 2007, Sterzenbach et al., 2013).

Though ten TLRs are known, TLR-4 and TLR-5 are essential for initiating immune defense mechanisms against *Salmonella* (Carpenter and O'Neill, 2007). Toll-like receptors-4 recognizes the O-antigen of the LPS, whereas, TLR-5 recognizes bacterial flagellin (Carpenter and O'Neill, 2007; Thiennimitr et al., 2011). Once PAMP is identified an increase in inflammatory mediators are induce, including cytokines, which activate innate immune cell production, and chemokines, which stimulate the transmigration of immune cells to the site of pathogenic infection. (Wray and Wray, 2000) The pattern recognition results in the production of pro-inflammatory cytokines (i.e., Tumor necrosis factor-Alpha [TNF-α], Interleukin-6 [IL-6], Interferon-Gamma [IFN-γ], etc.) which signal a host defense pathways including macrophage activation, neutrophil recruitment, and the epithelial release of antimicrobials into the intestinal lumen (AndrewsPolymenis et al., 2010; Thiennimitr et al., 2011; Figueira and Holden, 2012).

During pathogenic infection, lymphocytes release pro-inflammatory cytokines such as IFN-γ, as part of the adaptive immune system (Carroll and Forsberg, 2007). Interferon-gamma is the primary macrophage activating factor of the pro-inflammatory cytokines in circulation (Young and Hardy, 1995). Though the name Interferon steams from IFN-γ negatively effecting viral replication, exposure to this cytokine results in signaling for specialized immune system function through critical in the stimulation, differentiation, and function of monocyte/macrophages (Young and Hardy, 1995; Schroder et al., 2004). In addition to being IFN-γ producers, lymphocytes are also drastically and profoundly affected by this cytokine (Dinarello, 2000). The cytokine, IFN-γ, activates lymphocyte differentiation and enhances the immune cell activity (Young and Hardy, 1995). With these effects on lymphocytes, IFN-γ increases sensitivity and responsiveness to the specific pathogens in the adaptive immune system (Schroder et al., 2004). Several major impacts IFN-γ has on neutrophils, include increasing phagocytosis function and enhancing the superoxide production for pathogenic lysing (Dinarello, 2000).

The cellular component of the innate immune system consists of phagocytic cells referred to as leukocytes or white blood cells (WBC; Carroll and Forsberg, 2007). These WBC include neutrophils, monocytes, macrophages, and dendritic cells which engulf the pathogen for elimination (Carroll and Forsberg, 2007). Shortly after the pathogen enters the digestive tract, the organism is engulfed by macrophages where the pathogen will be circulated throughout the

lymphatic system, activating the adaptive immune response; however, *Salmonella* have mechanisms previously stated to use this phagocytosis to migrate into the intestinal lumen while evading detection (Nieto et al., 2016). *S. Typhimurium* prefers macrophages as the intercellular host cell, evading detection using the O-antigen SVC (Fields et al., 1986; Nieto et al., 2016). Within the macrophage, *S. Typhimurium* acquires nutrients to complete replication of new bacteria cells (Thiennimitr et al., 2011). Once the pathogen has achieved maximum replication, apoptosis of the macrophage cell is triggered by Caspase-1 referred to as pyroptosis (Cookson and Brennan, 2001; Miao et al., 2011). This programmed cell death releases *S. Typhimurium* from macrophages allowing phagocytosis of other pathogen killing cells such as neutrophils (Thiennimitr et al., 2011). Increasing production of the pro-inflammatory cytokine IFN-γ allows enhances the pathogen killing ability of the macrophages once the immune system moves from innate to adaptive immunity (Young and Hardy, 1995). The jejunal-ileal Peyer's patches in the bovine ileum have a follicle-associated epithelium, composed largely of these macrophage cells that cover the domed villi which are surrounded by other nutrient absorbing villi (Santos et al., 2002).

Once released from the macrophages, infection of the jejunal-ileal Peyer's patches results in an expression of chemokines which activates an influx of neutrophils enters vascular circulation eventually migrating to the inflamed tissues (Burton et al. 1995; Raffler et al., 2005; Nunes et al., 2010). This transmigration process is possible through adhesion molecules present on both the WBC and the epithelial tissue (Burton et al. 1995; Raffler et al., 2005). Expression of L-selectin on the surface of WBC facilitates migration from the vascular wall to the inflamed tissue via receptor–ligand binding from L-selectin expressed on the WBC and Mac-1 expressed in the vascular tissue (Burton et al., 1995).

Once neutrophils infiltrate the target tissue the phagocytosis mechanism is employed to engulf the invading cells and use ROS to induce oxidative killing of bacteria (Thiennimitr et al., 2011). As previously stated, though the primary function of ROS is to kill bacteria, the side effect has been shown to allow *Salmonella* to out-compete the host's microbial levels (Hensel et al., 1999). A negative effect of the increase in neutrophil concentrations is the loss of epithelial integrity due to necrosis and the characteristic scours of *Salmonella* by exudative mechanisms (Nunes et al., 2010). The necrosis of the intestinal lining due to the massive infiltration of

neutrophils allow the bacteria further attachment and invasion due to the lack of microvilli of the apical cell surface (Santos et al., 2002).

1.4.2. The Adaptive Immune System

The jejunal-ileal Peyer's patch is a primary lymphoid organ of B cell lymphocyte development, therefore, once the antigen of *S. Typhimurium* becomes exposed, the adaptive immune system takes action (Yasuda et al., 2006). The adaptive immune system is categorized into the cell-mediated or humoral immunity in which a specialized immunologic response acts directly against the specific antigen or the production of specific antibodies of a pathogen previously encountered (Carroll and Forsberg, 2007). In the cell mediated defense, antibodysecreting cell precursor, B cell lymphocytes, can directly recognize native antigens in the mucosal lymph tissue through B cell specific receptors (Banchereau and Steinman, 1998). However, T-cell lymphocytes need antigen presenting cells to process and present antigens of specific pathogens to them using the T-cell antigen receptors (Goodnow, 1997; Banchereau and Steinman, 1998). The T-cell antigen receptors recognize fragments of antigens bound to molecules of the major histocompatibility complex (MHC) which are separated into two types, MHC class I and MHC class II (Banchereau and Steinman, 1998). These two binding proteins which stimulate a response of the cytotoxic T cells (CTLs) and helper T cells, which, once activated, directly kill a target cell through antibody production (Banchereau and Steinman, 1998). Dendritic cells are efficient stimulators of both B and T cell lymphocytes, which capture and process antigens, presenting MHC complexes on their surface before migrating to the lymphoid organs such as the lymph nodes (Banchereau and Steinman, 1998). This specialized function of dendritic cells helps lymphocytes activation by being a mobile sentinel presenting antigens to T-cell located in lymph tissues throughout the body (Banchereau and Steinman, 1998). These third-party antigen presenting cells are the key between an innate and adaptive immune response.

Helper T cells aid in the proliferation and differentiation of lymphocyte cells, as well as produce a cytokine profile for both the cell-mediated and humoral response (Fearon and

Locksley, 1996). This differentiation can develop helper T cells into CTLs when the adaptive immune system is activated (Fearon and Locksley, 1996). Antibodies are expressed through CTLs known as immunoglobulins on the surface of the cell and contribute to the destruction of the pathogenic cell through the tubular insertion into the bilayer of the phospholipid vesicles of bacteria (Muller-Eberhard, 1986). Therefore, detection of the pathogen leads to the productions of antibodies from the lymphocytes which rid the body tissues of bacteria cells, while in turn preparing the immune system for a future pathogenic infection from *S. Typhimurium* (Thiennimitr et al., 2011).

1.4.3. The Febrile Response

Along with leukocyte signaling, pro-inflammatory cytokines stimulate a multitude of reactions such as fever, lethargy, and loss of appetite (Carroll et al., 2009). The febrile response is a highly developed immune defense with primitive roots, sufficiently destroying many bacteria cells within the host (Matthew, 1986). During an increase in host body temperature, bacteria become under attack by enzymatic processes associated with negatively alternating the formation of the bacterial protective coat of bacteria (Carroll and Forsberg, 2007). Although specified pathogens evoke a specialized immune responsive, characteristic inflammation and heat are associated with adaptive qualities of the non-specific defense mechanisms (Coutinho and Chapman, 2011). Though it is well understood that the febrile response can thermally suppress bacterial growth, it has been found to have effects on cytokine production as well (Downing et al., 1987). Previously, Downing et al (1987) isolated and cultured mononuclear cells isolated from adult rhesus monkeys experiencing hyperthermia (2°C above control temperature) and observed as much as a 16-fold increase in IFN-γ production relative to those of the same animal before the heat treatment. Comparable results were obtained when a febrile response was induced with a systemic injection of nonviable Escherichia coli, which similarly to SAL, includes a LPS layer (Downing et al., 1987) Later, Downing et al. (1987) evaluated lymphocytes function in humans from hyperthermic individuals, and observed as much as 10-fold increase of IFN-γ than cells withdrawn at basal core temperatures from the same individuals.

Though the febrile response is needed for increase immune function, maintaining a rectal temperature of even 1°C above normal increases metabolic energy cost by 10% to 15% (Kulger, 1986). In addition, energy is needed in the production of cytokines, leukocytes, and other actions

of the immune system, redirection nutrient utilization away from growth and towards the immune system (Carroll et al., 2011; Sanchez et al., 2013). Though nutrients are needed to perform immune functions, bacterial infection typically decreases feed intake (Lohuis et al., 1988; Cullor, 1992; Steiger et al., 1999; Waldron et al., 1999; Elsasser et al., 2008; Sanchez et al., 2013).

1.5. Effects of Dexamethasone administration on the Immune System

1.5.1. Introduction

Natural and synthetic glucocorticoids (GC) remain at the forefront of anti-inflammatory and immunosuppressive therapies in humans. Similarly, in cattle synthetic GC are used in post parturition treatment of mastitis and other anti-inflammatory therapies. In research, the administration of synthetic GCs such as DEX, are given to mimic the effects of immunosuppressive similar to what is observed during stress (Oldham and Howard, 1992). The stress of these a single event increases cortisol which act to briefly enhance the immune system for defense (Oldham and Howard, 1992; Burton et. al., 1995). However, if stress persists moving from an acute to chronic experience, the immune system becomes suppressed increasing the susceptibility for an animal to contract a pathogenic infection (Burton et al., 1995; McGuirk and Peek, 2003). Similarly, to natural GCs, when DEX is administered daily for a prolonged time, there is a negative impact on the immune system seen in a chronic stress event (Oldham and Howard, 1992; Burton et al., 1995; Lopparelli et al., 2012, Hughes et al., 2017). For immunosuppression effects, GC treatment manifests itself by diminished phagocytic activity, decreased pro-inflammatory cytokine stimulation and production, reduction of the febrile response, and decreased responsiveness to adhesion molecules (Barton et al., 2000; Sapolsky et al., 2000; Seekamp et al., 2001). To evaluate the immune status of animals receiving synthetic GCs, mechanisms of the inflammatory response must be well understood and explored.

1.5.2. Dexamethasone Overview

Dexamethasone is classified as a synthetic GCs that affects the hypothalamic pituitary adrenal (HPA) axis similarly to what is observed in stress, in which cortisol exerts a negative feedback on the hypothalamus and decreases the production of recruiting hormones that active immune function (Anderson et al., 1999). Natural GCs have two main actions that influence

immune function, when a stressor is present GCs enhance the synthesis of catecholamines, which control physiological mechanism such as, vasoconstriction, increased pulse, and pupil dilation (Munck et al., 1984). The other affect in a suppressive action to prevent damage of the immune cells on healthy tissues (Munck et al., 1984). This suppressive action observed in natural GCs is the main function of the synthetic GC such as DEX. However, DEX is roughly 30 times more potent than endogenous cortisol, therefore dosage and frequency of administration can wreak havoc on the immune system function with prolonged recovery periods (Roth and Kaeberle, 1981). Of the many biologic effects of GCs, one is suppression of pituitary-adrenal function through the production of cortisol which performs a negative feedback on the system (Melby, 1974). This negative feedback runs concurrently with anti-inflammatory actions of DEX (Melby, 1974). The plasma concentration of cortisol is regulated by ACTH stimulation of the adrenal cortex and will have negative feedback on the HPA axis (Carroll and Forsberg, 2007). When a synthetic exogenous GC such as DEX is administered, the release of endogenous cortisol is completely down regulated because of the negative feedback mechanism (Burton et al., 1995). Anti-inflammatory effects influenced by DEX peak roughly 8-hour post single inter-muscular injection (Pruett et al., 1987). In cattle, DEX, has been shown to have a half-life of 12 hours and total blood cells concentrations will return to their basal concentrations within 48 hours post intra-muscular or intravenous injections (Pruett et al., 1987, Burton et al., 1995). However, the function of adaptive immune cells may exhibit a prolonged down regulated depending on dosages and frequency (Oldham and Howard, 1992; Burton et al, 1995)

1.5.3. Dexamethasone effect on Rectal Temperature

The manifestation of DEX can be observed as reduction of fever, increased appetite, and reduction of inflammation. As previously discussed, increased body temperature is part of the innate defense mechanism of the host against invading pathogens such as *Salmonella* (Lohuis et al., 1988), and the anti-inflammatory effects of DEX administration inhibits the febrile response in various species following inoculation of the pathogen itself or a live strain vaccine (Raekallio et al., 2005; Danek, 2006, Hughes et al., 2017).

1.5.4. Dexamethasone effect on Total Blood Count

At the site of injury or invasion of a pathogen, the host's immune response is the primary mode of defense, setting upon action to expel the foreign invader ultimately restoring homeostasis in the body (Coutinho and Chapman, 2010). Some of the first immune cells to take action are the neutrophils which engulf the bacteria cells, eliminating the threat (Thiennimitr et al., 2011). Several studies have demonstrated an increase in bovine neutrophils as a result of the administration of DEX (Pruett et al., 1987; Anderson et al., 1999; Lopparelli et al., 2012, Hughes et al., 2017). Though increased concentrations of circulating neutrophils indicate an immune system stimulation, in the case of DEX induced immunosuppression, neutrophilia results from inhibition of adhesion molecules expression, therefore decreasing margination and migration of neutrophils from vascular circulation into inflamed tissues (Sousa, 2005). Neutrophils are functional in the recruitment of inflammatory factors, activation of the adaptive immune system, and aid in the phagocytic process of the immune response (Janeway et al., 1996). In a study by Hughes et al. (2017), suppression of the phagocytizing function in neutrophils was reported in cattle receiving daily administrations of DEX. During phagocytosis of pathogens, neutrophils increase their oxygen consumption through an NADPH-oxidase that generates superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2; Dahlgren and Karlsson, 1999). These oxygen metabolites activate other reactive oxygen species that are strongly anti-microbial by releasing the reactive oxygens into the pathogenic cell, termed oxidative burst (Dahlgren and Karlsson, 1999). Intensity of the oxidative burst was also suppressed in cattle receiving frequent DEX injections (Hughes et al., 2017). Hughes et al. (2017) suggests that adhesion molecules are a critical function in the immune response aiding in the translocation of immune cells to the infected target tissues.

Dexamethasone also has negative effects on the adaptive immune system (Burton et al., 1995; Hughes et al., 2017). Data has shown that DEX administration not only depresses circulating lymphocytes but has various effects on different lymphocyte subsets (Oldham and Howard, 1992). A decrease in the blood circulating T-cell lymphocytes has been reported from several studies where immunosuppression was elicited using DEX (Pruett et al., 1987; Oldham and Howard, 1992; Anderson et al., 1999; Hughes et al., 2015). This may be explained by the function of adhesion molecules has on lymphocytes. Bovine blood T-cell lymphocytes express

adhesion molecules as a homing receptor, allowing these immune cells to leave the vascular endothelia and transmigrate back to the lymph nodes (Burton and Kehrli, 1996). Another function of DEX is suppression of lymphocyte responsiveness to the mitogen factors, phytohaemagglutinin and pokeweed mitogen leading to a decreased amount of lymphocyte proliferation (Oldham and Howard, 1992). Lastly, B-cell lymphocytes show signs of decreased responsive ness due to the down-regulation of MHC class II, which activate the cytotoxicity of T-cell lymphocytes (Nonecke et al., 1997). Overall DEX exhibits a suppressive action on both the function and transmission of several immune blood cells in bovine circulation.

1.5.5. Dexamethasone effect on Adhesion Molecules

Neutrophilia results from inhibition of endothelial margination through the vascular wall (Lan et al. 1998). The translocation of neutrophils into inflamed peripheral tissue involves a twostep regulation process of the surface adhesion molecules, L- selectin and Mac-1 (Barton et al. 1995). L-selectin is an adhesion molecule expressed on the surface of WBC such as neutrophils (Barton et al. 1995). L-selectin possesses a lectin domain that interacts with carbohydrate moieties on glycoproteins and glycolipids of the vascular endothelium (Bosworth et al. 1993). This mechanism creates a migrating pool of neutrophils rolling along the vascular tissue seeking inflammatory signals for translocation into target tissues (Burton et al., 1995). When the intercellular adhesion molecule, MAC-1 is expressed on vascular tissues, the two adhesion molecule bind allowing migrating neutrophils to transmigrate through vascular tissue to the targeted inflamed tissues (Raffler et al., 2005). Research by Burton et al. (1995) found that the administration of DEX resulted in a short term decrease in expression of L-selectin on the surface of bovine neutrophils. This activation-induced shedding of L-selectin is proposed to occur by proteolytic cleavage of the molecule close to its transmembrane domain (Lipsky et al. 2002). An anti-inflammatory activity of GCs, including DEX, is to decrease the size of the margination pool of neutrophils by causing both massive shedding of L-selectin and decreased affinity of L-selectin for the vascular endothelium (Burton et al, 1995). The rapid onset and recovery of neutrophil L-selectin expression also suggests that the down-regulating effect of DEX on L-selectin is tightly regulated and disappears once the system in void of DEX (Burton et al., 1995). In the same study, down regulation of MAC- 1 was also observed; however, the

decrease in this adhesion molecule was delayed and occurred over a long period (Burton et al., 1995). Anti- inflammatory mechanisms by DEX prevent neutrophil margination by shedding Lselectin off the surface of the neutrophil and to reduce the number and/or affinity of expressed MAC-1 (Burton et al, 1995). Because T-cell lymphocytes use this L-selectin to exit the epithelial tissue and return to the lymph nodes, the circulating concentrations of lymphocytes are also negatively affected by the down-regulation of these adhesion molecules (Bosworth et al., 1993; Burton and Kehrli, 1996). In conclusion, DEX inhibits the expression of both L- selectin on the surface of WBC and lymphocytes, as well as, the expression on the MAC-1 expression on the vascular endothelium, increasing susceptibility to infections.

1.5.6. Dexamethasone effect on Transcription Factors and Cytokines

Synthetic GCs mostly affect the inflammatory response either directly or indirectly transforming to the transcriptional effects which alter transcription of numerous genes in WBC, both in up and down regulation (Ashwell et al., 2000; McEwen et al., 1997). During inflammation, macrophages (the first phagocytic WBC present) release pro-inflammatory cytokines such as IL-6, TNF-α, and IFN-γ, which stimulate defense mechanisms including fever, production of acute phase proteins, alterations in plasma mineral concentrations, and proliferation of lymphocytes, while negatively impacting the animal’s health resulting in lethargy and loss of appetite (Carroll et al., 2009). However, GCs have been shown to inhibit the production of many cytokines including IL-2 (Bettens et al., 1984), TNF (Waage and Bakke, 1988), IL-1 (Lee et al., 1988) in humans, IFN-γ in cattle (Hughes et al., 2017) and IL-3 (Culpepper and Lee, 1985) in mice. As previously discussed, TLR-4 is responsible for gram negative LPS identification and cell-signaling for the adaptive immune cells to fight the pathogen through activating cytokines (Eichera et al., 2003). Monocytes, macrophages, and dendritic cells of cattle contain mRNA for bovine TLR-2 and bovine TLR-4 causing the cytokine signaling once a pathogen is detected (Werling and Jungi, 2003). The administration of DEX has been found to suppress bovine lymphocyte blastogenesis and polymorphonuclear WBC function (Roth and Kaerberle, 1981). A decrease in IFN-γ concentration should follow a suppression of lymphocytes after DEX administration since IFN-γ is largely produced by lymphocytes (Dinarello, 2000). Many studies in cattle have demonstrated diminished concentrations of IFN-γ in response GC administration (Fischer et al., 1997; Kehrli et al., 1999; Hickey et al., 2003; Kim

et al., 2011, Hughes et al., 2017). One of the primary functions of IFN-γ is to inhibit viral replication of pathogens within the host (Young and Hardy, 1995). In a study by Richeson et al. (2016), found that viral replication was enhanced in animals receiving DEX treatments, which may be directly related to the diminished concentrations of IFN-γ that were observed in these treatments. In conclusion, DEX has a direct effect on cytokines through transcription factors.

1.5.7. Conclusion

Exogenous GCs such as DEX can be used to mimic a natural stressor based on several immunological factors. Total blood cell count is affected with an increase of neutrophils in circulation but the inability for them to translocate through the vascular wall into the affected tissue due to the down-regulation of adhesion molecules. Lymphocytes concentrations are depressed due to decreased responsiveness to mitogens which signals the lymphocyte cell to multiply in the presence of infection. The role of activation/ inhibition of pro and antiinflammatory cytokines are also affected by synthetic GCs. Overall, synthetic GCs have similar pharmacological actions on immune factors as those found in natural GCs when a stressor is present.

1.6. Presence of *Salmonella* in the Host

1.6.1. *Salmonella* in the Outside Environment

Salmonella is one of the leading foodborne illness in the U.S. with the three illnesscausing serotypes listed as *S. Enteritidis* (16.5%), *S typhimurium* (13.4%), and *S. Newport* (11.4%) (FoodNet, 2016). Humans and domesticated animals have been the focal point of *Salmonella* prevalence, however there are a wide array of environmental sites which facilitate the survival of this pathogen (Miner et al., 1967; Murray, 2000; Sanchez et al., 2002; Tizard, 2004; Renter et al., 2006). *Salmonella* thrive in various wet environments such as agricultural runoff, excretions by animals wild or domesticated, and fresh water (Miner et al., 1967; Murray, 2000; Sanchez et al., 2002; Tizard, 2004; Renter et al., 2006). Sediments may be protecting *Salmonella* from environmental stress, therefore, water contaminated with animal feces has the potential for proliferation and be the distribution point of *Salmonella* to other hosts (Fish and Petttibone, 1995; Murray, 2000). The farm environment also facilitates the spread of *Salmonella* through recycling of manure-lagoon waste water for pasture fertilization, contamination of feeds, inadequately controlled rodents and wild bird populations, contaminated farm equipment failing

to sanitize between jobs (Murray, 2000). In a studied by Ply-Forshell (1995), *Salmonella* survived in manure pit for 35 days following introduction.

Though human infection of *Salmonella* is commonly contributed to contamination of meat-producing animals, these individuals can be ingesting foods contaminated by fecal sources including eggs, vegetables, fruit, and nuts (Gopinath et al., 2012). In the feedlot setting, frequent movement of the animals results in the pulverization and aerosolization of the contents on the pen floor. Though feed-yards have developed management practices to such as sprinkler systems to decrease the aeration of dirt; however the close proximity of the pens to one another and the copious amounts of wind currents over the facility makes it difficult to constantly control the potential bacterial contamination (Von Essen and Auvermann, 2005; McEachran et al., 2015). In the outdoor environment, *Salmonella* can live for 4 to 5 years in water, soil, dust, and moist areas devoid of direct sunlight, including foods (McGuirk and Peek, 2003). Since the sources for contamination of environmental sites are so diverse, the absolute elimination of *Salmonella* in the environment is impossible.

1.6.2. *Salmonella* in Cattle

The host-host transmission of *Salmonella* in cattle is primarily by the fecal-oral route (Gopinath et al., 2012). A common management practice is transportation of animals to different facilities. Animals can easily be infected during transport and may disseminate infection among other calves or cattle at the new facilities (Wray et al., 1991). Introduction of infected calves into new herds can unknowingly lead the transmission of *Salmonella* (Wray et al., 1990). Additional factors labeled as stressors, such as transportation and weaning may also exacerbate the spread of disease or increase susceptibility (Carroll and Forsberg, 2007). As previously stated, feces serve as the primary contamination point for *Salmonella* transmission, however the wide array of contaminated environmental sources leaves cattle open to exposure. Another risk factor with cattle are the high shedders termed super-shedders whom may shed up to 10^{10} CFU *Salmonella* per gram of feces and pose a risk of contamination in the food chain (De Jong and Ekdahl, 1965; Gopinath et al., 2012). These super-shedders can also be primary contributors to environmental spread of zoonotic pathogens like *Salmonella* to other host animals (Gopinath et al., 2012). In an experiment by Clinton and Weaver (1981), feeder calves were able to transmit S. Typhimurium to 80% of naïve calves within one week and 23% of those became asymptomatic

carriers (Clinton and Weaver, 1981). Asymptomatic carriers are cattle who are shedding significant amounts of *Salmonella* cells though the animals itself is showing no clinical symptoms of salmonellosis (Clinton and Weaver, 1981); thus, it is critical to identify infected animals and employ farm management practices to treat the situation before the spread of the pathogen.

A USDA-APHIS *Salmonella* surveillance study in 2009 found that 9.2% of cow-calf operations have at least one cow positive for *Salmonella* by fecal culture, however of the cows sampled, only 0.5% of cows were *Salmonella*-positive. This leads to the theory that *Salmonella* is uncommon in the U.S. cow-calf operations. *Salmonella* is a major concern in the beef and dairy industries. A USDA-APHIS *Salmonella* surveillance study in 2011 found that 39.7% of dairy operations had at least one cow positive for *Salmonella* by fecal culture and 13.8% of cows sampled were *Salmonella*-positive, which is an upward trend from the same surveillance study preformed in 1996 and 2002. This increase may be contributed to the parallel increase in the size of the overall dairy herd. In 2007, dairy herds with fewer than 100 cows were 24.3% *Salmonella*positive, herds with 100-499 cows were 44.7% *Salmonella*-positive, and herds with 500 or more cows were 48.7% *Salmonella*-positive (USDA, 2011). A study by Cummings et al. (2009), found that cattle may shed *Salmonella* for more than one year following the recovery of clinical signs of salmonellosis. Along with super-shedders, long-term shedders may also play a major role in *Salmonella* transmission in cattle herds. In a study by Well et al. (2001), fecal samples were collected from representative cows on 91 dairies and 97 cull dairy cow markets in 19 states. In this study 18.1% in cows to be culled across USA dairies and 14.9% for cull dairy cows at markets across the USA were *Salmonella*-positive (Wells et al., 2001). In similar studies the USDA-APHIS released a *Salmonella* surveillance study in 2014 that found of cattle feedlots in the USA, *Salmonella* was cultured positive in 9.1% in fecal samples, 35.6% of pens, and 60.3% of feedlots.

Region and season have also been shown to have an effect of *Salmonella* presence. In a study by Barkocy-Gallagher et al. (2003) included fecal sampling to determine the prevalence of *Salmonella* from feces at three fed-beef slaughter plants during four separate seasons. The authors determined fecal prevalence to be 2.1%, 9.1%, 2.8%, and 2.5% during spring, summer, fall and winter, respectively. This study suggests prevalence peaks during warmer months (summer and fall), and declines in colder months (winter and spring). Furthermore, several

studies suggest a regional variation in *Salmonella* prevalence in North America (Sorensen et al., 2002; Barkocy-Gallagher et al., 2003; Kunze et al., 2008). In a large study by Sorensen et al. (2002), which included fecal samples of cattle housed in Canadian feedlots, *Salmonella* was recovered from 0.2% of cattle prior to entry into the harvest facility (Sorensen et al., 2002). As previously stated, Barkocy-Gallagher et al. (2003), reported peak fecal prevalence of 9.1% during the summer and fall months in the mid-western. While a study by Kunze et al., 2008 resulted in 30.3% recovery of *Salmonella* in the fecal swabs samples from six feedlots over all four seasons. Feed-lots are the final destination of beef animal production prior to the harvest facility; therefore, the necessary steps must be taken to impede *Salmonella* transmission into the food supply chain.

1.6.3. *Salmonella* in Ground Beef

Salmonella infected cattle pose a threat for humans primarily by being a reservoir for the pathogen to enter the food-supply chain, in turn infected human hosts. In a 2 year study of commercial ground beef from seven regions of the United States, *Salmonella* was recovered from 4.2% of ground beef samples (Bosilevac et al., 2009). Using a different detection method FSIS (2011) progress report suggests that the overall prevalence in ground beef is 2.4% in the United States. When evaluating retail ground beef, Zhao et al., (2001) investigated packaged ground beef from seven regionally different cities, reporting a *Salmonella* prevalence of 3.5%. A similar study found 3.8% *Salmonella*-positive samples over a 12 month period in a Seattle, Washington (Samadpour et al., 2005).

As previously discussed, cattle are natural carriers for *Salmonella* and the pathogen is commonly found on the hides. Hide removal as well as evisceration are points of the harvesting process in which the contamination of the carcass is a potential hazard (Brichta-Harhay et al., 2008). The prevalence of *Salmonella* on beef carcasses, although low, have resulted in many interventions throughout the harvesting and fabrication processes as a means to eliminate the pathogen contamination of beef carcasses. Rivera-Betancourt et al. (2003), showed the potential for contamination with a study evaluating the prevalence of Sal*monella* on the hides of cattle, and on the carcass, both pre-evisceration and post-interventions at two different plants in the U.S. Carcass swabs taken prior to evisceration showed a 23.3% and 26.8% prevalence in Plants A

(Southern plant) and B (Northern plant), respectively. Prevalence of *Salmonella* post intervention steps was 0% in Plant A, and 0.8% in Plant B.

In a study previously discussed, Barkocy-Gallagher et al. (2003) evaluated the prevalence of *Salmonella* on hides, and carcass pre- evisceration and post-intervention processes. *Salmonella* was recovered from 61.4%, 91.6%, 97.7%, and 27.7% of hide samples during spring, summer, fall and winter, respectively (Barkocy-Gallagher et al.,2003). The prevalence of *Salmonella* on pre-evisceration carcasses resulted in 3.0%, 19.7%, 24.9%, and 4.1% during spring, summer, fall and winter, respectively (Barkocy-Gallagher et al.,2003). During the summer sample period, *Salmonella* was recovered from only 0.3% post-intervention carcasses, whereas the other sampling periods resulted in a prevalence of 0.1% (Barkocy-Gallagher et al., 2003). This research suggests that the steady prevalence of *Salmonella* in ground beef samples may not be directly related to hide contamination, but by anatomical areas not reached by the harvest intervention steps.

1.6.4. *Salmonella* in Lymph Nodes

Recent research suggests that the lymph nodes of pathogen infected cattle could be the point of ground beef contamination (Arthur et al., 2008). As previously discussed, the lymphatic tissue highly involved in immune function of an infected animal, subsequently aiding in the elimination of the pathogen. Cattle possess large numbers of lymph nodes that are interwoven in the fatty tissue making them a potential point of contamination for ground beef. These lymph nodes are harbored within the animal tissue allowing them to escape chemical and thermal antimicrobial carcass interventions. *Salmonella*-positive lymph nodes could explain the persisting levels of ground beef contamination.

Salmonella is noted for its ability to invade leukocytes and survive within host lymphoid tissues (Thiennimitr et al., 2011). Research suggests that prevalence of *Salmonella* in lymph nodes of seemingly healthy cattle entering the harvest facility can range between 1.6 and 88% (Arthur et al., 2008; Gragg et al., 2013; Haneklaus et al., 2012; Koohmaraie et al., 2012). In a study by Gragg et al., (2013), *Salmonella* was isolated from 100% (hide), 94.1% (feces), 91.2% (mesenteric), 76.5% (subiliac), 55.9% (mandibular), and 7.4% (mediastinal) of samples. This data suggests that the prevalence of *Salmonella* in particular lymph nodes may be dependent on

the pathogen's translocation across various points of the GI tract. For example, the mesenteric lymph nodes are closely associated with the small intestine, a common entry point for macrophage invading *Salmonella* cells. The mandibular lymph nodes are another example as they are positioned near the mouth, a point of entry for *Salmonella*. In a study by Loneragan et al. (2018), cattle were infected with *Salmonella* using skin pricks in specific anatomical locations, to access systematic lymphatic drainage of the pathogen. The data showed a correlation between the anatomical location of inoculation and the specific peripheral lymph node that was located near the site of exposure.

The FSIS research efforts of *Salmonella* testing were key in the recommendation for the National School Lunch Program to purchase ground beef for school lunches that are free of larger lymph nodes such as the popliteal, subiliac, and the subscapular (FSIS, 2017). However, given the extensive number of lymph nodes in the body, complete elimination of the lymph tissue prior to ground beef production is simply not a feasible solution overall.

1.6.5. *Salmonella* in Synovial Fluid

Patients with advanced arthritis usually are treated with a steroid to decrease the inflammation of the joint, therefore seizing some of the joint pain associated with the disease. In a study by Granfors et al., (1990), the synovial cells from nine patients with reactive arthritis following *Salmonella enteritidis* or *Salmonella Typhimurium* infection were examined for *Salmonella* antigens. They reported that four patients had monoclonal antibodies whose western blots indicated *Salmonella* LPS in the synovial cells. In several case studies collected by Warren (1970), *Salmonella* was cultured from the joints in 52 cases where the patient with known arthritis and undergoing steroidal treatment presented with diarrhea, increased WBC concentration, and swollen joints. These results conclude that synovial fluid in the joints of immune suppressed patients can harbor *Salmonella* cells.

In a study by Broadway et al., (2015), *S. Typhimurium* inoculated pigs were sampled for the prevalence the pathogen in the feces, lymph nodes (Ileocecal, subiliac, popliteal, and mandibular), and synovial fluid (coxofemoral, shoulder, and stifle) preformed in two phases (Broadway et al., 2015). Phase 1 had a fecal prevalence of 58.8%, which was greater than that of Phase 2 (31.6%) (Broadway et al., 2015). The ileocecal lymph node had the greatest prevalence

at 41.67% for Phase 1 and 37.00% for Phase 2 (Broadway et al., 2015). Both mandibular and subiliac lymph node prevalence was low with Phase 1 equating 2.78% in and 0% in Phase 2 (Broadway et al., 2015). The synovial fluid yielded one positive sample from Phase 1 (Broadway et al., 2015). This pig was noted as having an abscess on the naval prior to harvest. A compromised immune system could be the reason *Salmonella* was able to invade this atypical anatomical location. In beef, the joints separated during fabrication allows the synovial fluid to come in contact with the meat used in ground beef trim. If this fluid contains *Salmonella* the jointing process could potentially contaminate the equipment used and trim sourced for ground beef.

1.7. Conclusion

To investigate the translocation of *Salmonella* in the gastrointestinal tract, the following study utilized the use of DEX for immunosuppression to mimic the effects of stress on the immune system. The decreased immune response elicited by a stress-like event has been shown to have adverse effects on pathogen migration into lymph tissues and possible atypical areas such as synovial fluid. In a study by Broadway et al. (2017), the translocation of *Salmonella* to synovial fluid was observed in a pig suffering from a large naval abscess. This situation could possibly be the result of an immunosuppressed individual allowing the pathogen to infected further tissues.

1.7. References

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Chapter 2.

Steers inoculated with *Salmonella* exhibit dissimilar hematology and body temperature profiles based on their exposure to a synthetic glucocorticoid

2.1. Abstract

The objective of this study was to determine the effects of daily DEX infusions on whole blood count and body temperature in response to experimental, oral inoculation with naladixic acid resistant *Salmonella* Typhimurium (SAL). Weaned Holstein steer calves (n = 20; BW = 102 ± 2.7kg) received DEX (n = 10; 0.5 mg/kg BW) or saline (CON; n = 10; 0.5 mg/kg BW) for 4 d (from d -1 to d 2) prior to oral inoculation of SAL ($3.4x10^6$ CFU/animal) via milk replacer on d 0. Fecal swabs for SAL shedding were obtained daily and samples were confirmed positive beginning 24 h post inoculation (d 1) to harvest (d 5). Dexamethasone administration was achieved via indwelling jugular catheters fitted simultaneously with rectal temperature (RT) recording devices on d −2 relative to inoculation and placed in individual pens in an environmentally controlled facility. Whole blood was collected at -24, -12, and 0 h; and 8-h intervals from 8 to 120 h for hematology. There was a time x treatment ($P < 0.01$) for RT such that the DEX treatment resulted in a decreased RT on d -1 to 2. A treatment × time interaction ($P < 0.05$) was observed for total WBC, neutrophils, lymphocytes, eosinophils, and monocytes. There was an increased WBC and neutrophils in DEX steers ($P < 0.01$) and lymphocytes increased following DEX administration ($P < 0.05$) at 24 and 48 h, respectively. Monocytes initially increased ($P < 0.01$) in response to the -24 h DEX administration but slowly decreased beyond CON group levels following the 48 h DEX administration. These data may be interpreted as either a mild immunosuppression due to multiple DEX exposures, or a combination of both mild immunosuppression and immune recovery when juxtaposing DEX with *Salmonella* inoculation. Further analysis is warranted to better elucidate the immune status of these steers given the complex interactions between GC administration and pathogenic, enteric bacteria inoculation.

2.2. Introduction

Salmonella bacteria is a considerable cause of morbidity and mortality, affecting an estimated 9.1% of cattle in the feed-lot setting (NAHMS, 2011). This stage of cattle production has been shown to exhibit the highest prevalence of animals shedding *Salmonella* (NAHMS, 2008; NAHMS, 2011). Cattle experience a wide range of psychological and physical stressors in the feedlot, perhaps contributing the presences of *Salmonella* through increased fecal shedding due to mild immunosuppression (Carrasco and Vande Kar, 2003; Charmandari et al., 2005; Carroll et al., 2007; Buckham Sporer et al., 2008). In cattle, the exposure to stressors such as co-commingling, weaning, transportation, and injury can result in decreased function of the immune system (Carrasco and Vande Kar, 2003; Charmandari et al., 2005; Carroll et al., 2007; Buckham Sporer et al., 2008; Hodgson et al., 2005). Therefore, better understanding of the interactions between a chronically stressed immune function and *Salmonella* infection should be assessed. During an immunosuppression event, glucocorticoids (GCs) act on the immune system through diminished phagocytic activity, decreased pro-inflammatory cytokine stimulation and production, and decreased responsiveness to adhesion molecules (Barton et al., 2000; Sapolsky et al., 2000; Seekamp et al., 2001). In research, synthetic GC adminiatration, such as DEX, have been given to mimic the effects of immunosuppression similar to what is observed during stress event (Oldham and Howard, 1992; Burton et al., 1995). Multiple studies have concluded that daily administration of DEX has resulted in insult to the immune system (Pruett et al., 1987; Oldham and Howard, 1992; Burton et al., 1995; Anderson et al., 1999; Hughes et al., 2017). Therefore, the objective of this study is to evaluate the effects of *Salmonella* infection during daily DEX infusions.

2.3. Materials and Methods

All procedures in this study were reviewed and approved by the USDA-ARS, Livestock Issues Research Unit's Institutional Animal Care and Use Committee (IACUC protocol #1807S) and Auburn University Institutional Animal Care and Use Committee (IACUC protocol #2018002R).

2.3.1. Animals and Housing

Weaned Holstein steer calves (n = 20) of similar age were obtained from a dairy in the Texas Panhandle and arrived at the USDA-ARS Livestock Issues Research Unit in Lubbock, TX on d -6. Upon arrival, calves were placed into individual stanchions with enough space for normal standing and lying behaviors in an environmentally controlled facility (average air temperature of 17.4 ± 6°C) in two groups. Calves rested overnight and in stanchions with ad libitum access to feed and water. Indwelling jugular catheters and RT recording devices (Reuter et al., 2010) were placed in the steers the following morning (d -5) to facilitate intravenous administration of DEX and serial blood collection for analysis and to record body temperatures at 5-min intervals. The calves were evaluated on feed disappearance using weigh back of feed each morning prior to feeding for each day throughout the study. The calves were fed a standard calf starter ration ad libitum, with free access to water throughout the study. Fecal samples were collected from each calf upon arrival and each subsequent day (for 5 d; d -6 to d -2) during the dietary/facility adaptation period to verify animals were SAL negative. Animals were humanely euthanized at the conclusion of the study.

2.3.2. Treatments and Blood Collection

Steers were weighed on d -2 (BW = 102 ± 2.7 kg) prior to random assortment to 1 of 2 treatment groups. The two treatment groups consisted of 1) Immunosuppressed (DEX; BW = 101.6 ± 3.5 kg) induced by repeated intravenous administration of 0.5 mg/kg BW of DEX (d -1 to d 2), or 2) control (CON; BW = 102 ± 9.0 kg) in which an equivalent amount of saline was administered. Beginning on d −1, blood was collected via jugular catheter into a 4-mL evacuated sampling tube containing 7.2 mg EDTA (Vacutainer: Becton, Dickinson and Company, Franklin Lakes, NJ) for determination of complete blood count (CBC) using an automated hemocyotometer (Procyte Dx Hematology Analyzer; IDEXX Laboratories, Inc., Westbrook, ME) at −24, −12, and 0 h and in 8-h intervals from 0 to 120 relative to inoculation. Serum was harvested from the blood collection and stored in duplicate aliquots at −80°C, and serum proinflammatory cytokine (IFN-γ) was assayed (Ciraplex; Quanterix Biosystems Inc., Billerica, MA) at the USDA-ARS laboratory using a bovine-specific singleplex sandwich ELISA.

Bacterial Cultures of *Salmonella* enterica serotype *Typhimurium* (ATTC-BAA-186) were adapted for growth in the intestinal tract and made resistant to nalidixic acid using methodology from Broadway et al. (2015). Oral inoculation of naldixic acid resistant *Salmonella* Typhimurium (3.4×10^6 CFU/animal) was administered via milk replacer on d 0.

2.3.3. Fecal Sample Collection

Following arrival to the facility, fecal samples were collected rectally using a transport swab (Fisher Scientific UK Ltd, Loughborough LE11 5RG, UK) with modified Liquid Stuarts (TS) to determine if *Salmonella* was present in each calf (n = 20). Following inoculation of SAL, fecal samples were taken daily (d -2 to 5) to monitor shedding.

2.3.4. Statistical Analysis

All data were analyzed in SAS 9.4 (SAS Inst. Inc. Cary, NC) and LSMEANS were separated using Tukey-Kramer adjustment with $\alpha = 0.05$. Feed disappearance, RT, CBC, and cytokine data were analyzed as repeated measures with steer nested within treatment. For RT data, the 5 min intervals were averaged to 1 h intervals prior to analysis. Fixed effects of treatment, time, and treatment by time were evaluated in the models. Fixed effects of treatment, time, and treatment by time were evaluated in the models.

2.4. Results

2.4.1 Detection and Enumeration of *Salmonella*

Fecal prevalence prior to the inoculation period was 0% for both the CON and DEX treatments. Post inoculation 19 of the 20 (95%) animals were shedding SAL on d 1 and by d 4, all 20 of the 20 (100%) animals were shedding on d 4.

2.4.2 Rectal Temperature

A treatment by time interaction was observed for RT ($P < 0.01$), with the SAL inoculation increasing the RT in the CON steers but not the DEX steers (Fig. 2.1). Dexamethasone administered pre-inoculation decreased the RT in the DEX steers staring at -24 h and continuing until 64 h post inoculation at which point the RT began to elevate. Steers in the DEX group exhibited the characteristic febrile response (> 40°C) associated with pathogenic

infection; however, the CON steers experienced an elevated RT beginning 32 h post inoculation and persisted until harvest (120 h; d 5).

2.4.3. Feed Disappearance

A treatment by time interaction was observed for the feed disappearance (P < 0.01), with a slight increase in feed intake was observed in the DEX treatment starting on 0 h following the first DEX infusion and remained elevated until 72 h relative to SAL inoculation, however the two treatment groups were not different (Fig. 2.2). Feed intake decreased ten-fold in the DEX steers at 96 h and remained depressed for the length of the study. The CON steers experienced a marked decrease (P < 0.01) in feed intake starting at -24 h and continuing for the remainder of the study. There was also a trend for the CON steers to have a decreased (P < 0.01) feed intake at 72 h with a steady increased (P < 0.01) noted for the remainder of the study.

2.4.4. Complete Blood Count

Dexamethasone also elicited a treatment by time interaction (P < 0.01) in total white blood cells (WBC), with DEX steers having a two-fold increase in WBC concentrations relative to CON steers staring at 12 h (P < 0.01) after the first DEX infusion and remaining elevated until 16 h post DEX treatment (P < 0.01). The observed increase of WBC is primarily comprised of neutrophils which also exhibited treatment by time interaction (P < 0.01). Neutrophil count increased in DEX steers four-fold following the first (P < 0.01) DEX treatment and remained elevated 16 h post treatment (P < 0.01) (Fig. 2.4.). As previously stated, the WBC and neutrophil concentrations were elevated following the daily administration of DEX, however, 16 h post final infusion the levels drastically decreased for 24 h past the CON levels (Fig 2.3.). Both WBC and neutrophils levels remained decreased for the remainder of the study.

Monocytes initially increased (P < 0.01) in response to treatment in DEX steers however following the inoculation of SAL, decreased steadily until 80 h (P = 0.54) where monocyte levels were within basal range (Fig 2.5.). In the absence of DEX, the monocytes levels increased in the DEX treatment for the remainder of the study. It is important to note that both treatment groups were well above the normal reference range of 0.01 to 2.11 1,000 cells per/µL for the entirety of the study (Panousis et al., 2018). *Salmonella* infection did impact the monocyte concentrations of both DEX and CON groups, increasing in response to the inoculation.

A treatment by time interaction (P = 0.02) was observed for lymphocytes. Lymphocyte concentrations were similar in both the DEX and CON group until a slight peak was observed on 32 h following the third DEX infusion but returned to near CON levels by 48 h (Fig. 2.6.). A second peak was elicited following the final DEX infusion, however, a sharp decline returned to basal at 64 h. A third and final peak was observed in slower progression from 72 h to 88 h followed again by a decline back to basal concentration at 112 h. It is important to note that the slight increases in lymphocyte concentrations for the DEX steers at were within the normal reference range of 1.8 to 9.4 1,000 cells per/µL (Panousis et al., 2018) across both treatment groups.

2.4.5. Cytokines

A treatment x time interaction (P < 0.01) was observed for interferon-gamma (IFN-γ). Prior to the SAL challenge, there was no difference between the two treatments (Fig.2.7). Concentrations of IFN-γ for the CON group were < 60 pg/ml and remained at this concentration until 32 h post inoculation. At 32 h post inoculation, an increase (P = 0.92) in IFN-γ concentration was observed, elevating at 48 h and remained elevated as a result of SAL infection until 72 h post inoculation. Concentrations of IFN-γ for the DEX treatment were < 60pg/ml until 64 h post inoculation, where a sharp increase was observed at 72 h (P = 0.28), 80 h (P < 0.01), and 88 h (P = 0.01) followed by a steady decline for the remainder of the study. Neither treatment group returned to baseline by 120 h.

2.5. Discussion

Exposure to a plethora of stress-events is a common occurrence for cattle (Kim et al., 2011). These stressors can be separated into one of three categories, physical stress, psychological stress, or a combination of both (Kim et al., 2011). Physical stress can present itself in starvation scenarios, injury, or pathogenic infections, whereas, psychological stress is mental strain inflicted on animals through weaning, transport, and handling. These stressors are commonly combined and can act on one another resulting a negative effect on the immune system (Kim et al 2011). In particular, co-mingling scenarios where animals are transported to a new environment and introduced to other animals of different backgrounding practices (Duff et al., 2007). This poses a threat to animals through psychological stress, animals transported for

long hours, as well as physical stress, pathogenic exposure increases. The stress of these events increases cortisol, a natural glucocorticoid, which act to briefly enhance the immune system for defense (Oldham and Howard, 1992; Burton et. al., 1995). However, in the current research blood was collected using indwelling jugular catheters as to not obstruct the signals of synthetically induced stress via DEX. If stress persists moving from an acute to chronic experience, the immune system becomes suppressed increasing the susceptibility for an animal to contract a pathogenic infection (Burton et al., 1995; McGuirk and Peek, 2003). Previous research suggests the administration of a synthetic glucocorticoid such as DEX, can elicit an immune response mimicking that of natural stress (Pruett et al., 1987; Oldham and Howard, 1992; Burton et al., 1995; Anderson et al., 1999; Lopparelli et al., 2012, Hughes et al., 2017). When DEX is administered daily for a prolonged time, there is a negative impact on the immune system seen in a chronic stress event (Oldham and Howard, 1992; Burton et al., 1995; Lopparelli et al., 2012, Hughes et al., 2017). With the introduction of a pathogen such as SAL, the immune system raises a defense to rid the body of the infection (Carroll et al, 2007). However if the immune system is suppressed by chronic stress, SAL has the ability to wreak havoc on the body while avoiding detection (Burton et al., 1995; Lopparelli et al., 2012, Hughes et al., 2017).

In this current study, the administration of daily DEX influenced the immune response during the inoculation and infection of SAL. Once the pathogen infects the body, the innate immune system engulfs the invader and marks them for disposal (Thiennimitr et al., 2012). However, in a chronic stress response, the immune system will decline in function (Miller et al., 2002). During the glucocorticoid-induced immunosuppression, an influx of neutrophils in vascular circulation is observed, however, these cells have the inability to translocate through the vascular wall into the affected tissue due to the down-regulation of adhesion molecules on the endothelial cells (Burton et al. 1995; Raffler et al., 2005). Therefore, though a large number of neutrophils are present in circulation, without the expression of the adhesion molecules, neutrophils have decreased margination and migration into inflamed tissues. White blood cells are a combination different phagocytic cells, composed largely of neutrophils (Burton et al., 1995). Comparable with previous research, neutrophils levels in this study were also elevated by four-fold in the DEX treatment as compared to the CON treatment mimicking that seen in the WBC, which consistent with previous research (Oldham and Howard, 1992; Burton, 1995;

Anderson, 1999; Hughes, 2017). Following the effects of DEX, the WBC and neutrophil levels declined over a 24 h span. This two fold decrease in circulating neutrophils may be from the activation of the adhesion molecules on the vascular wall, allowing the over produced neutrophils to leave the vascular system and enter the infected tissues. Although the DEX greatly affected the production of WBC,

The initial increase and steady decline of monocytes reveals the manifestation of DEX, as they decrease production of monocytes to avoid excess of potentially damaging phagocytes (Melby, 1974). Glucocorticoids have a suppressive function to aid in the defense against the over production of phagocytic cells by the immune system (Carroll and Forsberg, 2007). When these cells are produced in excess, there is an increased probability of WBC engulfing and destroying cells of healthy tissue, therefore, obstructing the healing process (Carroll and Forsberg, 2007). In the absence of DEX, the monocyte concentrations steadily increased in the DEX treatment from 80 h the remainder of the study (120 h). The same increase was also observed in the monocyte concentrations of the CON steers.

Chronic stress, from natural and synthetic glucocorticoids, commonly presents immunosuppression with lymphopenia and neutrophilia (Pruett et al., 1987; Burton et al., 1995; Anderson et al., 1999; Lopparelli et al., 2012). Similarly, Oldham and Howard (1992) administered DEX (0.5mg/kg per day) for 20 consecutive days to ten calves and observed decrease in responsiveness of lymphocytes to mitogens following administration. Without the induction of mitosis via mitogen factors, lymphocytes do not duplicate, leaving a decreased concentration of circulating lymphocytes (Oldham and Howard, 1992) However, in contrast with earlier research, lymphopenia was not achieved in this study. Lymphocyte concentrations were not significantly different between treatment groups. Three slight peaks were observed in the DEX group and this could indicate the administration of DEX evoked an immune system enhancement similar to previous studies of short-term stress where an increase in circulating lymphocytes were observed (Pruett et al., 1987; Hughes et al., 2017). These peaks may be associated with an enhance immune response reacting to *Salmonella*. It is important to note that the slight increases in lymphocytes concentrations for the DEX steers were within the normal reference range of 1.8 to 9.4 1,000 cells per/µL (Panousis et al., 2018) across both treatment

groups. The inoculation of SAL did not appear to have an impact on lymphocyte concentrations of both the DEX and CON treatments, as they remained steady within this study.

During pathogenic infection, lymphocytes release pro-inflammatory cytokines such as IFN-γ, as part of the adaptive immune system (Carroll and Forsberg, 2007). The effects of IFN-γ result in a heighted immune system function through critical in the stimulation, differentiation, and function of monocyte/macrophages (Young and Hardy, 1995; Schroder et al., 2004). As in previous research, the exposure to stressors or administration of glucocorticoids elicit diminished concentrations of IFN-γ (Fischer et al., 1997; Kehrli et al., 1999; Hickey et al., 2003; Kim et al., 2011; Hughes et al., 2015) which corresponds to the data found in this study. When immune function is shifting from innate to adaptive, IFN-γ increases sensitivity and responsiveness to the specific pathogens resulting in a specialized attack (Schroder et al., 2004).

Along with leukocyte signaling, pro-inflammatory cytokines stimulate a multitude of reactions such as fever, lethargy, and loss of appetite (Carroll et al., 2009). In the current study, steers in the CON group exhibited the characteristic febrile response (> 40°C) associated with pathogenic infection 32 h post inoculation; however, the DEX steers exhibited baseline RT until 64 h post inoculation where a sharp increased to febrile concentrations was observed. This timeline is consistent with the conclusion that clinical signs arise within 12 – 48 h post oral inoculation of the serotype *Typhimurium* (Watson et al., 1998). Although specified pathogens evoke the specific immune responsive, characteristic inflammation and heat are associated with adaptive of non-specific defense mechanisms (Coutinho and Chapman, 2011). A marked increase in RT in the DEX groups corresponds to the increase of the IFN-γ concentrations in this study. Though it is well understood the febrile response can thermally suppress bacterial growth, it has been found to have deeper effects on cytokine production (Hasday et al., 2000; Lee et al. 2012). Downing et al (1987) isolated and cultured mononuclear cells isolated from adult rhesus monkeys experiencing hyperthermia (2°C above control levels) and observed as much as a 16fold increase in IFN-γ production relative to those of the same animal before the heat treatment. Comparable results were obtained when a febrile response was induced with a systemic injection of nonviable *Escherichia coli*, which similarly to SAL, includes a LPS layer (Downing et al., 1987) Later, Downing et al. (1987) evaluated lymphocytes function in humans from hyperthermic individuals, and observed as much as 10-fold increase of IFN-γ than cells

withdrawn at basal core temperatures from the same individuals. Similar to these earlier studies, a correlation between RT and IFN-γ activity was noted in this study. The CON group had an increase in IFN-γ concentrations at 32 h where a drastic increase in RT induced by SAL infection was also observed. In contrast, the anti-inflammatory actions of synthetic glucocorticoid, such as DEX, inhibits this febrile response experienced during parthenogenesis (Wiśniewski et al., 1992; Danek, 2006; Hughes et al., 2015). In the current study, the DEX treated steers showed diminished concentrations of IFN-γ until 16 h post final DEX infusion, which is similar to other studies (Chiang et al., 1990; Nonnecke et al., 1997; Fischer et al. 1997; Hughes et al., 2017). However, a simultaneous increase was also observed in the DEX treatment where both IFN-γ concentrations and RT spiked at 64 h following the degradation of DEX in the body. These results demonstrate that increased RT results in lymphocytes that are primed for an elevated stimulation of IFN-γ activity, therefore increasing potentiation of immune functions against SAL infection.

Though the febrile response is needed for increase immune function, maintaining a RT of even 1°C above normal increases metabolic energy cost by 10% to 15% (Kulger, 1985; Carroll and Forsberg, 2007). In addition, energy is needed in the production of cytokines, leukocytes, and other actions of the immune system, redirection nutrient utilization away from growth and towards the immune system (Carroll et al., 2011; Sanchez et al., 2013). Though nutrients are needed to perform immune functions, bacterial infection typically decreases feed intake (Lohuis et al., 1988; Cullor, 1992; Steiger et al., 1999; Waldron et al., 1999; Elsasser et al., 2008; Sanchez et al., 2013). Data from the present study agrees with previous research in that feed intake was decreased momentarily in the CON steers at 72 h post inoculation. Though previous studies use DEX to mimic stress, (Oldham and Howard, 1992; Burton et al., 1995), the use of DEX has also been used in the promotion of beef cattle growth (Brethour, 1972; Dicke et al., 1974). When DEX is administered, the inhibitory factors on the immune system are evoked and the metabolism of nutrients are once again shifted to growth (Courtheyn et al., 2002; Gottardo et al., 2008). This conclusion supports the present data in which, feed intake is increased following the first DEX infusion then is drastically reduced 24 h post final infusion once inhibition of the suppressed immune system in halted.

2.6. Conclusions

These results may show a mild immunosuppression event where the DEX steers had an inhibited immune function until the body began naturally degrading DEX, in turn, activating the immune system. Although this theory is plausible, a combination of a mild immunosuppression juxtaposing with slight immune recovery between daily DEX administrations seems more likely. Although many immune functions were decreased as a result of DEX exposure, lymphopenia was not achieved in this study possible showing an adaptive immune strike against *Salmonella* infection. Furthermore, slight peaks were observed in the lymphocyte concentrations. Although these peaks were within the normal reference range, slight increases in IFN-γ concentrations were observed simultaneously. Further analysis is warranted to better elucidate the immune status of these steers given the complex interactions between GC administration and pathogenic, enteric bacteria inoculation.

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2.8. Tables and Figures

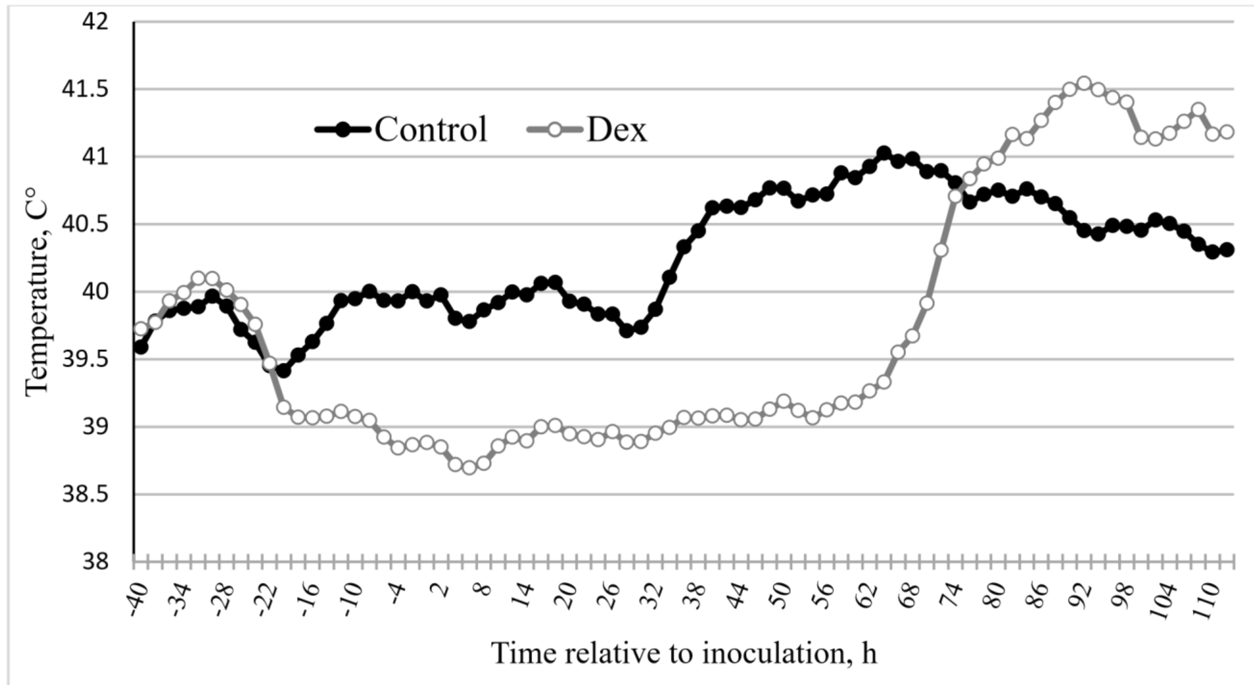

Figure 2.1. Effect of dexamethasone (DEX) on the Rectal Temperature (RT) in the Dex treatment in conjunction with the inoculation of naldixic acid resistant *Salmonella Typhimurium* (3.4×10^6 CFU/animal) received by both the Dex and Control treatments. In the Dex treatment, dexamethasone was administered at 0.5 mg/kg BW on d -1 through d 2. A treatment by time interaction ($P < 0.01$) was observed. Following SAL inoculation, the RT increased in the Control steers beginning at 32 h and remaining elevated for the remainder of the study. The RT in the Dex treatment decreased at -24 h remained attenuated until 64 h where a sharp febrile response (> 40°C) was observed.

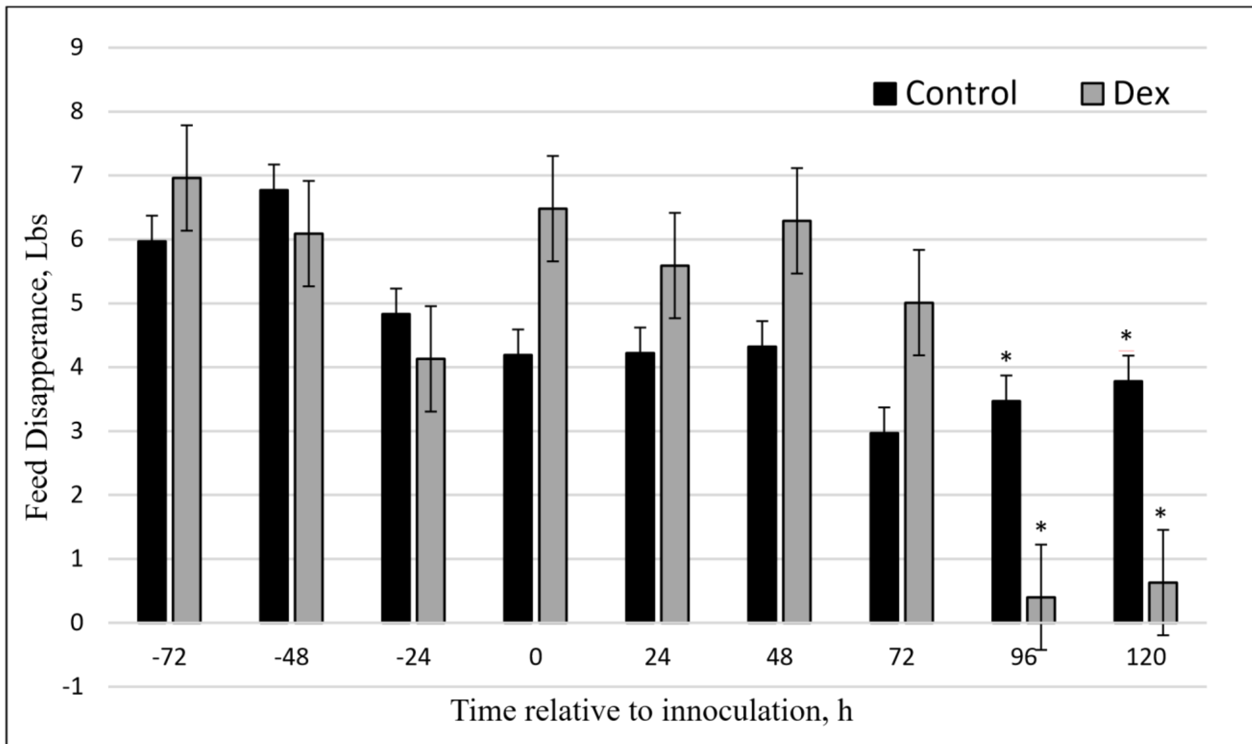

Figure 2.2. Effect of dexamethasone on the Feed Disappearance of the Dex group. In the Dex treatment, dexamethasone was administered at 0.5 mg/kg BW on d -1 through d 2. Both treatments received naldixic acid resistant *Salmonella Typhimurium* ($3.4x10^6$ CFU/animal) orally. Control treatment of steers were significant higher in feed intake at 96 h and 120 h as compared to Dex. Standard error of the mean 0.47 for both treatments. Alpha = 0.05

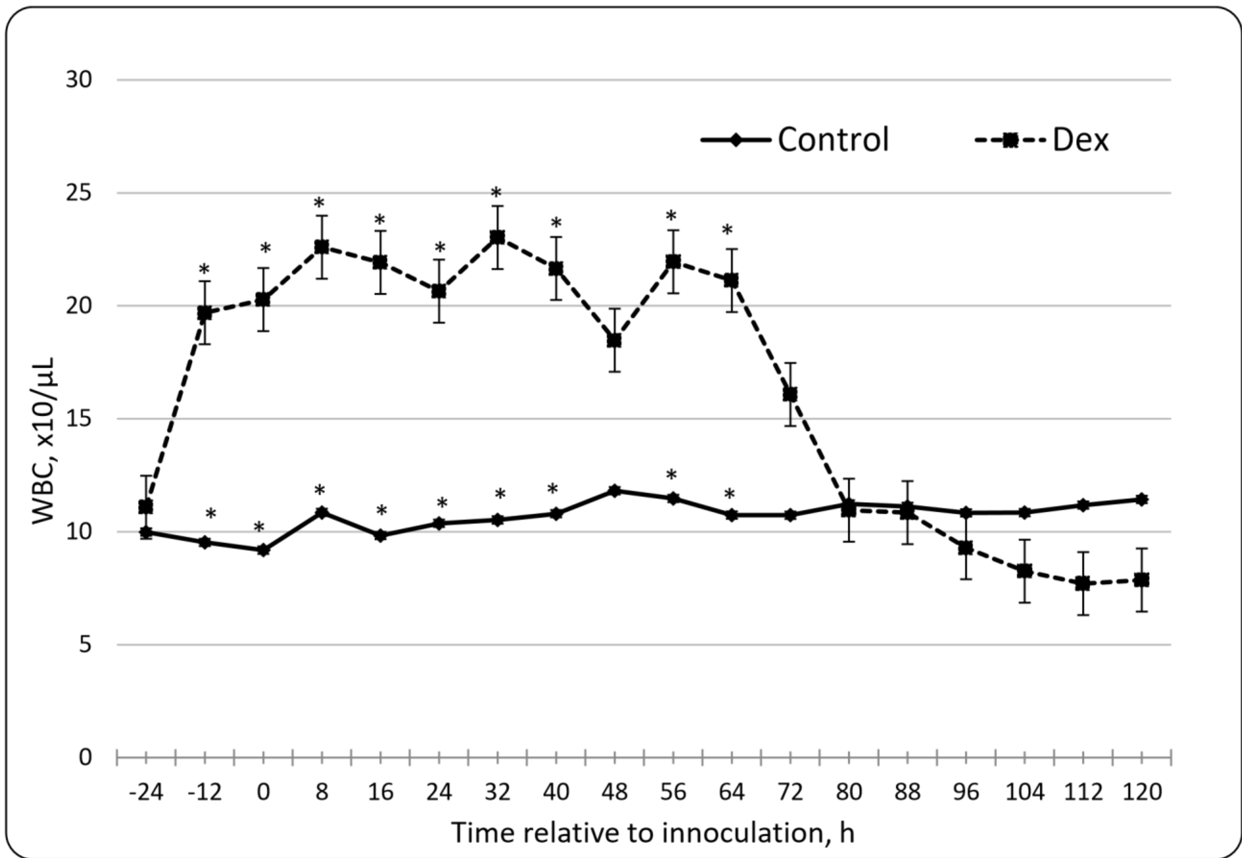

Figure 2.3. Effect of dexamethasone on the White Blood Cells (WBC) concentrations of the Dex group. In the Dex treatment, dexamethasone was administered at 0.5 mg/kg BW on d -1 through d 2. Both treatments received naldixic acid resistant *Salmonella Typhimurium* ($3.4x10^6$ CFU/animal) orally. Dex treatment of steers were significant higher in WBC at -12 h, 0 h, 8 h, 16 h, 24 h, 32 h, 40 h, 56 h, and 64 h as compared to Control. Standard error of the mean 1.34 for both treatments. Alpha = 0.05

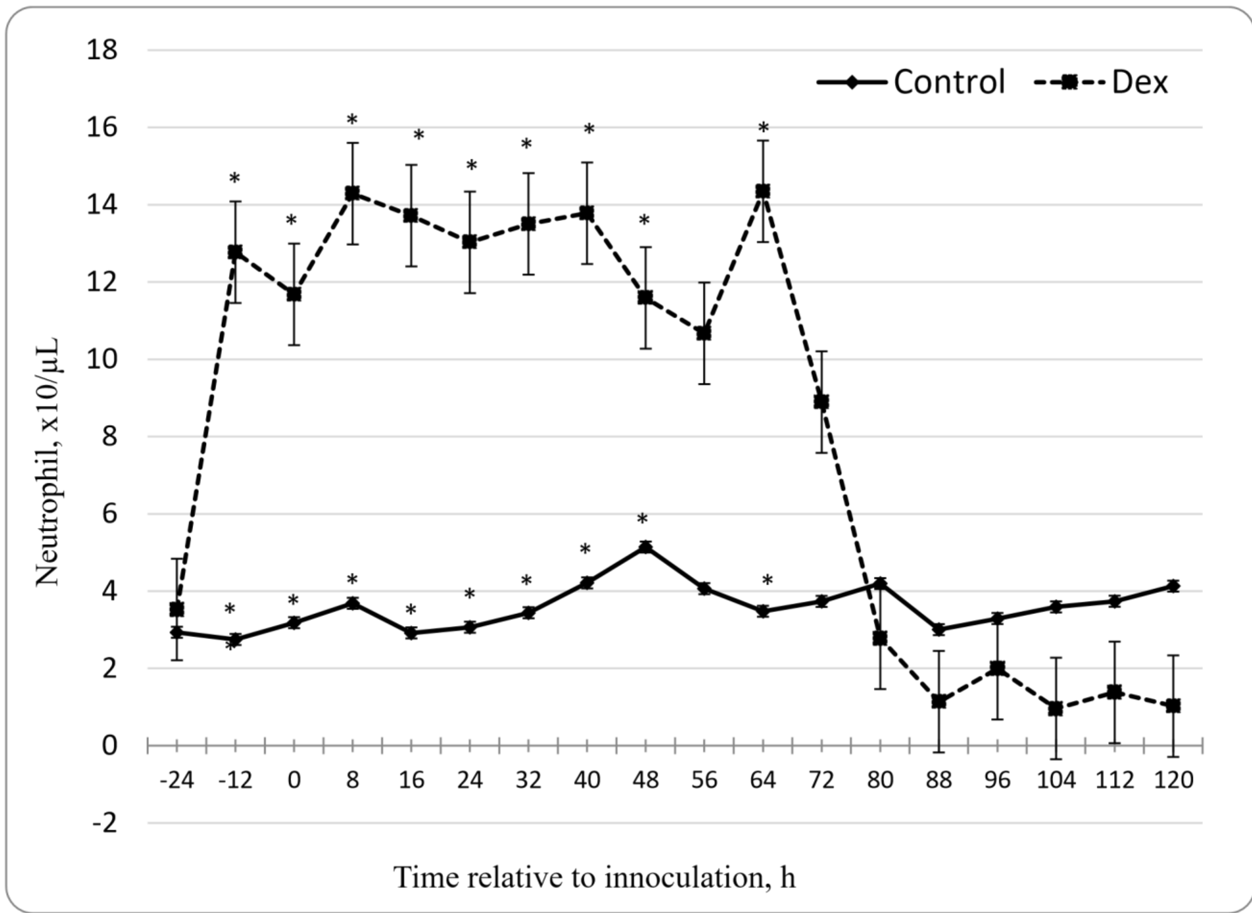

Figure 2.4. Effect of dexamethasone on the Neutrophil concentrations of the Dex group. In the Dex treatment, dexamethasone was administered at 0.5 mg/kg BW on d -1 through d 2. Both treatments received naldixic acid resistant *Salmonella Typhimurium* ($3.4x10^6$ CFU/animal) orally. Dex treatment of steers were significant higher in neutrophils at -12 h, 0 h, 8 h, 16 h, 24 h, 32 h, 40 h, 56 h, and 64 h as compared to Control. Standard error of the mean 0.87 for both treatments. Alpha = 0.05

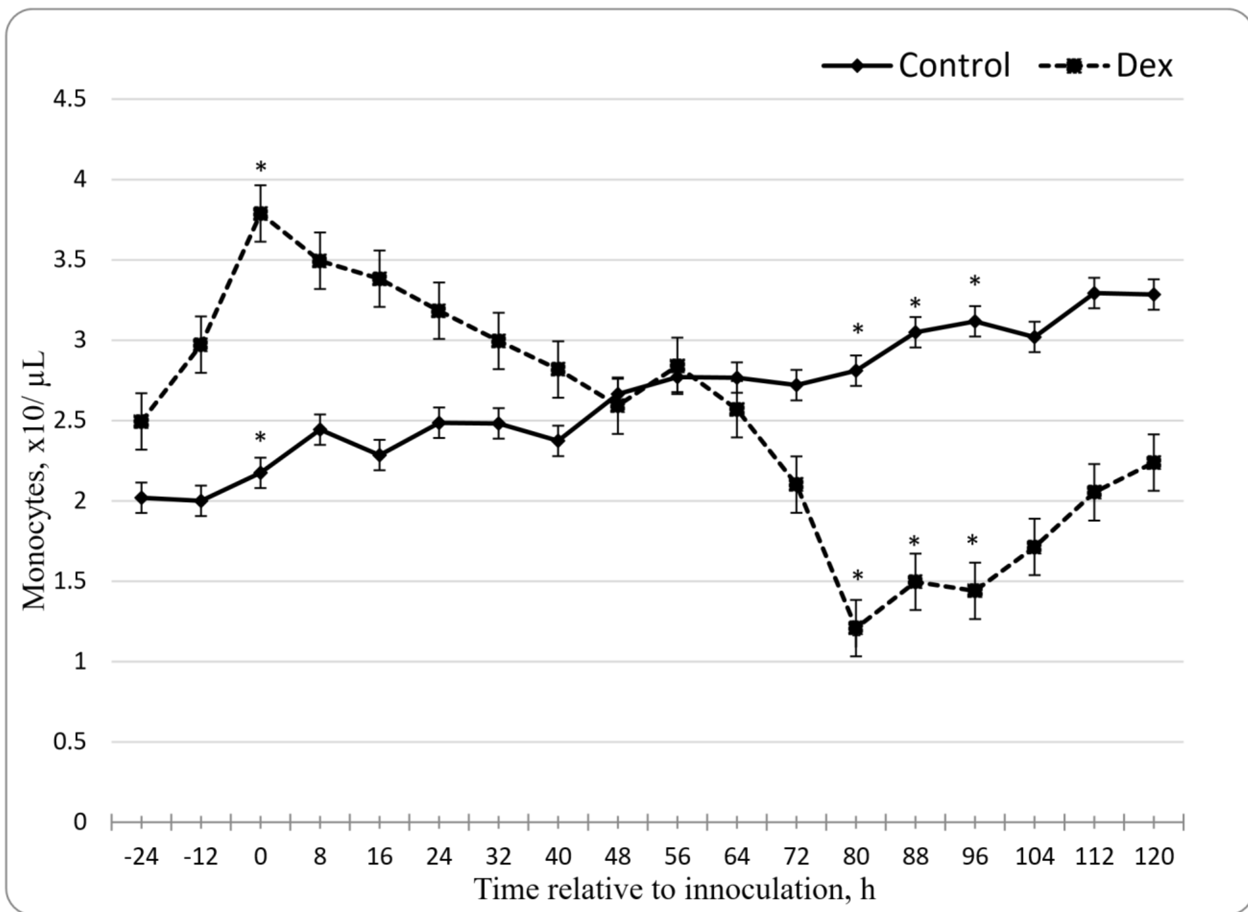

Figure 2.5. Effect of dexamethasone on the Monocytes concentrations of the Dex group. In the DEX treatment, dexamethasone was administered at 0.5 mg/kg BW on d -1 through d 2. Both treatments received naldixic acid resistant *Salmonella Typhimurium* ($3.4x10^6$ CFU/animal) orally. Dex treatment of steers were significant higher in monocytes at h 0, h 80, h 88, and 96 h as compared to Control. Standard error of the mean 0.20 for both treatments. Alpha = 0.05

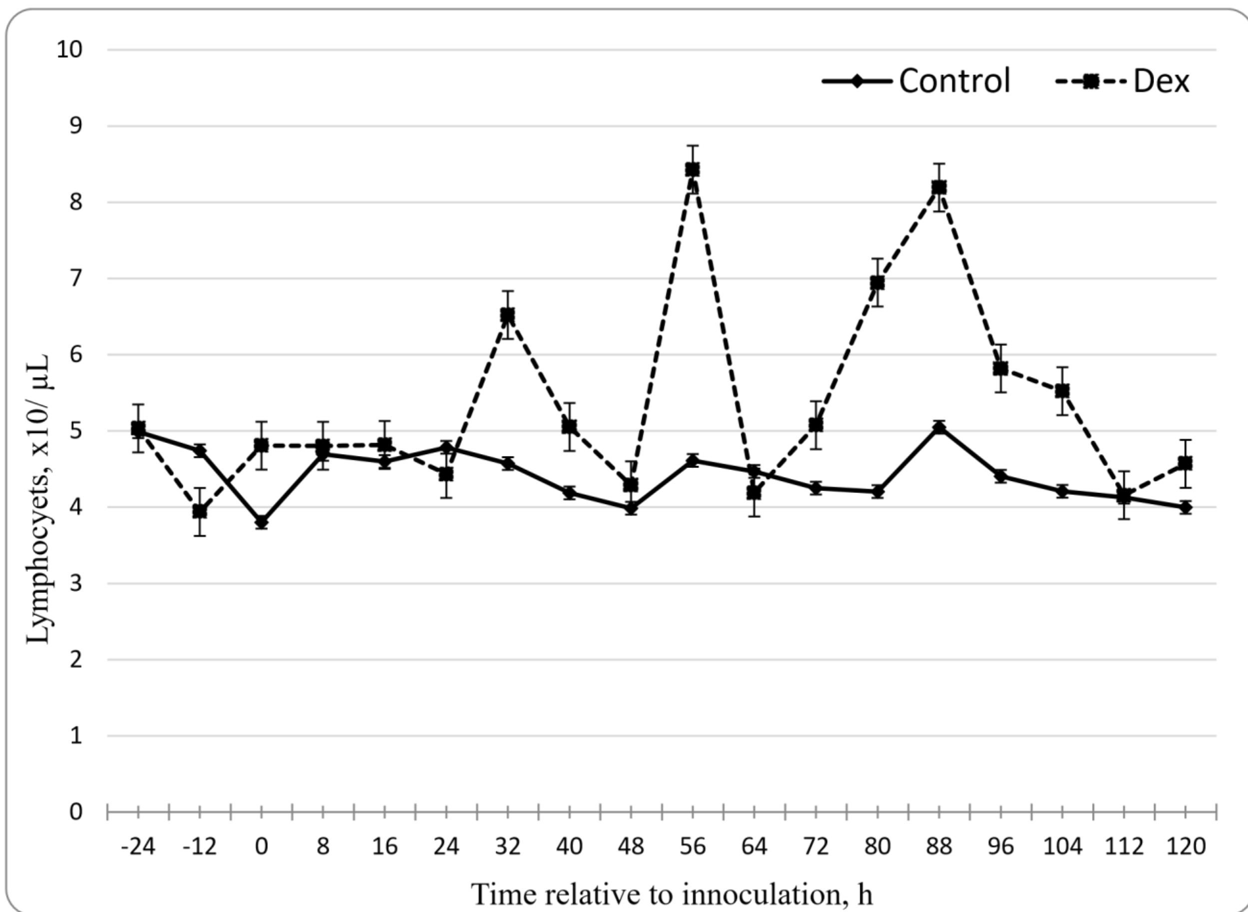

Figure 2.6. Effect of dexamethasone on the Lymphocyte concentrations of the Dex group. In the DEX treatment, dexamethasone was administered at 0.5 mg/kg BW on d -1 through d 2. Both treatments received naldixic acid resistant *Salmonella Typhimurium* ($3.4x10^6$ CFU/animal) orally. Dex treatment of steers wasn't significant higher in lymphocytes any time point as compared to Control. Standard error of the mean 0.49 for both treatments. Alpha = 0.05

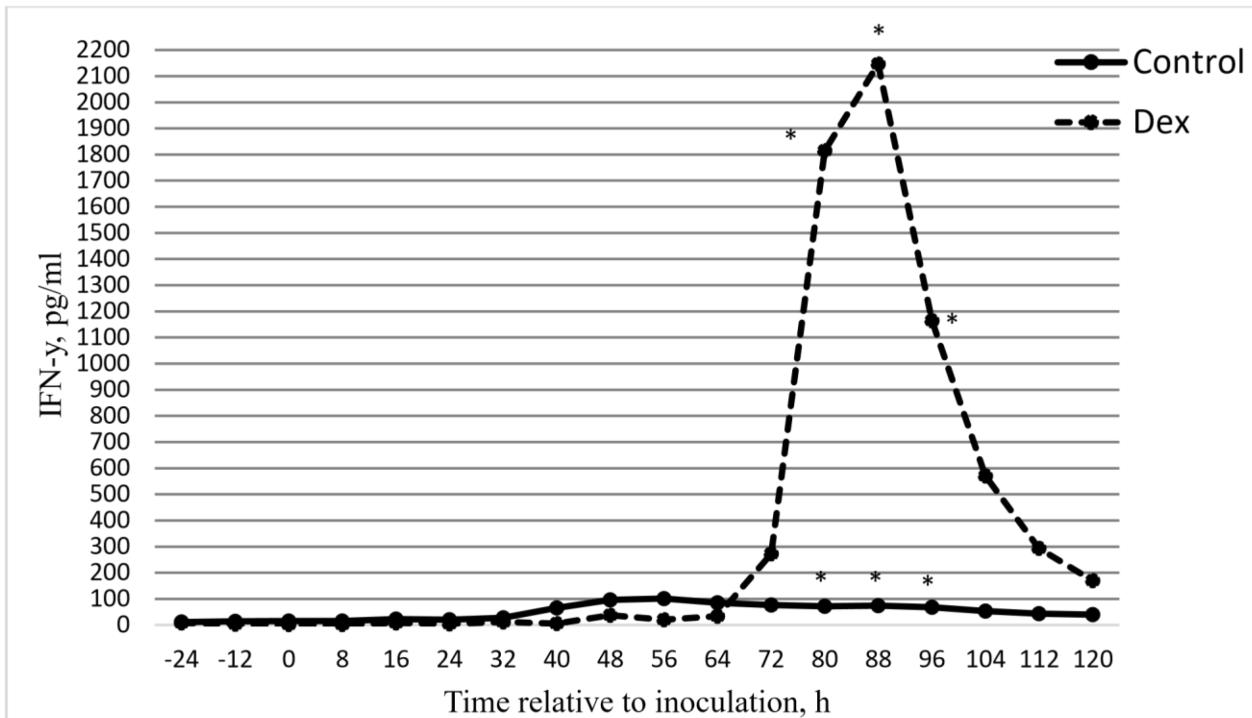

Figure. 2.7. Effect of dexamethasone on the IFN-γ concentration of the Dex group. In the Dex treatment, dexamethasone was administered at 0.5 mg/kg BW on d -1 through d 2. Both treatments received naldixic acid resistant *Salmonella Typhimurium* ($3.4x10^6$ CFU/animal) orally. Dex treatment of steers were higher in monocytes at 80 h, 88 h, and 96 h as compared to Control. Standard error of the mean 57.98 for the Control and 58.02 for the Dex treatment. Alpha = 0.05

Chapter 3.

Translocation of orally inoculated *Salmonella* following mild immunosuppression in Holstein calves and the presence of the *Salmonella* in ground beef samples.

3.1. Abstract

The objective of this study was to determine if immunosuppression via daily DEX infusion altered *Salmonella* translocation from the GI tract. Weaned Holstein steer calves (n = 20; BW = 102 ± 2.7kg) received DEX (n = 10; 0.5 mg/kg BW) or saline (CON; n = 10; 0.5 mg/kg BW) for 4 d (from d -1 to d 2) prior to oral inoculation of naldixic acid resistant *Salmonella* Typhimurium (SAL; 3.4×10^6 CFU/animal) on d 0. Fecal swabs were obtained daily, and blood was collected for hematology. Upon harvest (d 5), the ileum, cecal content, lymph nodes (ileocecal, mandibular, popliteal, and prescapular), and synovial (stifle, coxofemoral and shoulder) swabs were collected for the isolation of the inoculated strain of SAL. White blood cells and neutrophil concentrations were elevated ($P < 0.01$) in the DEX treatment following DEX administration event. Following inoculation, 100% of DEX calves shed the experimental strain of SAL for 5 d, 90% of CON calves shed from d 1 to 3, and 100% of CON calves shed from d 4 to 5. Greater ($P < 0.01$) concentrations of SAL were quantified from the cecum of DEX calves (3.86 ± 0.37 log CFU/g) than CON (1.37 ± 0.37 log CFU/g); There was no difference in SAL concentrations between DEX and CON calves in ileal tissue ($P = 0.07$), nor ileocecal ($P = 0.57$), mandibular ($P = 0.12$), popliteal ($P = 0.99$), or prescapular ($P = 0.83$) lymph nodes. *Salmonella* was isolated from the stifle joint of one calf in the CON group; however, SAL was not isolated from any other joint fluids sampled. Of the stifle samples collected, 3.3% were positive for SAL indicating the opportunity of contaminating the meat during hind quarter fabrication. While more research is needed to elucidate the interactions of immunosuppression and pathogen migration patterns, these data confirm that orally inoculated SAL can translocate from the GI and be harbored in atypical locations representing a food safety risk.

3.2. Introduction

Salmonella is a foodborne pathogen of increasing concern in the U.S. as foodborne infection rates remain high and legislative action against foods containing this pathogen are in continuous discussion (ODPHP, 2010). In 2014, the CDC released a Food Safety Progress

Report for the Healthy People 2020 Target rate in which there was a failure to mitigate SAL from 2006- 2014 (ODPHP, 2014). In December 2013, following recalls associated with ground beef, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) released the *Salmonella* action plan, which included 10 action items for the agency. Following this report of that plan, USDA-FSIS changed its SAL verification testing program for beef, initiated exploratory SAL testing in pork, and modernized poultry inspection regulations. Item 8 in the plan is to explore the contribution of lymph nodes associated with SAL contamination. *Salmonella* may be more difficult to control because of the suspected location of the pathogen. Non-typhoidal SAL serotypes cause gastroenteritis which is concentrated in specific locations in the gastrointestinal tract including the ilium, colon, and the mesenteric lymph nodes (Barham et al., 2002; Arthur et al., 2008; Stevens et al., 2009; Koohmaraie et al., 2012; Gragg et al., 2013; Broadway et al., 2015). Research has shown that SAL has also been quantified inside bovine peripheral lymph nodes as well (Gragg et al., 2013). *Salmonella* cells located within lymph nodes may not be reduced by typical antimicrobial or thermal interventions applied to the surface of a carcass during the harvest process. Many of the current intervention strategies are based on the understanding that pathogens commonly enter ground beef products by way of surface contamination of carcasses through fecal material on the hides (Fegan et. al, 2005; Koohmaraie et. al, 2012). While many lymph nodes are removed in typical dressing procedures, some remain intact to the carcass, interwoven in the adipose and muscular tissue. Once the wholesale cuts are cut to specifications, the trim containing the many smaller lymph nodes are the primary source for ground beef.

In 2015, Broadway et al. investigated the migration of the pathogen into atypical carcass reservoirs and SAL was recovered in the synovial fluid of an immunocompromised pig. Researchers concluded that immunosuppressed animals may not show clear clinical signs of infection and can be a greater risk for migration of the bacteria into areas of the carcass not treated with any antimicrobial intervention techniques prior to or during fabrication, which could significantly impact food safety. In addition, the manifestations of SAL infection throughout the animal’s immune system vary depending on the three important factors: the infectious dose, specific serotype of SAL, and immune statues of the host animal (Griffin and McSorley, 2011).

In previous research, administration of the exogenous glucocorticoid, DEX, has been successful in mimicking a chronic stressor, thus, inducing immunosuppression in cattle (Estrada et al., 1999; Lippolis et al., 2006; Hughes et al., 2017). Multiple studies have concluded that the daily administration of DEX have resulted in insult to the immune system (Pruett et al., 1987; Oldham and Howard, 1992; Burton et al., 1995; Anderson et al., 1999; Hughes et al., 2017). Therefore, the objective of this study was to determine if immunosuppression via daily DEX infusion altered SAL translocation from the GI tract.

3.3 Materials and Methods

All procedures in this study were reviewed and approved by the USDA-ARS, Livestock Issues Research Unit's Institutional Animal Care and Use Committee (IACUC protocol #1807S) and Auburn University Institutional Animal Care and Use Committee (IACUC protocol #2018002R).

3.3.1. Animals and Housing

Weaned Holstein steer calves (n=20) of similar age were obtained from a dairy in the Texas Panhandle and arrived at the USDA-ARS Livestock Issues Research Unit in Lubbock, TX on d -6. Upon arrival, calves were placed into individual stanchions with enough space for normal standing and lying behaviors in an environmentally controlled facility (average air temperature of 17.4 ± 6°C) in two groups. Calves rested overnight and in stanchions with ad libitum access to feed and water. Indwelling jugular catheters and rectal temperature (RT) recording devices (Reuter et al., 2010) were placed in the steers the following morning (d -5) to facilitate intravenous administration of DEX and serial blood collection for analysis and to record body temperatures at 5-min intervals. Feed disappearance was monitored daily. The calves were fed a standard calf starter ration with free access to water throughout the study. Fecal samples were collected from each calf upon arrival and each subsequent day (for 5 d; d -6 to d 2) during the dietary/facility adaptation period to verify animals were not actively shedding *Salmonella* prior to inoculation.

3.3.2. Treatments and Blood Collection

Steers were weighed on d -2 (BW = 102 ± 2.7 kg) prior to random assortment to 1 of 2 treatment groups. The two treatment groups consisted of 1) Immune suppressed (DEX; BW = 101.6 ± 3.5 kg) induced by repeated intravenous administration of 0.5mg/kg BW of DEX (d -1 to d 2), or 2) control (CON; BW = 102 ± 9 kg) in which an equivalent amount of saline was administered. Beginning on d −1, blood was collected via jugular catheter into a 4-mL evacuated sampling tube containing 7.2 mg EDTA (Vacutainer: Becton, Dickinson and Company, Franklin Lakes, NJ for determination of CBC using an automated hemocyotometer (Procyte Dx Hematology Analyzer; IDEXX Laboratories, Inc., Westbrook, ME) at −24, −12, and 0 h and in 8-h intervals from 0 to 120 relative to inoculation.

Bacterial Cultures of *Salmonella* enterica serotype *Typhimurium* (ATTC-BAA-186) were adapted for growth in the intestinal tract and made resistant to nalidixic acid using methodology from Broadway et al. (2015). Oral inoculation of naldixic acid resistant *Salmonella* Typhimurium (3.4×10^6 CFU/animal) was administered via milk replacer on d 0.

3.3.3. Fecal Sample Collection

Following arrival to the facility, fecal samples were collected rectally using a transport swab (Fisher Scientific UK Ltd, Loughborough LE11 5RG, UK) with modified Liquid Stuarts to determine if *Salmonella* was present in each calf (n = 20). Following inoculation of the lab strain of nalidixic acid resistant SAL, fecal samples were taken daily (d -2 to 5) to monitor shedding.

3.3.4. Gastrointestinal Sample Collection

Steers were humanely euthanized 120 h after inoculation with SAL. Viscera was removed from the carcass and the cecum fluid was aseptically collected. The ileocecal and subiliac were also collected upon the viscera necropsy. To confirm the presence of the inoculated SAL presence in the ilium tissue, and gastrointestinal content, the tissue samples were trimmed and macerated at 10 g sample to 100 ml phosphate buffered saline (PBS). Serial dilutions were performed, and samples were plated.

3.3.5. Lymph Node Collection

Mandibular, subiliac, and popliteal lymph nodes were aseptically collected from the left side of the carcass. Samples were subjected to a surface disinfectant dip in 100°C water for 3 seconds, macerated and placed into a filter bag prior to homogenization and SAL detection (Gragg et al., 2013; Broadway et al., 2015).

3.3.6. Synovial Fluid Collection

Synovial fluid from three anatomical joints (i.e., shoulder, coxofemoral, and stifle) on the left side of the carcass were aseptically collected on all carcasses. These joints were selected due to the locations representing an area were the synovial membrane of a joint that may be ruptured exposing potentially contaminated synovial fluid during fabrication that could lead to possible cross contamination of the muscle tissue (Broadway et al., 2015).

Using anatomical knowledge of joints location, a sterile scalpel was used to cutting through the tissues exposing the joint. Both the joint of the animal and the instruments used were disinfected with 70% ethanol and flame prior to incision. Once visible an incision in the synovial membrane was made, a sample of synovial fluid was taken using a sterile transport swab (TS). The swab was fully saturated prior to removal from the joint. Samples were then processed for SAL detection.

3.3.7. Ground Sample Collection

From the opposite half of the carcass, the fore quarter and hind quarter were split, suspended on an overhead rail, and chilled overnight at 0 ± 0.5 °C The forequarter and hind quarter were fabricated mimicking commercial carcass fabrication procedures. The trim obtained during fabrication was then ground through a meat grinder (Grinder KG-12-FS, Houston, TX) with a 10mm plate attachment once and again through a 4.5 mm plate. Forequarter and hind quarters of each carcass were kept separate and the grinder sanitized in between each sample. Ground beef samples were taken using a random grab method (Koohmaraie et al., 2012) and combined for a composite sample for each fore quarter and each hind quarter for every carcass.

The sampling pattern was performed in duplicates with four samples from each carcass. Samples were then cultured for the inoculated strain of SAL.

3.3.8. *Salmonella* Detection

Fecal samples were taken using a TS and enriched in 10 ml Tetrathionate (TT) Broth with iodine supplement at 37°C for 20 - 24 h. Samples were then transferred to 10 ml Rappaport Vassiliadis (RV) broth enrichment and incubated at 42°C for 20- 24 h. Following enrichment, samples were streak plated on selective media Brilliant Green agar (BGA), Luria-Bertani agar (LB), and Xylose Lysine Tergitol-4 (XLT) all supplemented with nalidixic acid (25 µg/ml) prior to incubation at 37°C for 20-24 h (Koohmaraie et al., 2012; Gragg et al., 2013; Broadway et al., 2015). Synovial fluid samples were processed as previously described for fecal samples. Lymph node samples were trimmed of surrounding adipose tissue and fascia, weighed, placed into individual filter collection bags containing 100 ml of PBS (Nasco, Atlanta, GA). In this solution, samples were stomached at 230 RPM for 2 min. (Stomacher 400 Circulator, Davie, FL). Lymph nodes samples were directing plated on BGA, LB, and XLT and quantified as CFU/g following incubation. Ground beef was weighed into 25 g composite samples and placed into a filtration bag with 225 ml of PBS, stomached, and transferred to 10 ml TT broth. Samples were then processed as previously stated for the tissue samples.

Biochemical confirmations were performed on presumptive SAL positives. Colonies were streaked onto a Triple Sugar Iron slant (TSI), indole slant, and Maconkey agar prior to incubation at 37C for 20-24 h (Llana et al., 2012; Broadway et al., 2015). Following incubation, slants were accessed for color development. Colonies taken for presumptive SAL positives were assessed for instant color development with the addition of oxidase drops (Vieira-Pinto et al., 2005). Unless otherwise noted, all media and agar were from Difco Laboratories (Sparks, MD). Reagents and antibiotics were obtained from Sigma Chemical Co., St. Louis, MO.

3.3.9. Statistical Analysis

All data were analyzed in SAS 9.4 (SAS Inst. Inc. Cary, NC) and LSMEANS were separated using Tukey-Kramer adjustment with $\alpha = 0.05$. Complete blood count and RT were analyzed as repeated measures with steer nested within treatment. Bacterial counts (*Salmonella*)

were converted to $\log_{10}$ to achieve normality. For RT data, the 5 min intervals were averaged to 1 h intervals prior to analysis. Fixed effects of treatment, time, and treatment by time were evaluated in the models.

3.4. Results

3.4.1. Rectal Temperature

A treatment by time interaction was observed for RT ($p < 0.01$), with the SAL inoculation increasing the RT in the CON steers staring at 32 h and remaining elevated until harvest (120 h; Fig. 2.1). The RT in the DEX treatment decreased ($p < 0.01$) 4 h post DEX administration and remained attenuated until 64 h where a sharp febrile response (> 40°C) was observed.

3.4.2. Complete Blood Count

Dexamethasone did elicit a treatment by time interaction ($p < 0.01$) in total WBC, with DEX steers having increased WBC concentrations relative to CON steers staring at 12 h ($p < 0.01$) after the first DEX infusion and remaining elevated until 16 h post DEX treatment ($p < 0.01$). The observed increase of WBC is primarily comprised of neutrophils (Fig. 2.3), which also exhibited treatment by time interaction ($p < 0.01$). The DEX steers displayed an increased neutrophil count following the first ($p < 0.01$) DEX treatment and remaining elevated 16 h post treatment ($p < 0.01$) (Fig. 2.4).

3.4.3. Lymph Nodes

The number of calves from each treatment group that tested positive for the presence of orally inoculated naldixic acid resistant *Salmonella* Typhimurium in tissues sampled is presented in Table 3.1. Fecal prevalence prior to the inoculation period was 0% for both the CON and DEX treatments. Post inoculation, 19 of the 20 (95%) animals were shedding SAL on d 1 and by d 4, 20 of the 20 (100%) animals were shedding. At the time of necropsy, SAL was isolated from 20 of the 20 (100%) of the ileocecal lymph nodes, 13 of the 20 (65%) of the subscapular, 13 of the 20 (65%) of the subiliac, 14 of the 20 (70%) of the mandibular, and 15 of the 20 (75%) of the popliteal lymph nodes. Of the 20 steers, 9 (45%) were positive for SAL in all lymph nodes sampled. In the ilium tissues, 20 of the 20 (100%) of the ileum and 18 of the 19 (94.7%) of the cecum fluid were positive for SAL.

A treatment by tissue interaction ($p < 0.01$; Fig 3.1.) was observed in the gastrointestinal and lymph node tissue samples. The DEX cecum content not only resulted in greatest concentration of SAL (3.85 ± 0.37 log CFU/g) but was also significantly different when compared to the CON cecum content ($p < 0.01$). It does not appear, with the exception of the cecum content, that mild immunosuppression of calves had an effect on the amount of SAL recovered in tissues in the experimentally infected calves (Fig. 3.1.). Between treatments, (DEX and CON), the cecum content was the only tissue that was different ($p = 0.031$), with DEX calves having a greater concentration of SAL. While there were numerical increases in SAL concentrations in the cecum ($p < 0.01$) of the DEX calves, there were no differences between the two treatments groups for the ileum tissue ($p = 0.07$), subiliac ($p = 0.99$) ileocecal ($p = 0.57$), mandibular ($p = 0.12$), popliteal ($p = 0.99$), or subscapular ($p = 0.83$) lymph nodes.

3.4.4. Synovial Fluid

Salmonella presence in the synovial fluid was not quantified, however accessed on a positive or negative basis. Orally inoculated SAL was isolated in 0 of the 20 (0%) of shoulder synovial fluid, 1 of the 20 (5%) of stifle synovial fluid, and 0 of the 20 (0%) of coxofemoral synovial fluid. Of the synovial swabs collected (n = 60; 3 samples/calf), one joint was positive from a calf in the control group. Two grab samples were taken and combined for a composite sample for each fore quarter and each hind quarter for every carcass. This sampling set was performed in duplicates with four samples taken from the carcass 2 in the forequarter and 2 in the hindquarter). *Salmonella* present in the ground beef samples were also accessed on a positive and negative basis. The prevalence of SAL in the ground beef samples was recovered in 7 of the 80 (8.75%) samples taken.

3.5. Discussion

The source of *Salmonella* in the fecal matter of cattle plays a major role in the crosscontamination of beef carcasses at the abattoir and ultimately the food supply (Fegan et. al, 2005; Koohmaraie et. al, 2012). *Salmonella* infection in cattle occurs when a susceptible animal ingests the bacteria, either through the fecal–oral route directly or by contaminated food and/or water from other animals shedding the organism, thus consuming a source of infection (Wray et al., 1990; Rhen et al., 2007; Stevens et al., 2009; Byers et al., 2011). While the calves in this

study were in a controlled environment, many factors can negatively influence infection and fecal shedding of SAL such as transportation (Barham, et al., 2001; Carroll et. al, 2007; Koohmaraie et. al, 2012). Studies have suggested that feed withdrawal, transportation, and the commingling of animals prior to harvest can also negatively affect the contamination of cattle with bacterial pathogens such as SAL in response to the stress event (Corrier et. al, 1990; Beach et al., 2002). The stress of these events increases cortisol, a natural glucocorticoid, and briefly enhance the immune system (Oldham and Howard, 1992; Burton et. al., 1995). If stress persists moving from an acute to chronic experience, the immune system becomes suppressed enhancing the

symptoms of salmonellosis such as scours expanding the risk of cross contamination from hides covered in fecal material to the carcass (Burton et al., 1995; McGuirk and Peek, 2003). Literature suggests, synthetic glucocorticoids can result in a negative immune response when given consecutively over a prolonged period of time (Pruett et al., 1987; Oldham and Howard, 1992; Burton et al., 1995; Anderson et al., 1999; Lopparelli et al., 2012, Hughes et al., 2017). Therefore, the shedding of SAL due to natural or synthetic stress may lead to cross contamination of cattle pre-harvest via holding pens, feed, or water.

After a SAL cell has been ingested, there are several mechanisms that allow it to invade the host's intestinal epithelium (Ewen et al., 1997; Naughton et al., 2001; LaRock et al., 2015). In this study SAL was quantified in greater concentrations in the gastrointestinal tissues (ileum and cecum) and one of the lymph nodes that lie in the small intestine tissue (ileocecal). These results are to be expected as the transmission of SAL is through gastrointestinal tract which is the site of the epithelial binding (LaRock et al., 2015). Once bound, SAL begins pathogenesis leading to mucosal membrane infection, systemic spread throughout the body, and Salmonellosis symptoms (Giannasca et al., 1996). The lymphatic system is involved in pathogen detecting, using a filtration mechanism through the lymph nodes to sequester bacteria, viruses, and other foreign invaders, ending in the destruction of foreign bodies by the lymphocytes (Swartz, 2001). SAL is equipped with the ability to briefly evade the function of the immune system and proliferate within lymph tissues of hosts (Nieto et al., 2016).

Once the pathogen is identified by the immune system, the internal temperature increases and the body produces WBC such as neutrophils to destroy the invader (Pruett et al., 1987;

Oldham and Howard, 1992; Burton et al., 1995; Anderson et al., 1999; Lopparelli et al., 2012, Hughes et al., 2017). In this current study, the characteristic inflammation and heat are associated with adaptive of non-specific defense mechanisms, is observed in the CON treatment post inoculation of SAL (Coutinho and Chapman, 2011). In contrast, the anti-inflammatory actions of synthetic GCs such as DEX, inhibits this febrile response experienced during parthenogenesis (Wiśniewski et al., 1992; Danek, 2006; Hughes et al., 2015). When evaluating the CBC data, an elevated WBC count was observed in the DEX treatment compared to the CON, which is indicative to an immune enhancement event. However, in a chronic stress response the immune system will decline (Miller et al., 2002). This can be characterized by an increase in neutrophils circulating in the vascular system (Oldham and Howard, 1992; Burton, 1995; Anderson, 1999; Hughes, 2017). During the immunosuppressed event, an influx of neutrophils in circulation is observed but have the inability to translocate through the vascular wall into the affected tissue due to the down-regulation of adhesion molecules (Burton et al. 1995; Raffler et al., 2005). In this study, neutrophils concentrations were increased in the DEX treatment as compared to the CON treatment starting 8 h post infusion and remained elevated 16 h post DEX administration (Fig. 2.4), which consistent with previous research (Oldham and Howard, 1992; Burton, 1995; Anderson, 1999; Hughes, 2017). *Salmonella* did not appear to have an impact on WBC or neutrophil concentrations, as they remained steady in CON steers before and after inoculation.

As discussed, interventions in the abattoir have been developed and implemented to control the pathogen risk at different critical control points throughout the harvesting process (Hanson et al, 2016). While these intervention steps are effective at reducing SAL present on the carcass surface, SAL is also known to harbor inside the musculoskeletal tissues (Gragg et al., 2013; Broadway et al., 2015). Several studies investigated SAL in the mesenteric (ileocecal) lymph nodes of beef cattle (Samual et al., 1979, Samual et al., 1981, Sofos et al., 1999; Arthur et al., 2008). In this study, the ileocecal lymph node was found to harbor the greatest amount of SAL colonies when compared to other the lymph nodes evaluated (Figure 1). This could be because of the anatomical location of the ileocecal resting in the connective tissues of the small intestine thus filtering the contents transmitted directly from the gastrointestinal tract. It is known, however, that gastrointestinal tract and connected lymph nodes, such as the ileocecal, are discarded during the evisceration process, thus, not poising a food safety hazard. Large numbers

of lymph nodes are interwoven within the adipose tissue of the carcass which is a common material for ground beef trim (Gragg et al., 2013). Preliminary research suggests that prevalence of SAL in lymph nodes of seemingly healthy cattle entering the harvest facility can range between 1.6 and 88% (Arthur et al., 2008; Gragg et al., 2013; Haneklaus et al., 2012; Koohmaraie et al., 2012). Surveillance measures have estimated that the prevalence of SAL in ground beef products may range between 2.0 and 4.2% in the U.S. (Bosilevac et al. 2009; FDA 2011, Samadpour et al., 2005; Zhao et al., 2002). Though in this study the contamination percentage exceeds the normal range, 100% of the animals used were shedding SAL before harvest. With positive confirmations of SAL within the lymph nodes, it is likely that the contamination could come from these tissues. Therefore, contaminated lymph nodes could explain the continuous prevalence of SAL within trim and ground beef, even after the cycle of affective intervention steps.

During the many commercial fabrication processes, the ileum tissue and ileocecal lymph node will be removed however, the mandibular, subscapular, subiliac, and popliteal lymph nodes will remain in interwoven within the tissues of the carcass (Arthur et al., 2008). As of 2016, FSIS began research on the presence of SAL in ground beef samples. Although there was a high percentage of lymph nodes that were positive in the carcasses in the current study, the SAL was not recovered in the meat samples at the same level using the grab sample method. The observed data suggests that the grab method for ground beef sampling may not be a correct quantification of overall presence of SAL in a ground beef sample. These results could be used to evaluate the way SAL is quantified. The FSIS research efforts of SAL testing were key in the recommendation for the National School Lunch Program to purchase ground beef for school lunches that are free of larger lymph nodes such as the popliteal, subiliac, and the subscapular (FSIS, 2017). However, given the extensive number of lymph nodes in the body, complete elimination of the lymph tissue prior to ground beef production is simply not a feasible solution overall. The joints separated during fabrication allows the synovial fluid to come in contact with the meat potentially contaminating the equipment used and trim sourced for ground beef. The grind sample results infer that the presence of SAL can come from outside sources such as synovial fluid and lymph nodes that avoid intervention contact, thus remaining interwoven in the

trim during the grind process. These observations highlight the need for investigation into the complex translocation factors of SAL throughout the host.

3.6. Conclusion

These results indicate that the lymph nodes closer to the ileum or site of infection, will result in greater concentrations of SAL. Otherwise, the presence of SAL in the lymph nodes indicates lymphatic spread of the organism, which reflects an increased risk of ground beef contamination. The previously stated data confirms that orally inoculated SAL can translocate from the gastrointestinal tract of immunosuppressed cattle and be harbored in peripheral lymph nodes and synovial fluid which represents a food safety risk. Therefore, the cause for this pathogen migration of this phenomenon needs to be further researched. There is a substantial likelihood of the stifle joint fluid to come in contact with meat during hind quarter fabrication. Therefore, further studies need to be performed to determine routes of SAL in translocation to the synovial fluid.

3.7. References

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3.8. Tables and Figures

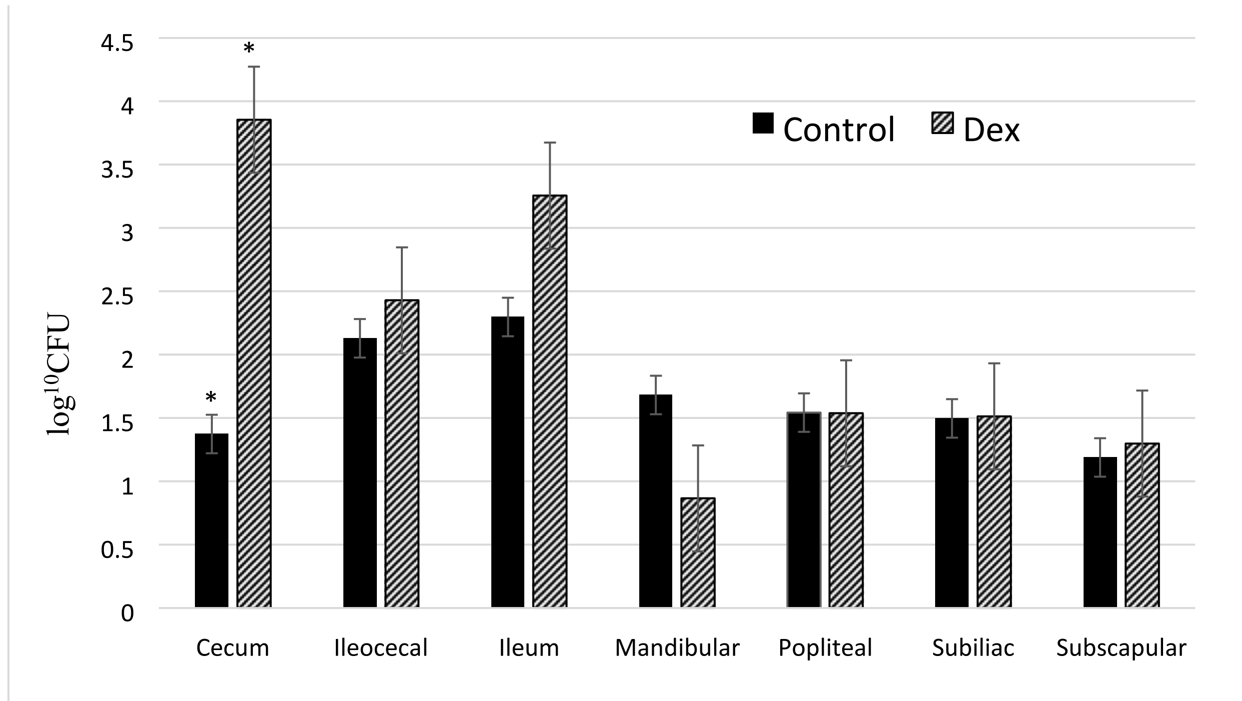

Figure 3.1. The presence of orally inoculated naldixic acid resistant *Salmonella Typhimurium* in the gastrointestinal tissues (Ileum and cecum) and lymph nodes (ileocecal, mandibular, popliteal, subiliac and subscapular). While there were numerical increases in SAL concentrations in the cecum of the Dex calves, there were no statistical differences between the two treatments groups for the ileum tissue (P = 0.07), subiliac (P = 0.99) ileocecal (P = 0.57), mandibular (P = 0.12), popliteal (P = 0.99), or subscapular (P = 0.83) lymph nodes. Standard error of the mean 0.37 for both treatments. Alpha = 0.05

Table 3.1. Frequency and percentage of calves from each treatment group that tested positive for the presence of orally inoculated naldixic acid resistant *Salmonella Typhimurium* in tissues sampled.

Tissue	CON	DEX	Percentage
Fecal	9/10 – 10/10*	10/10	100%
Ileocecal Lymph Node	10/10	10/10	100%
Subscapular Lymph Node	6/10	7/10	65%
Subiliac Lymph Node	8/10	6/10	70%
Mandibular Lymph Node	9/10	5/10	70%
Popliteal Lymph Node	8/10	7/10	75%
Ileum	10/10	10/10	100%
Cecum Fluid	8/9	10/10	94.7%
Stifle Synovial Fluid	1/10	0/10	5%
Coxofemoral Synovial Fluid	0/10	0/10	0%
Shoulder Synovial Fluid	0/10	0/10	0%
Forequarter Grind	1/10	2/10	15%
Hindquarter Grind	2/10	3/10	25%

*9/10 day 1 to 3 and 10/10 d 4

Chapter 4.

Discussion and Future Directions

4.1. Conclusion and Future Research

These results indicate that contamination of specific lymph nodes is reliant of the sit of SAL infection. Otherwise, the presence of SAL in the lymph nodes indicates customary lymphatic spread of the bacteria through the lymphatic tissues of the bovine system. This action may be elevated by the mild immunosuppression event where the DEX steers had an inhibited immune function, therefore slowing the attack of the immune defense on the pathogen. Although this theory is plausible, results of lymph nodes quantification showed no significant difference between the two treatment groups. However, a combination of a mild immunosuppression juxtaposing with slight immune recovery between daily DEX administrations seems more likely due to common suppression traits of DEX influence on neutrophils, monocytes, and cytokines, with the contrast of no effect of the lymphocytes in the study. With a potential increase in SAL positive lymph nodes elicited by an immunosuppressive event, such as chronic stress, the risk of further contamination of ground beef also increases. The data confirms that orally inoculated SAL can translocate from the gastrointestinal tract of immunosuppressed cattle and be harbored in peripheral lymph nodes and synovial fluid of cattle. There is a substantial likelihood of the synovial fluid from the stifle joint to come in contact with meat during hind quarter fabrication posing a potential food safety concern. Therefore, the cause for this atypical pathogen migration to be further researched along with the mechanisms behind this translocation process.

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