USE OF ASPIRIN TO INTENTIONALLY INDUCE GASTROINTESTINAL TRACT BARRIER DYSFUNCTION IN FEEDLOT CATTLE

by

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Dedicated to all of my friends and family

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ABSTRACT

The beef cattle industry segmentation causes stress on cattle as they are weaned and transported to a feedlot or stocker operation. Through this stress and others (acidosis, feed restriction, heat), gastrointestinal tract (GIT) barrier dysfunction occurs, which can lead to numerous additional disorders, such as liver abscesses or laminitis. In order to develop therapies that can minimize leaky gut, a better understanding of how compromised GIT integrity affects the health and growth of beef cattle is needed. Currently, the most common way to study leaky gut is to induce GIT barrier dysfunction by imposing a stress, which can include transportation, feed restriction, pathogen challenge, or heat stress, and then using various markers to measure GIT permeability. However, these methods are resource intensive, cause unwanted side effects for the animal (sickness, decreased growth), and there is large variation among animals in their response to stress, thus these models are less than ideal. To fully understand the GIT without ambiguity, a clearer picture of which effects are from the stress and which effects are from a compromised GIT is needed. In this thesis, the use of aspirin to intentionally induce GIT barrier dysfunction in feedlot cattle was evaluated through Cr-EDTA kinetics, tight junction protein mRNA expression, and blood serum inflammatory indicators. The long-term effects of GIT barrier dysfunction was evaluated through cattle performance and carcass physiology. Cattle receiving aspirin can enter into the food chain at the end of research, which makes research less costly, and aspirin more attractive as a model for inducing leaky gut. Appearance of Cr-EDTA in urine is commonly used as an indicator of GIT barrier permeability.

Chapter 2 includes two complimentary experiments that evaluate the efficacy of aspirin at inducing leaky gut. Experiment 1 used sixteen crossbred heifers (425.0 ± 8.6 kg) in two experimental periods and allotted by BW to 0, 50, 100, or 200 mg/kg BW aspirin. Each treatment had four heifers that received the same aspirin dose during each period, which were separated by 4 wks. Heifers were fed a 49.4% corn silage, 50.6% concentrate diet. Aspirin boluses were administered orally to animals. The 200 mg/kg BW aspirin treatment was dosed as 100 mg/kg BW aspirin 36 and 24 h prior to Cr-EDTA dosing (1 L; 180 mM). The 50 and 100 mg/kg BW treatment were dosed 24 h prior to Cr-EDTA dosing. Every 3 h for 48 h, urine was collected and analyzed for Cr using atomic absorption spectroscopy. At 0, 24, and 48 h, serum was collected and analyzed for lipopolysaccharide binding protein (LBP), interleukin-6 (IL-6), serum amyloid A (SAA),

haptoglobin, and aspartate amino transferase (AST). Experiment 2 used sixteen crossbred steers (576.0 ± 14.2 kg), fed a similar diet for 6 wks. Steers were allotted by BW to the 0 and 200 mg/kg BW aspirin treatments (8 steers/treatment) 36 h before slaughter. Twenty-four h after the last aspirin dose, steers were slaughtered, jejunal tissues were collected and tight junction mRNA expression was determined. Data were analyzed using the MIXED procedure of SAS. Aspirin linearly increased Cr absorption ($P = 0.02$) and elimination ($P = 0.04$) rates and linearly decreased mean retention time of Cr ($P = 0.02$). Aspirin tended to increase serum LBP ($P = 0.09$), but did not affect any other serum inflammatory marker ($P \geq 0.16$). Aspirin tended to increase jejunal claudin-1 mRNA expression ($P = 0.10$), but did not affect expression of other jejunal tight junction mRNA ($P \geq 0.20$). Results from this study indicate that aspirin disrupts GIT barrier function in beef cattle and has potential as a model in GIT permeability research.

Chapter 3 evaluated the long-term effect of GIT barrier dysfunction on animal performance and physiology. Ninety-six Simmental x Angus steers (355.0 ± 14.8 kg) were allotted by body weight and breed into two treatments: control (no aspirin); and aspirin fed at 50 mg/kg/d. Steers were housed in 16 pens (8 pens/treatment) with 6 steers in each pen. Weight was recorded monthly and blood was collected on d 159 and serum was analyzed for LBP, IL-6, SAA, haptoglobin, and AST. Data were analyzed using the MIXED procedure of SAS. Aspirin tended to decrease average daily gain (ADG, $P = 0.10$) and did decrease hot carcass weight ($P = 0.05$) and rib-eye area ($P = 0.01$), while increasing fat thickness ($P = 0.02$), marbling score ($P = 0.003$), and yield grade ($P = 0.01$). Aspirin tended to increase percent KPH ($P = 0.10$). Aspirin had no effect on body weight, dry matter intake, gain:feed, days on feed, dressing percentage, liver abscess score, or percent liver abscesses. Aspirin tended to increase serum LBP ($P = 0.07$), but had no effect on serum concentrations of IL-6, haptoglobin, SAA, and AST ($P \geq 0.30$). This study indicates that aspirin induced leaky gut has negative impacts on feedlot performance and carcass leanness. The negative impact of aspirin induced leaky gut on animal performance suggests that leaky gut caused by other factors (subacute acidosis, stress) may be a significant problem for the feedlot industry.

Overall, aspirin can be used as a model for leaky gut research in both short-term and long-term studies in cattle. The versatility of this model to be used in both short-term and long-term, while allowing animals to enter into the food chain, shows the value that this model brings to research. Additional research is needed to fully understand the mechanisms of leaky gut, and how

to mitigate the negative effects of leaky gut. The aspirin model is a new method in cattle to further research.

CHAPTER 1. REVIEW OF LITERATURE

1.1 Introduction

Appreciation is growing for the importance of gastrointestinal health in domesticated farm animal production. The main purpose of the gastrointestinal tract (GIT) is to allow nutrients to be absorbed for use by the body, while keeping toxins and other opportunistic microbes inside. Stress can upset this balance between absorption and exclusion which compromises GIT integrity. Gastrointestinal tract barrier dysfunction (leaky gut) allows pathogens and toxins to escape the first line of defense (epithelium) and initiate an adaptive immune response by the host, which diverts energy away from productive purposes. Recent research has linked compromised GIT to toxins and microbes weakening the immune system, and potentially entering the blood stream (Lambert, 2009), but further research is needed to confirm this in beef cattle. In livestock systems, stress that compromises the GIT can arise from weaning (Boudry et al., 2004; Moeser et al., 2007), feed restriction (Zhang et al., 2013a; Wood et al., 2015), excess heat (Baumgard and Rhoads, 2013; Pearce et al., 2013a,b), acidosis (Emmanuel et al., 2007; Khafipour et al., 2009a; Minuti et al., 2014), transportation (Wan et al., 2014), and oxidative damage (Lambert, 2009). The combination of weaning and transportation stressors have negative effects on the immune system in newly received beef cattle (Blecha et al., 1984). Gastrointestinal tract dysfunction can lead to many diseases, such as laminitis (Nocek, 1997; Vermunt, 1992), liver abscesses (Vermunt, 1992; Nagaraja and Chengappa, 1998), acute interstitial pneumonia (Loneragan et al., 2001), and inflammation (Kvidera et al., 2017b). These diseases decrease efficiency and can be fatal, which is costly to producers.

The cost of leaky gut is unclear; however, costs for many of the diseases thought to be related to leaky gut have been quantified and are significant. Liver abscesses occur in 12-32 percent of feedlot cattle (Nagaraja and Lechtenberg, 2007), which cause carcass losses of about $38 to $52/hd (Brown and Lawrence, 2010; Reinhardt and Hubbert, 2015). Heat stress decreases BW gain by 7 to 17 kg/hd and can increase death loss by 15 to 51 animals/ 10,000 hd (St-Pierre et al., 2003). The financial burden from heat stress across species is between $1.69 and $2.36 B, and 40-60 percent can be associated with the dairy industry, while the remainder is accounted for in the beef, swine, and poultry industries (Key and Sneeringer, 2014). Acute interstitial pneumonia has been reported

to have a mortality rate in feedlots of 0.03 to 0.15 percent of received cattle (Vogel et al., 2015). In addition, the GIT and the lung are connected through the mucosal immune system (mucosal associated lymphatic tissue), thus pulmonary inflammation can be driven by gut microbiota (He et al., 2012). Bovine respiratory disease (BRD) accounts for 70-80 percent and 40-50 percent of all feedlot morbidity and mortality respectively (Hilton, 2014), which lessens the value of these cattle from $23.23 to $151.18 when compared to healthy cattle (Smith, 2009). Some of the effects of the stress can be mitigated with proper management; however, many stressors are challenging to mitigate through management alone. In order to develop therapies that can minimize leaky gut, an understanding of how compromised GIT integrity affects health and growth of beef cattle is needed.

1.2 GIT Anatomy

Digesta in the lumen are separated from the lymphatic and portal circulation by two distinct epithelia in the reticulo-rumen and the hindgut (Figure 1.1). The contents of the reticulo-rumen and omasum are separated from circulation by 4 layers of stratified squamous epithelium (SSE) and the abomasum, small intestine, cecum, and large intestine are separated from circulation by a single layer of columnar epithelium (CE, Steele et al., 2016). Finger-like projections, called papillae, protrude from ruminal epithelium to produce more surface area for short-chain fatty acids (SCFA) and minerals to be absorbed (Steele et al., 2016). The 4 layers of the SSE are the stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. The first layer, the stratum corneum directly interacts with ruminal and omasal contents and some microbes can colonize on the surface (Steele et al., 2016). However, microbes do not generally penetrate the second layer – the stratum granulosum (Steele et al., 2016). The stratum granulosum cells are secured together with junctional complexes (tight junctions, adherin junctions, and desmosomes), which act as a gate to prevent pathogens from progressing into the hepatic vein (Steele et al., 2016). Tight junction proteins decrease from the stratum granulosum moving towards stratum spinosum (Garcia et al., 2017). The cells of the stratum spinosum and the basale are responsible for metabolism of butyrate to beta-hydroxybutyrate to ketones (Steele et al., 2016). The stratum basale houses the lamina propria and sub-epithelium, which do not have an immunological function like in the intestine (Liang et al., 2019). For example, the specialized and immune cells, such as Peyer's patches, Paneth cells, dendritic cells, lymphocytes, goblet cells, and enteroendocrine cells that are found in the small intestine, are lacking within the rumen epithelium, because there are multiple

cell layers for protection due to the SSE (Steele et al., 2016). However, both the lamina propria and sub-epithelium contain part of the nervous system (Liang et al., 2019). When ruminal papillae are damaged or inflamed, sloughing of papillae in the corneum occurs (parakeratosis) and ruminal wall integrity decreases allowing bacteria to enter into the hepatic vein (Steele et al., 2009; Hinders and Owen, 1965). Liver abscesses, caused by F. necrophorum, are the most common sequalae to subacute ruminal acidosis, and is a classic example of "leaky gut".

The lower gut differs significantly from the reticulo-rumen and omasum (Figure 1.1). Whereas, the rumen is well-protected by multiple epithelial layers (~85 µm thick), the intestinal lining is relatively thin (1 layer of cells ~20 µm thick) and is protected by mucus (Steele et al., 2016). A difference between the SSE and CE, is that the CE has multiple cells side by side rather than stacking on top of one another like in the SSE. The six major layers in the intestine are the mucus layer, epithelium, lamina propria, muscularis mucosa, and submucosa (Steele et al., 2016). The first defensive mechanism used by the lower GIT is mucus, because it prevents pathogens from adhering to the intestinal barrier (Atuma et al., 2001). Mucus is secreted by Goblet cells located in the epithelium and is composed of a combination of mucin proteins. In non-ruminants, the primary mucin found in mucus is mucin 2 (MUC2, Atuma et al., 2001). There are two mucus layers - the firmly adherent mucus layer and the loosely adherent mucus layer (Atuma et al., 2001). These layers vary in thickness depending on the location of the GIT (Atuma et al., 2001).

The intestinal epithelium has finger-like projections, called villi in order to increase absorptive surface area. Cell types within the small intestine include: enterocytes which absorb nutrients, goblet cells that produce mucins for mucus protection, intestinal enteroendocrine cells that produce gastrointestinal hormones, Paneth cells that produce antimicrobial peptides (beta-defensin), microfold (M) cells that transport antigens from the lumen and deliver them to gut-associated lymphoid tissue (GALT) located in Peyer's patches, and tuft cells that play a signaling role in the immune response (Steele et al., 2016; Umar, 2010). Enterocytes and goblet cells predominate the villus surface (Umar, 2010). Older cells undergo apoptosis and shed off from the tip of the villus, while new proliferative (stem) cells produce six different cell types at the base of the villus (crypt); new cells will migrate towards the tip of the villus (Umar, 2010). Intestinal epithelial cells are secured together using three different types of junction proteins: desmosomes, adherent junctions, and tight junctions (Steele et al., 2016). In addition, gap junctional complexes allow the exchange of small metabolites and ions between adjacent cells (Meşe et al., 2007). The

lamina propria has arterioles and venules for blood flow, a diffuse lymph tissue that is rich in lymphocytes, macrophages, and dendritic cells, and organized lymph tissue called Peyer's patches (Steele et al., 2016). Mesenteric lymph tissue and Peyer's patches are part of the immune system within the GALT (McGhee and Fujihashi, 2012). Peyer's patches are one of the first lines of defense against potential pathogens and unique to the intestinal mucosa. The intestinal wall protects the extracellular space from bacteria and endotoxins produced by bacteria in the lumen of the intestine (Anderson and Van Itallie, 2009).

1.3 Tight Junction Proteins

Epithelial cells are held tightly together by 3 types of junctional complexes (Figure 1.2): tight junctions, adheren junctions, and desmosomes, (Anderson and Van Itallie, 2009; Robinson et al., 2015). In addition, gap junctional complexes allow the exchange of small metabolites and ions between adjacent cells (Meşe et al., 2007). Tight junctions predominate in the small intestine, whereas desmosomes primarily act as the permeability barrier of the SSE of the rumen (Baldwin, 1998; Gaebel et al., 1989; Graham and Simmons, 2005). Among the major junctional complexes, only tight junctions have the ability to control the selective paracellular permeability for ions, water, and other small molecules (Robinson et al., 2015). The role of tight junctions are to regulate paracellular movement of water and ions and solutes across the epithelia (Bazzoni et al., 2000). Just as importantly, tight junctions keep bacteria and other potentially pathogenic microbes from crossing into the extracellular space. Therefore, tight junctions are the major determinant of mucosal epithelial permeability. The tight junctions are composed of different groups of proteins called claudins, occludins, zonula occludens (ZO), junctional adhesion molecules (JAM), tricellulin, and cingulin (Anderson and Van Itallie, 2009; Robinson et al., 2015). Knowledge of tight junction function has expanded from human models to non-ruminant models, and in recent years to ruminant models (Furuse et al., 1998). The vast complexity of the 40 known tight junction proteins is why further research needs to be done to completely understand the function of each (Anderson and Van Itallie, 2009). Claudins 1, 3, 4, 5, 8, 9, 11, and 14 are known to decrease paracellular permeability in the intestinal tract; whereas, claudins 2, 7, 12, and 15 increase paracellular permeability (Robinson et al., 2015). The ZO are known to anchor other tight junction proteins, so if there is damage to the ZO, then the damage could be reflected in the structure of other junction proteins (Bazzoni, 2000). This example shows the complexity of paracellular

regulation, and that current research is only starting to scratch the surface. The less studied adheren junctions initiate and stabilize cell to cell adhesion, intracellular signaling, and transcriptional regulation (Hartsock and Nelson, 2008). The most well studied glycoprotein within the adheren junction class is E-cadherin, which aids in the formation, maintenance, and overall function of the adheren junction (Hartsock and Nelson, 2008). Desmosomes' main function is to adhere and maintain tissue integrity (Garrod and Chidgey, 2008). Desmosomes are specialized for strong adhesion, so function failure can lead to increased permeability and diseases, such as leaky gut (Garrod and Chidgey, 2008). In addition to adhesion, desmosomes act as signaling centers for cell proliferation and differentiation (Garrod and Chidgey, 2008). The main proteins that are part of the gap junctional complexes are connexins and innexins (Meşe et al., 2007).

Desmosomes primarily act as the permeability barrier of the SSE of the rumen (Baldwin, 1998; Gaebel et al., 1989; Graham and Simmons, 2005). Towards the surface (*s. corneum, s. granulosum*) of the of the rumen epithelium, the cells become filled with keratins that are expressed in pairs which form heterodimers called intermediate filaments (Bragulla and Homberger, 2009; Magin et al., 2007). These intermediate filaments are linked together by desmosomes (Green and Simpson, 2007; Holthöfer et al., 2007). Desmosomes are calcium dependent adherins (cadherin) composed of multiple proteins (desmosglein, desmocollin, desmoplakin, plakoglobin and plakophilin). The intermediate filaments are then crosslinked to cornified cell envelope proteins by transglutaminase enzymes (Candi et al., 1999). The insoluble barrier formed provides mechanical strength to the epithelium as well as protection against abrasive forces during the mixing of feed and the immune challenge (Green and Simpson, 2007; Holthöfer et al., 2007; Trevisi et al., 2014) posed by the diverse microbial population in the rumen. Cadherin desmoglein (DSG) plays a role in formation of desmosomes, which perform a similar role as tight junctions that hold cells tight to one another (Steele et al., 2011).

1.4 Mucosal Immunity

The mucosal immune system protects against toxic elements that enter the body through mucous membranes of the gut, skin, nasal and oral cavities as well as the female reproductive tract. The mucosal immune system is part of the innate immune response (local, no memory) in the GIT and can be divided into inductive and effector sites based on their anatomical and functional properties. The mucosal inductive sites are collectively called mucosa-associated lymphoid tissue

(MALT) and include gut-associated lymphoid tissues (GALT), nasopharyngeal-associated lymphoid tissue (NALT) and lymphoid sites. Mucosa-associated lymphatic tissue forms the largest mammalian lymphoid organ to guard organs from an abounding supply of pathogenic material. The MALT is populated with phenotypically and functionally distinct accessory cell subpopulations, B cells, and T cells that then move into effector sites (Holmgren and Czerkinsky, 2005). Mucosal effector sites include the lamina propria regions of the GI, upper respiratory and reproductive tracts as well as secretory glandular tissues. These sites contain antigen-specific mucosal effector cells such as IgA-producing plasma cells, and memory B and T cells (McGhee and Fujihashi, 2012). Approximately 80% of immunocytes (T and B cells, dendritic cells, and macrophages) in humans are from the innate immune system and accumulated in various MALTs (Holmgren and Czerkinsky, 2005). Gut associated lymphoid tissue includes Peyer's patches, dendritic cells, and T and B cells (Holmgren and Czerkinsky, 2005).

The innate immune response is initiated when the body recognizes antigens such as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Any damaged or dying cell will release DAMPs that will further stimulate cellular communication for an immune response. A form of PAMP that stimulates an immune response are the phospholipids that make up the outer membranes of bacteria (Raetz and Whitfield, 2002). A form of DAMP is any endogenous molecular structure that can be inside or outside of the cell that are liberated when tissue is damaged (De Lorenzo et al., 2018). The innate immune responses help neutralize invaders from an abundant supply of pathogenic material. Conventionally, antigens have been assumed to enter the intestinal immune system through the M-cells that line the Peyer's Patches, which then pass on the antigen to underlying cells of the innate immune system (McGhee and Fujihashi, 2012). The cells of the innate immune system including neutrophils, macrophages, dendritic cells, natural killer cells (NK), eosinophils, basophils, and mast cells express pattern recognition receptors, such as toll-like receptors (TLR), that enable them to detect DAMPs and PAMPs (Ahluwalia et al., 2017). Activation of these TLRs start the nuclear-translocated transcription factor nuclear factor kappa B (NF-κB) signaling pathway (Serasanambati and Chilakapati, 2016) which enables the expression of pro-inflammatory cytokines (Garcia et al., 2017) and induces inflammation. Pro-inflammatory cytokines, chemokines, and antimicrobial peptides, then recruit more immune system cells to the site. The dendritic cells, following engagement with pattern recognition receptors will present antigens to lymphoid tissue that then

starts the T & B cell adaptive immune response (McGhee and Fujihashi, 2012). T cells only target and phagocytose pathogens inside of cells, whereas, B cells produce antibodies that can phagocytose pathogen-infected cells. From the Peyer's patches, T and B cells can relocate to intestinal and extra-intestinal sites such as lymphoid tissues or bronchial epithelium, providing pathogen specific memory to those sites (He et al., 2012). As part of the adaptive immune system, T and B cells are signaled to the area of inflammation to phagocytose pathogens and infected cells to detoxify the body.

An adaptive immune response is initiated by an extensive innate response, which indicates that the pathogen is persisting past the capability of the innate immune system (Holmgren and Czerkinsky, 2005). Using B cells, T cells, cytokines, and antibodies, the adaptive (acquired) immune response provides the pathogen-specific memory for protection from subsequent infections with the same pathogen. The memory built into the adaptive immune response allows for more efficient defenses. Instead of every MALT location signaling a cascade of systemic reactions for the adaptive immune response, immunocytes that are activated at one site of the mucosa will communicate the immune response throughout other MALT locations for preparation of pathogen infiltration (Holmgren and Czerkinsky, 2005). The mammalian immune system attempts to conserve energy by not signaling for an adaptive immune response unless the pathogen continues to persist and evade the innate immune response.

1.5 Inflammation

Inflammation is the first response of specialized cells to the recognition of pathogenic microbes or tissue damage (Takeuchi and Akira, 2010). Inflammation can be caused by infection, bacterial or endotoxin exposure, physical injury, or new, abnormal tissue growth (Carroll and Forsberg, 2007). A proper immune response activates epithelial and immune cells to initiate an inflammatory response that aids tissue repair and healing, aiming to recover homeostasis. However, an unchecked release of pro-inflammatory cytokines can result in an unresolved inflammatory state that may fail to induce tissue repair and healing (Garcia et al., 2017). Thus, a balance is needed between pro-inflammatory and anti-inflammatory cytokines; an imbalance can lead to further signaling of acute phase proteins and diversion of energy away from productive purposes. A proper balance of inflammatory cytokines allows the animal to allocate energy towards what is needed. The immune system regulates inflammation through secretion of a series of pro-

inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines in cattle include interleukins (IL): IL-1, IL-6, tumor necrosis factor alpha (TNF-alpha), and interferon gamma (IFN-gamma), and the anti-inflammatory cytokines include IL-4, IL-10, and IL-13 (Carroll and Forsberg, 2007). Interleukin-8 is neither pro- nor anti-inflammatory, but rather recruits leukocytes to the inflamed region and promotes leukocyte phagocytosis (Kishimoto and Tanaka, 2015). The role of these pro-inflammatory cytokines are to regulate apoptosis of damaged cells, modify vascular endothelial permeability, and recruit blood cells to inflamed tissue (Garcia et al., 2017). Pro-inflammatory cytokines can also increase T cell development, B cell proliferation, and signal for increased cytokine production (Sims and Smith, 2010). Inflammatory cytokines also signal for the production of acute phase proteins in the liver, which is part of the adaptive immune system. Anti-inflammatory cytokines (IL-4, IL-10, IL-13) are also potent activators of B-lymphocytes (Dinarello, 2000).

After cells detect DAMP and PAMP, TLRs stimulate monocytes and macrophages to produce pro-inflammatory cytokines (Kishimoto and Tanaka, 2015; Carroll and Forsberg, 2007). Specifically, when TLRs are stimulated by endotoxins, they activate the NF-$_{\kappa}$B signaling pathway (Serasanambati and Chilakapati, 2016), which activates the expression of pro-inflammatory cytokines (Garcia et al., 2017). Nuclear factor kappa B (NF-$_{\kappa}$B) is located in the cytoplasm of every cell, and is activated by stress, environmental toxins, viruses, bacteria, inflammatory stimuli, cytokines, free radicals, carcinogens, tumor promotors, and endotoxins (Serasanambati and Chilakapati, 2016). After they are produced, pro-inflammatory cytokines stimulate liver hepatocytes to produce numerous acute phase proteins, such as serum amyloid A, haptoglobin and lipopolysaccharide binding protein (LBP). Acute phase proteins all have different functions, but the common main goal is to aid the immune system by slowing down, binding, or inhibiting invading pathogens from reproducing and growing.

Haptoglobin directly interacts with neutrophils' surface through two binding sites to aid the immune response (Ceciliani et al., 2012). Haptoglobin also suppress endotoxin-induced inflammatory effects, by promoting the production of anti-inflammatory cytokines and binds with iron that bacteria, such as *E. coli*, need to grow (Ceciliani et al., 2012). Serum levels of this acute phase protein can rise and fall in 24 to 48 h during an immune challenge. Serum albumin A, which is classified as an apolipoprotein, can be used for cholesterol uptake and removal from phagocytosed cell membranes at inflammatory sites (Ceciliani et al., 2012). In addition to

cholesterol uptake, SAA is a chemoattractant that mediates the migration, adhesion, and tissue infiltration of monocytes and neutrophils (Ceciliani et al., 2012). Intestinal mucins, whose function is to protect the intestine from endotoxins in periods of stress, increases in the presence of SAA (Ceciliani et al., 2012; Mack et al., 2003; Larson et al., 2003). For example, an increase of bovine mammary-associated serum amyloid A3 isoform upregulated gene expression for mucin-3 by 2.5-fold in the intestinal mucosa and decreased *E. coli* adhesion by 73 percent in a human model and *in vitro* (Mack et al., 2003; Larson et al., 2003). The final known function of SAA is the opsonization of gram-positive and gram-negative bacteria (Ceciliani et al., 2012). Haptoglobin and SAA also play broader roles in the immune system, which can be influenced by many other factors (Ceciliani et al., 2012).

Lipopolysaccharide binding protein's (LBP) role in the body is to bind with lipopolysaccharide (LPS) or lipoteichoic acid (LTA) to further signal for opsonization (Ceciliani et al., 2012). Both LPS and LTA are recognized as endotoxins, meaning they have toxic effects to the host after they are shed from lysed gram negative and gram positive bacteria, respectively. Lipopolysaccharide binding protein is a sensitive indicator for leaky gut, because it specifically binds with LPS. Lipopolysaccharide binding protein can enhance pro-inflammatory effects by 100 to 1000 fold (Ceciliani et al., 2012). A previous ruminant study connected changes in circulating LBP to jejunum villus height-to-crypt depth ratio (Kvidera et al., 2017b). The study suggests that LBP is positively correlated with the degree of intestinal damage and decreased barrier function (Kvidera et al., 2017b).

1.6 Lipopolysaccharide

A form of PAMP that stimulates an immune response are the phospholipids that make up the outer membranes of bacteria (Raetz and Whitfield, 2002). In gram-negative bacteria, the phospholipid membranes are glucosamine-based LPS (Raetz and Whitfield, 2002), whereas in gram-positive bacteria the phospholipid membranes are composed of LTA. Both LPS and LTA are recognized as endotoxins, meaning they have toxic effects to the host after they are shed from lysed bacteria. While LTA is considered a PAMP, most research focuses on LPS because of the greater endotoxic activity in LPS (Ceciliani et al., 2012). Gram-negative bacteria release a variety of LPS species from their outer membrane (Mueller et al., 2004).

Endotoxins (LPS and LTA) are released into the GIT when bacteria shed the outer cell wall during their cell division and when they die due to cell lysis. Endotoxins are normally present in a large amount in the rumen and in the hindgut of ruminants. In addition, the amounts of LPS will rise when ruminants are fed with diets rich in easily digestible carbohydrates, because Gram-negative bacteria experience growth benefits from such diets and thereby increased LPS shedding (Plaizier et al., 2012). Endotoxin concentrations will also increase in the GIT during microbial dysbiosis (e.g., acidosis, feed deprivation, death from antibiotic treatment). However, the rumen wall is usually impermeable to LPS and other large molecules, unless there are injuries and/or inflammation (Gómez et al., 2014; Yáñez-Ruiz et al., 2015). For example, some studies have reported increased peripheral LPS as a result of subacute ruminal acidosis (Khafipour et al., 2009b; Minuti et al., 2014) whereas other have not (Li et al., 2012; Plaizier et al., 2014). Toll-like receptor 2 and 4 are expressed along the length of the ruminant GIT (rumen to colon); however, expression of TLR2 and TLR4 in the rumen was significantly lower compared to the cecum and colon (Malmuthuge et al., 2012). During the transition period in dairy cattle, high grain feeding induced inflammation and increased LPS in mucosal tissues (Eckel and Ametaj, 2016). High-grain diets in dairy cattle can lead to multiple periparturient diseases, such as acidosis, metritis, laminitis, displaced abomasums, and fatty liver (Ametaj, 2017). There is a positive correlation between the level of concentrate in a diet and inflammation, particularly above 45% concentrate in the diet (Plaizier et al., 2012). In dairy cows and steers, visceral fat deposition is promoted by high-energy diets, which leads to chronic inflammation (Moisá et al., 2017; Gozho et al., 2006).

Endotoxins, in general, are temperature resistant, but not acid resistant (Ribeiro et al., 2010). Therefore, there should be substantial deactivation of LPS in the acidic abomasum (Ribeiro et al., 2010), but there is evidence for abomasal uptake of LPS (Wittek et al., 2004). Plazier et al. (2014) reported that the lowest level of LPS is in the jejunum, while the highest levels of LPS occur in the rumen, ileum, and hindgut. Bile, intestinal alkaline phosphatase, and antimicrobial peptides detoxify endotoxins in the small intestine (Mani, 2012; Hersoug et al., 2016). However, dysbiosis will increase LPS presence in the hindgut. Li et al. (2012) reported greater LPS concentrations in the hindgut and the rumen in subacute ruminal acidotic cattle when compared to non-acidotic cattle. Significant evidence from mice (Ghoshal et al., 2009; Williams et al., 2013) and cattle (Khafipour et al., 2009b) indicates that LPS cannot only cross the intestinal epithelium, but also damage the intestinal barrier.

The stratum corneum layer of the rumen epithelium is continually exposed to ruminal contents and the endotoxins present. Khiaosa-ard and Zebeli (2018) suggest that stratum corneum immune response to endotoxins is less "aggressive" compared to other layers and relies instead on a physical barrier (eg keratin) to protect from infiltration. A muted immune response in the stratum corneum likely prevents the immune system from being constantly activated and consuming energy. The stratum basale and spinosum appear to respond to endotoxin exposure. Zhang et al. (2016) isolated and pooled rumen epithelial tissue from the stratum basale and spinosum and observed that these inner-layered cells sensed and reacted to the addition of LPS, which caused an inflammatory response (increased IL-1β, IL-2, IL-6, and IL-8). In the rumen, an increase in TLR2 and TLR4, which detect endotoxins, have been correlated to higher resistance to ruminal acidosis (Chen and Oba, 2012). When TLRs are activated, they increase epithelial cell proliferation, antimicrobial peptide expression, maintenance of tight junctions, and IgA production (Abreu, 2010). Other regions with greater levels of TLR2 and TLR4 are the cecum and colon, in comparison to the rumen, jejunum, and ileum (Malmuthuge et al., 2012). Young cattle show the greatest levels of TLR10 in the ileum, indicating that LPS is transported to a larger extent through the hindgut rather than the upper gut regions (Malmuthuge et al., 2012). Greater TLR concentration may indicate that the digestive system is more prone to receiving high levels of pathogens (ileum, cecum, rumen, omasum). The ileum could potentially have higher TLR10 expression to help prevent LPS damage and translocation that resulted from a backflow from the hindgut.

Endotoxins are thought to traverse the GIT epithelium via two routes: the paracellular pathway through compromised tight junctions and the transcellular pathway through specific receptor-mediated endocytosis (Eckel and Ametaj, 2016). Transport through the paracellular route occurs when excessive production of either LPS or LTA damages tight junction proteins and disrupts their function (Eckel and Ametaj, 2016). Transport through the transcellular pathway occurs when GIT tissues become inflamed and TLRs and associated signaling proteins increase in concentration, facilitating endocytosis of endotoxins through the intestinal cell (Abreu et al., 2002; Abreu, 2010; Guo et al., 2013). The majority of endotoxins that enter through the transcellular route are lysed; however, endotoxin that enters through the paracellular route and some that enters through the transcellular route end up in the lamina propia. Endotoxin entering the lymphatic system or portal vein will be removed by local macrophages and trigger release of proinflammatory cytokines and acute phase proteins that will further neutralize the endotoxins.

Endotoxin that escapes local macrophages in the liver or lymph nodes, will enter into systemic circulation. When endotoxins do manage to enter circulation, a series of interactions occur with a combination of proteins to counteract the toxin (Lu et al., 2008; Ceciliani et al., 2012). Some of these proteins that will counteract LPS include LPS-binding protein (LBP), cluster of differentiation 14 (CD14), myeloid differentiation-2 (MD-2), and toll-like receptor 4 (Lu et al., 2008; Ceciliani et al., 2012). The acute phase protein, LBP, binds directly with LPS (Lu et al., 2008; Ceciliani et al., 2012). After binding with LPS, LBP mediates the association between LPS and CD14 (Lu et al., 2008; Ceciliani et al., 2012). In circulation, LPS that is bound to LBP and CD14 will facilitate the binding of high-density lipoproteins (HDL) and low-density lipoproteins to endotoxins (Eckel and Ametaj, 2016). Once bound, endotoxins move to hepatocytes or white adipose tissue (Eckel and Ametaj, 2016). Endotoxins that are bound to lipoproteins are endocytosed by liver hepatocytes and later released deactivated in the bile, or stored in the white adipose tissue and then removed by adipose macrophages (Figure 1.3), which release more cytokines into the systemic circulation

When LPS is bound to HDL, this complex is neutralized in hepatocytes, reducing the inflammatory response, which is known as the lipoprotein pathway (Eckel and Ametaj, 2016). The neutralized LPS will be excreted through bile and prevents an immune response (Eckel and Ametaj, 2016). Excessive inflammation in the liver is avoided by utilizing the lipoprotein pathway, which could be the preferred clearance method of ridding circulating LPS (Khiaosa-ard and Zebeli, 2018). The lipoprotein pathway eases the removal of LPS from circulation by facilitating the uptake of lipoproteins bound with LPS by adipocytes (Hersoug et al., 2016). An increase in LPS may lead to more binding to HDL, which delivers greater amounts of triglycerides to the liver. Over time, triglycerides can accumulate in the liver from the abundance of HDLs, causing fatty liver (Eckel and Ametaj, 2016). Cattle with fatty liver are inflamed and have an increased concentration of circulating acute phase proteins and cytokines (Eckel and Ametaj, 2016). Alternatively, plasma Ca binds with LPS, which pushes LPS towards tissue macrophage phagocytosis, causing more tissue inflammation (Khiaosa-ard and Zebeli, 2018). The shift in LPS clearance from macrophages to hepatocytes promotes LPS to be rapidly cleared in a large capacity, but this also promotes triglycerides to be deposited in hepatic tissue (Khiaosa-ard and Zebeli, 2018; Yao et al., 2016). In humans, non-alcoholic fatty liver disease is due to an increase in circulating LPS levels and an activation of the pro-inflammatory cascade (Szabo et al., 2010; Alisi et al., 2012).

Lipopolysaccharide is known for inducing lipolytic and lipogenic responses, but adipocytes are also used for intermediate storage of LPS that can be later neutralized by macrophages in adipose tissue (Hersoug et al., 2016). The combination of greater fat deposition and compromised gut integrity in cattle fed high grain diets can lead to greater LPS infiltration into adipocytes, increased macrophage activity, and heightened inflammation (Eckel and Ametaj, 2016). The inflammation and endotoxemia that comes from high-grain diets could be the cause for all of the previously mentioned diseases associated with leaky gut. In future studies, indicators of the onset fatty liver could play a role in determining endotoxemia and leaky gut. Obesity could stem from adipose tissue having increased LPS storage and lipid delivery (Hersoug et al., 2016). Lipopolysaccharide stored in fat reserves, could flood into circulation when body fat mobilization occurs and cause an activation of the immune system (Khiaosa-ard and Zebeli, 2018). Lipopolysaccharide can stay in the body and have long lasting negative effects on cattle. For example, dairy cattle with chronic inflammation have decreased longevity (Eckel and Ametaj, 2016). An acutely activated immune system will use more than a kilogram of glucose in 720 min, which pulls energy from production to maintain the immune system (Kvidera at al., 2017a).

1.7 GIT Histology

Intestinal histology changes when the gut barrier integrity is compromised. Villus height, crypt depth, and their ratio are commonly measured to determine the extent of damage to the intestine. Excessive shedding of villi can occur when toxin loads damage the epithelium, which results in shortened, stubby villi that do not absorb nutrients as well. A villus height decrease is associated with compromised gut integrity, because the mucosal surface area decreases, and has less nutrient absorption capacity, thus decreasing nutrient supply to the body (Kvidera et al., 2017b). A villus height to crypt depth ratio decrease can be indicative of a rapid decrease in cell proliferation and/or a decrease in crypt cell generation (Kvidera et al., 2017b). Overall, a combination of these histological measures can indicate that gut health and nutrient absorption is decreasing.

1.8 Diet Induced GIT Dysfunction

Acidosis can occur when cattle are improperly transitioned onto high grain diets. Acidosis can cause parakeratosis which compromises gastrointestinal barrier function and is a form of leaky gut (Pederzolli et al., 2018; Steele et al., 2009). Unhealthy compounds, such as LPS, move towards the portal vein and causes local and systemic inflammation acting in part of the immune system in cattle (Khafipour et al., 2009a,b; Plaizer et al., 2012). Steele et al. (2011) reported that in cattle experiencing grain-induced subacute ruminal acidosis, the thickness of the strata granulosum, basale, and spinosum were reduced and ruminal lesions increased. Ultrastructure of the ruminal epithelium viewed by a transmission electron microscope showed large spaces between levels of strata in cattle fed a high-grain diet (Steele et al., 2011). In the same study, researchers noticed a downregulation of cadherin desmoglein 1 in the granulosum layer of the rumen in dairy cows fed high-grain diets compared to cows fed high forage diets, which indicates that junctions in the desmosomes were compromised (Steele et al., 2011). The change in ruminal epithelium and tight junction expression are a cause for parakeratosis and leaky gut in cattle (Steele et al., 2011). In goats, high grain diets damaged ruminal epithelial cells, eroded intercellular junctions, and down-regulated claudin-4, occludin, and ZO-1 compared to non-grain diets (Liu et al., 2013; Liu et al., 2014). In the rumen of dairy cattle, claudin-1 and claudin-4 gene expression were up-regulated 24 h after acidosis was induced (McCann et al., 2016). There is a strong negative correlation between junction proteins (claudin-1 and desmoglein) and pH during acidosis (McCann et al., 2016). In Holstein steers, acidosis decreased pH in the reticulo-rumen, cecum, and the colon, decreased papillae width, upregulated TLR4 in the rumen, and occludin and tight junction protein 1 and 2 in the jejunum, and downregulated claudin 1 in the distal colon (Pederzolli et al., 2018). Acidotic sheep had more of the leaky gut marker lactulose leak into blood than control (Minuti et al., 2014). Ruminal epithelia and colonic epithelia allowed more H-mannitol during acidic conditions *in vitro* (Emmanuel et al., 2007).

High-grain feeding has been connected to increased levels of free LPS in rumen fluid and acute phase proteins in peripheral circulation (Khafipour et al., 2009a). Acidic conditions (pH = 4.5) compared to less acidic conditions (pH = 5.5 and 6.5) *in vitro* caused more permeation of LPS through ruminal and colonic tissues (Emmanuel et al., 2007). The combination of increased LPS and increased leakiness in cattle fed high-grain diets leads to immune activation and potential systemic problems. Intravenous dosed LPS is known to cause an adaptive immune response in

dairy and beef cattle (Kvidera et al., 2017a; Carroll et al., 2009). Ametaj et al. (2012) first showed that repeated oral doses of *E. coli* LPS will stimulate the innate immune system in dairy cattle. High concentration of LPS in digesta have been reported to induce cell apoptosis, disrupt ZO-1, and increase human intestinal epithelial cell permeability *in vitro* (Chin et al., 2006).

In general, low feed intake and acidosis cause changes in luminal conditions, such as decreased pH, decreased papillae length, width, and perimeter, reduced surface area, and lowered short chain fatty acid production in the rumen (Pederzolli et al., 2018). Gäbel and Aschenbach (2002) collected ruminal epithelia from sheep that had feed removed 48 h or 30 min prior to slaughter and observed that epithelia from sheep removed from feed for 48 h had increased 3-O-methyl-α-D-glucose permeation in an Ussing chamber experiment, which indicates that the sheep had compromised barrier function. Pederzolli et al. (2018) observed that restricting Holstein steers to 25% of their dry matter intake for 4 days decreased pH in the reticulo-rumen and proximal colon and decreased papillae length, width, and perimeter in the rumen. In addition, nutrient restriction increased claudin-1, occludin, ZO-1, ZO-2, and TLR4 expression in the rumen and the jejunum (Pederzolli et al., 2018). Occludin, tight junctional protein 1 and 2, and TLR4 were upregulated in the beginning of the colon when cattle were fed 25 percent of *ad libitum* dry matter intake (Pederzolli et al., 2018). Within 5 d after cattle were allowed *ad libitum* access to feed, expression of these same tight junction genes did not differ among treatments, indicating that GIT permeability reverted to the pre-challenge state (Pederzolli et al., 2018). Zhang et al. (2013b) compared beef cattle experiencing 75, 50, and 25 percent of DMI feed restriction to cattle not experiencing a feed restriction and showed that pH decreases as feed restriction increases. In addition, urinary Cr recovery after ruminal dosing was greatest in cattle who experienced the most amount of feed restriction (Zhang et al., 2013b), indicating that leaky gut incidence was greater.

Cells are constantly turning over from damage that occurs on a normal basis, but cell death and growth are in equilibrium. If excessive damage is occurring, then a longer period of time is needed to recover from the damage. In cattle that have been exposed to weaning, transportation, comingling, environmental change, and feed deprivation stressors it is common that it takes more than 2 wks for feed intake to return to expected levels (Hutcheson and Cole, 1986). Using a feed restriction model, researchers observed that at least 2 wks were needed to return to expected voluntary intakes (Zhang et al., 2013a). In sheep, when ruminal pH decreased after a high-grain diet was fed, the rumen took approximately 5 to 7 d for the epithelium to return to full electrolyte

(Na^+, Mg^{2+}, Cl^-) absorptive function (Gaebel and Martens, 1988; Etschmann et al., 2009). Two types of adaption occur in the rumen: functional, which is when the purpose of the rumen adapts and morphological which is when the structure changes. Doreau et al. (2003) suggested that 2 wks are needed to recover absorptive function of ruminal epithelia after 10 wks of feed restriction at 50% maintenance requirement in sheep. On average, the ruminal epithelial turnover is 16 d on a roughage diet and 11 d on a concentrate diet (Goodlad, 1981). The ruminal epithelia will take approximately 4 d to transition from a roughage diet to a concentrate diet (Goodlad, 1981). Rumen papillae length is longest in high roughage, and become shorter in high concentrate diets.

Liver abscesses have been connected to compromised gastrointestinal integrity (parakeratosis), which is partially a result of an acidic rumen (Vermunt, 1992; Nagaraja and Chengappa, 1998; Nagaraja and Lechtenberg, 2007). On average, liver abscess prevalence is 12% to 32% on most feedlots (Nagaraja and Lechtenberg, 2007). Steers have 1% to 3% greater prevalence than heifers, while Holstein steers have a 12% greater prevalence of liver abscesses compared to beef steers, (Nagaraja and Lechtenberg, 2007). *Fusobacterium necrophorum*, which is a normal resident of the rumen and entire digestive tract, is the main bacteria that is thought to initiate liver abscess (Tadepalli et al., 2009). *Fusobacterium necrophorum* crosses the damaged ruminal epithelium and infects the liver. Pro-inflammatory cytokines, such as IL-1, are released in response to *F. necrophorum's* presence in the body of cattle (Tadepalli et al., 2009). Histologically, liver abscesses are often surrounded by an inflammatory zone (Tadepalli et al., 2009). Cattle with liver abscesses or fatty liver will experience increased levels of aspartate aminotransferase (AST) in blood (Abd Ellah et al., 2004). Concentrations of AST are greater in cattle with liver abscesses, because liver abscesses cause damage; thus, aspartate aminotransferase can indicate if liver stress (abscess) is occurring from short-term or long-term challenges.

Wood et al. (2015) compared calves that were weaned to calves that were not weaned and observed a decrease in ruminal pH in calves that were weaned. As a result, calves weaned at 6 wks of age had greater 48 h urinary Cr excretion than calves that were not weaned (Wood et al., 2015). Transportation increased blood plasma SAA concentration in newly weaned beef cattle when compared to non-transported cattle (Arthington et al., 2003). Heat stress in pigs increased plasma endotoxins and decreased villus height, crypt depth, and villus height to crypt depth ratio (Pearce et al., 2013a). In the same study, occludin, ZO-1, and claudin 3 expression was upregulated on the seventh day of heat stress (Pearce et al., 2013a). Furthermore, blood haptoglobin and LPS

permeability increased in pigs experiencing a 7 d heat stress (Pearce et al., 2013a). Under heat stress situations, swine have shown an increase in claudin-3 and occludin, but not claudin-1 (Pearce et al., 2013b). Yu et al. (2010) determined in swine that villus height decreased in the duodenum and jejunum for 6 d after induction of heat stress and villus height decreased in the ileum 3 d after induction of heat stress. In the same study, crypt depth decreased in the jejunum for 3 d (Yu et al., 2010).

1.9 Disease Induced GIT Dysfunction

Pigs experimentally infected with rotavirus experienced villus atrophy in the caudal two-thirds of the small intestine, and villi were shortened, more blunt, and covered with cuboidal epithelial cells (Theil et al., 1978). Villi and enterocyte lining destruction in pigs experimentally infected with rotavirus resulted in diarrhea (Graham et al., 1984). The damage to intestinal cells reduces mucosal surface area and digestive enzymes, which can lead to nutrient malabsorption (Graham et al., 1984). Cryptosporidiosis also causes intestinal villi damage, malabsorption, and reduces NaCl absorption (Argenzio et al., 1996). In swine, cryptosporidiosis caused decreased ileum villus height by d 9 of infection and decreased villus surface area (Argenzio et al., 1990). Ileum crypt depth and villus height increased and decreased, respectively when pigs were challenged with cryptosporidiosis (Argenzio et al., 1990). The ileum villus height does not fully recover until after 9 d post-challenge (Argenzio et al., 1990). The histological results from animals experiencing gut integrity challenges have been used to indicate the decrease in absorptive efficiency. Total recovery of the jejunum, ileum, and colon took 12 d after infection with cryptosporidium (Argenzio et al., 1990). Some studies use *E. coli* (K88) to induce similar intestinal damage (Owusu-Asiedu et al., 2003; Bosi et al., 2004).

Cryptosporidiosis also impairs intestinal absorption and barrier function based on the increased ratio of lactulose/mannitol in experimentally infected calves (Klein et al., 2008). Hunt et al. (2002) noticed a decrease in villus surface area in calves experimentally infected with cryptosporidiosis that were not fed an additional bovine serum concentrate supplement. Calves experimentally infected with cryptosporidiosis had greatest intestinal leakiness at 4-6 d after infection (Hunt et al., 2002).

Johnes Disease (paratuberculosis) is caused by *Mycobacterium avium* subsp. Paratuberculosis, which is a slow growing intracellular bacterium that persists in macrophages in

the intestinal tract for numerous years prior to the onset of symptoms (Olsen et al., 2002). This disease is found in humans and cattle. The bacteria enters through M cells, and is phagocytosed by macrophages (Olsen et al., 2002). The bacteria slowly replicates in macrophages, because the bacteria is resistant to intracellular degradation (Olsen et al., 2002). The slow replication in macrophages causes an increase in inflammatory cytokines and antibody production (Olsen et al., 2002). Johnes symptoms can have a long onset, but can include chronic/intermittent diarrhea, weight loss, intestinal lesions (most commonly in distal ileum and sometimes in the large intestine), elevated TNF-alpha, and inflamed cells surrounding the Peyer's patches (Clarke, 1997). Cattle infected with Johnes expressed higher amounts of IL-1, IL-6, and IFN-gamma in ileal tissues (Lee et al., 2001). In addition to chronic inflammation, tight junction integrity has been demonstrated to deteriorate in the intestinal mucosa (Bannantine and Bermudez, 2013). The combination of chronic inflammation and deteriorated tight junctions in Johnes infected cattle could be causing chronic leaky gut due to mucosal deterioration (Bannantine and Bermudez, 2013).

Arthington et al. (2013) reported increased haptoglobin and reduced performance in cattle vaccinated against *Mannheimia haemolytica*. Rodrigues et al. (2015) observed that multiple vaccinations against infectious bovine rhinotracheitis virus, bovine respiratory syncytial virus, parainfluenza-3 virus, bovine viral diarrhea Types 1 and 2 viruses, and *Mannheimia haemolytica* increased plasma haptoglobin, serum TNF-alpha, and plasma insulin.

1.10 Measuring Leaky Gut

Ussing chambers have been used to determine GIT tissue permeability *in vitro*. The chamber is split in half with a tissue sample, which acts as a barrier to each half of the chamber (Li et al., 2004). If barrier function is compromised, molecular markers, such as dextrans, will pass through the tissue sample and be concentrated on the other half of the chamber (Li et al., 2004). If barrier function is intact, then fewer of the molecules will pass through the tissue sample (Li et al., 2004). These larger molecular markers can allow researchers to speculate if the endotoxins could pass through due to molecule size (Li et al., 2004). A challenge with this method of research is that tissue samples could degrade before the test is run, or if samples are not taken correctly.

The most common way to determine leaky gut in non-ruminant models is by dosing animals (or Ussing chambers) with sugars or polyethylene glycols and measure their appearance in urine (or the other side of the chamber). Dextrans are polysaccharides that come in numerous molecular

sizes (3 kDa to 2000 kDa), but could be digested through fermentation in the rumen (Wang et al., 2015). Intestinal permeability can be measured in non-ruminants by using fluorescein isothiocyanate-conjugated (FITC) dextran (Wang et al., 2015). Following administration of FITC dextran, plasma samples can be taken to analyze for the fluorescent marker, indicating if leakiness is occurring (Wang et al., 2015). The lactulose/mannitol test is a common method used to determine gut permeability. Mannitol is a 182 Da monosaccharide; whereas, lactulose is a 342 Da disaccharide (Wang et al., 2015). Lactulose and mannitol, in a known ratio, are given to the animal, and urine is collected for at least 24 h (Dastych et al., 2008; Wang et al., 2015). The ratio between the two sugars concentrations can give insight on gut leakiness. The lactulose/mannitol test commonly used *in vitro* to determine permeability in ruminants, but cannot be used *in vivo*, because these molecules could be fermented in the rumen, which prevents researchers from using this model (Wang et al., 2015).

The use of chromium-EDTA is a well-established technique for the *in vivo* measurement of gastrointestinal permeability in non-ruminants (Bjarnason, 1986; Hollander, 1992; Eutamene et al., 2007) and ruminants (Schweigel et al., 2005; Zhang et al., 2013a). Chromium-EDTA is not digested or metabolized, it has a high renal clearance rate (Bjarnason et al., 1986), and can permeate the paracellular pathway, eventually appearing in the urine (Schweigel et al., 2005; García-Lafuente et al., 2001; Ten-Bruggencate, 2006). In ruminants, approximately 3 percent of Cr-EDTA is absorbed and excreted in the urine in healthy animals (Shingfield et al., 2008). Quicker passage into urine and greater amounts in urine are an indication of greater GI leakage (and potential damage). The approximate mass of Cr-EDTA is 340 Da and the size is approximately 10 Å (García-Lafuente et al., 2001), whereas, endotoxin approximate mass is 2-70 kDa. Thus, Cr-EDTA is not an indicator of endotoxin translocation, but rather an indicator of gut barrier dysfunction (Magalhães et al., 2007).

1.11 Inducing Leaky Gut

Currently, the most common way to induce and measure GIT barrier dysfunction is by imposing a stress, which can include transportation, feed restriction, pathogen challenge, heat, or stress. However, these methods are resource intensive, cause unwanted side effects for the animal (sickness, decreased growth), and there is large variation among animals in their response to stress, thus these models are less than ideal. To fully understand the GIT without ambiguity, a clearer

picture of which effects are from the stress and which effects are from a compromised GIT is needed.

1.11.1 Inducing Leaky Gut: GSI Model

Kvidera et al., (2017b) studied leaky gut in dairy cattle independent of other stressors using the drug gamma secretase inhibitor (GSI), which has a side effect of inducing goblet cell metaplasia (Milano et al., 2004) and disrupting crypt cell differentiation. Disruption of crypt cells severely damages the intestinal barrier (Wong et al., 2004). Villus height decreased, crypt depth increased, and villus height to crypt depth ratio decreased in the jejunum of dairy cows treated with 1.5 mg/kg BW of GSI compared to control cows (Kvidera et al., 2017b). In addition, Kvidera et al. (2017b) observed that goblet cell area increased in the jejunum, ileum, and colon, which increased mucus production and limited bacterial adhesion to the intestinal wall of GSI-treated cattle. However, the additional mucus production increased the incidence of diarrhea, and more importantly, decreased absorptive enterocyte proliferation, which negatively impacted intestinal integrity (Kvidera et al., 2017b). Plasma LBP tended to increase in GSI-treated cows when compared to the control treatment (Kvidera et al., 2017b) indicating that there was endotoxin movement into the portal vein. Although haptoglobin and SAA increased daily during the study, no differences were noticed between cows treated with GSI and control cows (Kvidera et al., 2017b). Using GSI to intentionally induce leaky gut is a good model, because leaky gut can be studied without the effects from other stressors, but the product is not FDA approved in animals. Thus, the animals fed GSI cannot enter into the food chain, which does not allow the researcher to recover the animal cost of the research. A cheaper model for studying leaky gut independent of other stressors is needed.

1.11.2 Inducing Leaky Gut: Non-steroidal anti-inflammatory drug (NSAID) Model

Non-steroidal anti-inflammatory drugs (NSAID) inhibit the cyclooxygenase (COX)-1 and/or COX-2 isozymes through blocking the active site (Simmons et al., 2004). The blocked active site decreases prostaglandin (PG) E_2 and PGI_2 production (Simmons et al., 2004). Pain and inflammation have been linked to PGE_2 and PGI_2, which is why NSAIDS are effective at relieving pain and inflammation (Simmons et al., 2004). With regard to the GIT, PGE_2, which is produced

from COX-2, functions to release viscous mucus, which helps to protect the mucosal lining. In addition, PGE_2 is mainly responsible for healing gastric ulcers or small intestinal lesions (Takeuchi and Amagase, 2018). Common NSAIDS include aspirin, ibuprofen, indomethacin, or meloxicam. All NSAIDS inhibit COX-1 and COX-2 isozymes; the difference between different NSAID, is how effectively the active ingredient reacts with the active sites. Indomethacin is commonly used to induce GIT dysfunction and study leaky gut in lab animals. Anthony et al. (1993) observed that rats dosed with a single dose of 15 mg/kg of indomethacin experienced severe degeneration of their mucosa, submucosa, muscularis propria, and serosal tissues when compared to untreated rats. In addition, rats dosed with indomethacin experienced ulcers and necrotic mucosal tissue (Anthony et al., 1993). In another study in rats, acute inflammation was noticed in jejunoileal mucosa by 24 h of administration of 15 mg/kg of indomethacin and that by 72 h post treatment, intestinal inflammation intensified and multiple ulcers were noticed (Fang et al., 1977). However, indomethacin is not approved for use in food animals (FDA, 2020). Thus, research with indomethacin in food animals would be too costly, because researchers cannot recover the cost of animal by selling the animal into the food chain.

A cheaper, more viable model, may be using aspirin (acetylsalicylic acid) to induce GIT dysfunction. Aspirin seems to be as effective as other NSAIDS at inducing leaky gut. In humans, measurement of Cr-EDTA in urine was used to determine and compare GIT permeability after dosing volunteers twice with aspirin (1.2 + 1.2 g), ibuprofen (400 + 400 mg), and indomethacin (75 + 50 mg; Bjarnason et al., 1986). All forms of NSAIDs produced more urinary Cr-EDTA in volunteers when compared to volunteers that did not receive an NSAID, which indicates more gut permeability in NSAID-treated volunteers (Bjarnason et al., 1986). Aspirin blocks COX-1 and COX-2 activity by covalently binding to their active sites (Simmons et al., 2004). Aspirin can also induce the adhesion of neutrophils to endothelial cells within the mucosal capillaries by increasing intracellular adhesion molecule-1 expression, resulting in reduced blood flow and possible damage (Iijima, 2016). When COX-1 and COX-2 were inhibited by aspirin, gastric lesions occurred in rats (Simmons et al., 2004), and necrotic and apoptotic cells increased in gastric tissues in another study with rats (Hernández et al., 2016). Humans experienced increased leakiness in the entire GIT when they ingested at least 650 mg of aspirin based on sucrose, lactulose, and rhamnose measures in urine (Lambert et al., 2012). Gnauck et al. (2014) determined that 600 mg of aspirin induced GIT damage, which was determined by increased appearance of lactulose and mannitol in urine

and increased LPS levels in systemic circulation in humans. The maximum aspirin dosage in cattle recommended by the food avoidance residue database (FARAD) is 100 mg/kg every 12 h (Gingerich et al., 1975) with a 24 h meat and milk withdrawal period (Smith et al., 2008).

The effects of aspirin on leaky gut in cattle are unknown; however, there have been recent studies investigating the use of sodium salicylate, a compound related to aspirin, on dairy cattle inflammation and performance. Carpenter et al. (2016) dosed postpartum dairy cattle with 125 g/hd/d of sodium salicylate for 3 d and compared production measures to cows receiving a placebo. Results showed that daily milk production, 305-d milk yield, milk protein, and plasma haptoglobin increased in cows treated with sodium salicylate compared to untreated cows (Carpenter et al., 2016). Although these cattle had enhanced production measures, increased haptoglobin indicates that sodium salicylate may have caused inflammation in dairy cows. Farney et al. (2013) dosed early postpartum dairy cows with 1.95 g/L of sodium salicylate for 7 d and observed increased 305-d milk yield and milk fat in multiparous cows but decreased 305-d milk yield in primiparous cows (Farney et al., 2013), indicating that the benefits of aspirin could be greater in older cattle than younger cattle. It is possible that 7 d of sodium salicylate administration may start to cause inflammation and detrimental effects on production in some cattle. In contrast, Barragan et al. (2017a) dosed dairy cows 12 h postpartum with 4 treatments of 100 mg/kg of acetylsalicylic acid at approximately 12 h intervals, and reported decreased haptoglobin levels at 24 and 36 h postpartum compared to a placebo treatment. No differences were noticed at 48 h or 7 d after parturition (Barragan et al., 2017a). In a complimentary study, Barragan et al. (2019) dosed dairy cattle 12 h after parturition with 2 treatments of 200 mg/kg of acetylsalicylic acid 24 h apart and noted that haptoglobin concentrations were not different at 7 and 14 d in milk compared to untreated cows. Barragan et al. (2017b), showed improved daily milk yields and decreased somatic cell count in dairy cows treated 12 h after parturition with four doses of 100 mg/kg of acetylsalicylic acid 12 h apart. These three studies indicate that administration of aspirin in the short term could decrease inflammatory markers, thus bettering milk production measures. However, the effects of long-term administration of aspirin to cattle are unknown.

1.12 Methods to Mitigate Gut Barrier Breakdown

Many products claim to be beneficial to the digestive tract of livestock. In porcine models, plasma proteins fed to pigs have been reported to aid in the recovery of the intestinal lining during

a rotavirus challenge (Corl et al., 2007). Piglets fed plasma proteins during a rotavirus challenge had a less dramatic reduction in villus height/crypt depth ratio, greater mucosal protein concentrations, and improved growth compared to piglets not fed plasma proteins (Corl et al., 2007). When *Escherichia coli* (*E. coli*, K88) was used to disrupt normal barrier function, dietary plasma proteins aided in the diarrheal recovery time (Owusu-Asiedu et al., 2003) and reduced intestinal inflammation in pigs (Bosi et al., 2004). *Cryptosporidium parvum* is the most widespread pathogen in neonatal calves which causes cryptosporidiosis, a major enteric disease that causes acute and chronic diarrhea. When fed bovine serum concentrate (BSC), dairy calves infected with cryptosporidium experienced a reduction in diarrheal volume, improvement of intestinal permeability, and facilitation of crypt cell-mediated regeneration of villous surface compared to control calves not fed BSC (Hunt et al., 2002). In the intestine, IGF-I activates two intestinal stem cell populations, which promote growth of normal intestinal epithelium and crypt regeneration after irradiation in mice (Van Landeghem et al., 2015)

1.13 Conclusion

As cattle move from segment to segment in the beef industry, they are stressed and health issues arise. More specifically, cattle with GIT dysfunction can be susceptible to numerous different diseases, which cause production and economic losses. Technologies are needed to keep cattle healthy through the transitions and to prevent any production or economic losses. Many technologies and products might be able to improve gut health through various mechanisms; however, an effective model to test these technologies that does not involve placing the animal under stress has not been developed. Leaky gut research is more complicated in ruminants than non-ruminants, because ruminants have extensive pre-gastric fermentation, which limits the types of markers able to be used. The use of aspirin to induce GIT dysfunction seems promising, but little is known about the optimal dose, impact on animal health, or the long-term effects. Therefore, the objective of this study was to determine the optimal aspirin dosage to induce GIT dysfunction and the long-term impact of leaky gut on animal physiology.

1.14 References

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1.15 Tables and Figures

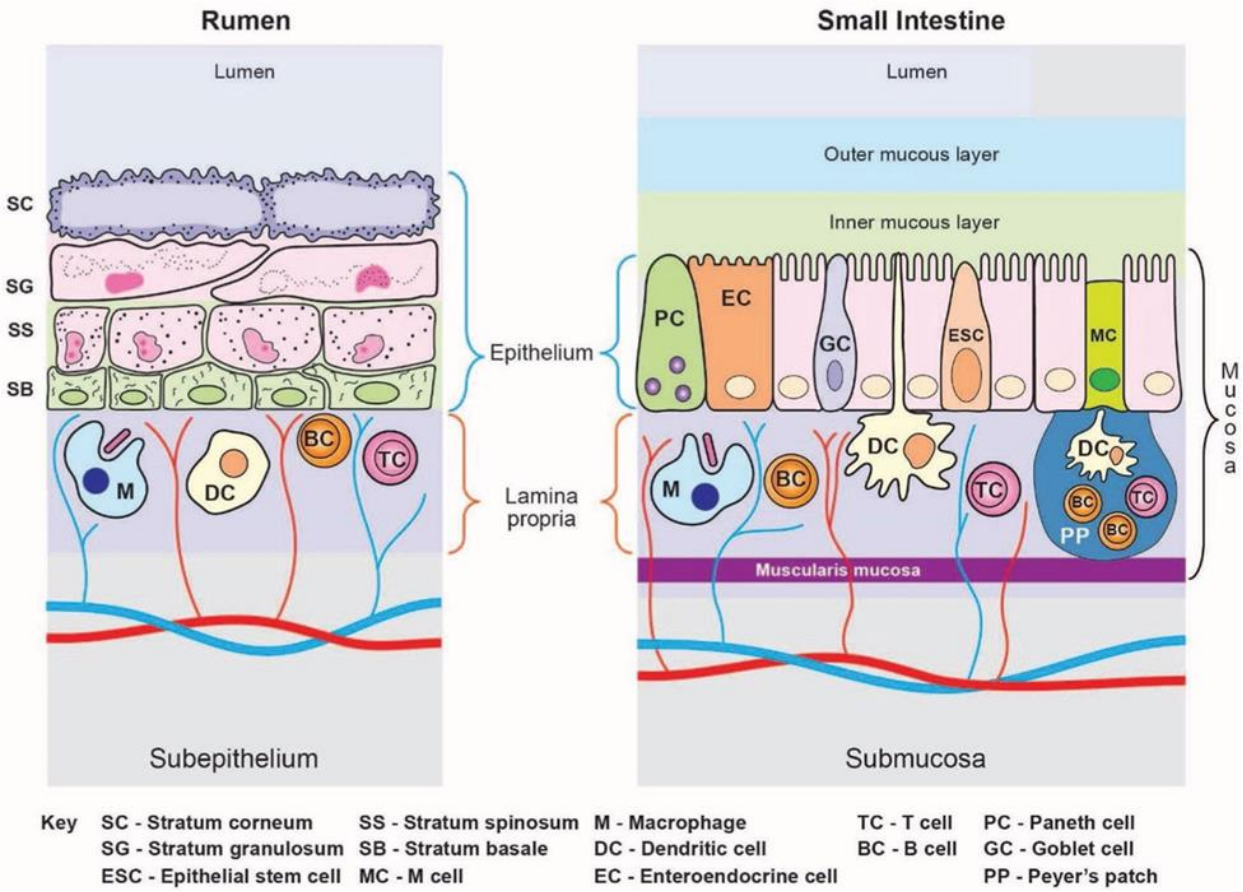

Figure 1.1. Comparison between ruminal cell wall and small intestinal cell wall of cattle (Garcia et al., 2017).

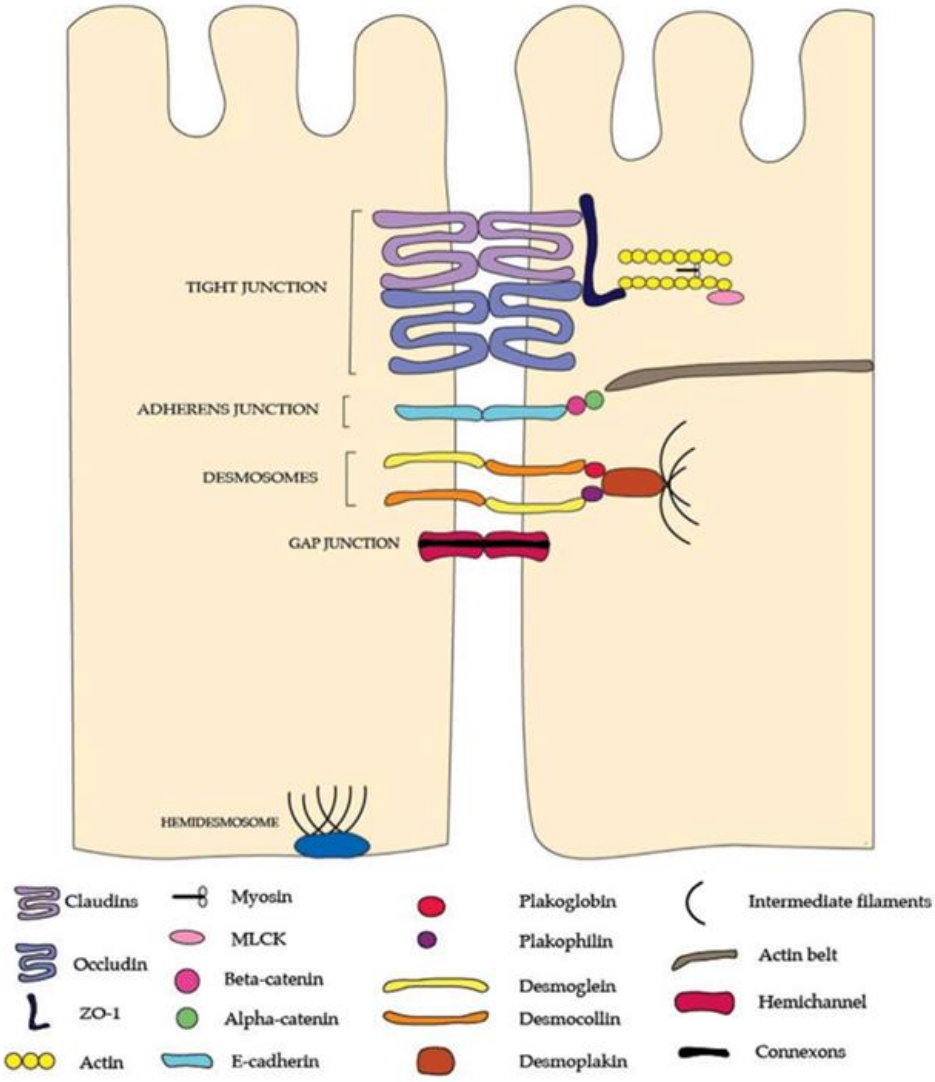

Figure 1.2. Illustration of structures of tight junction, adherens junction, desmosomes, and gap junction (Lee et al., 2018).

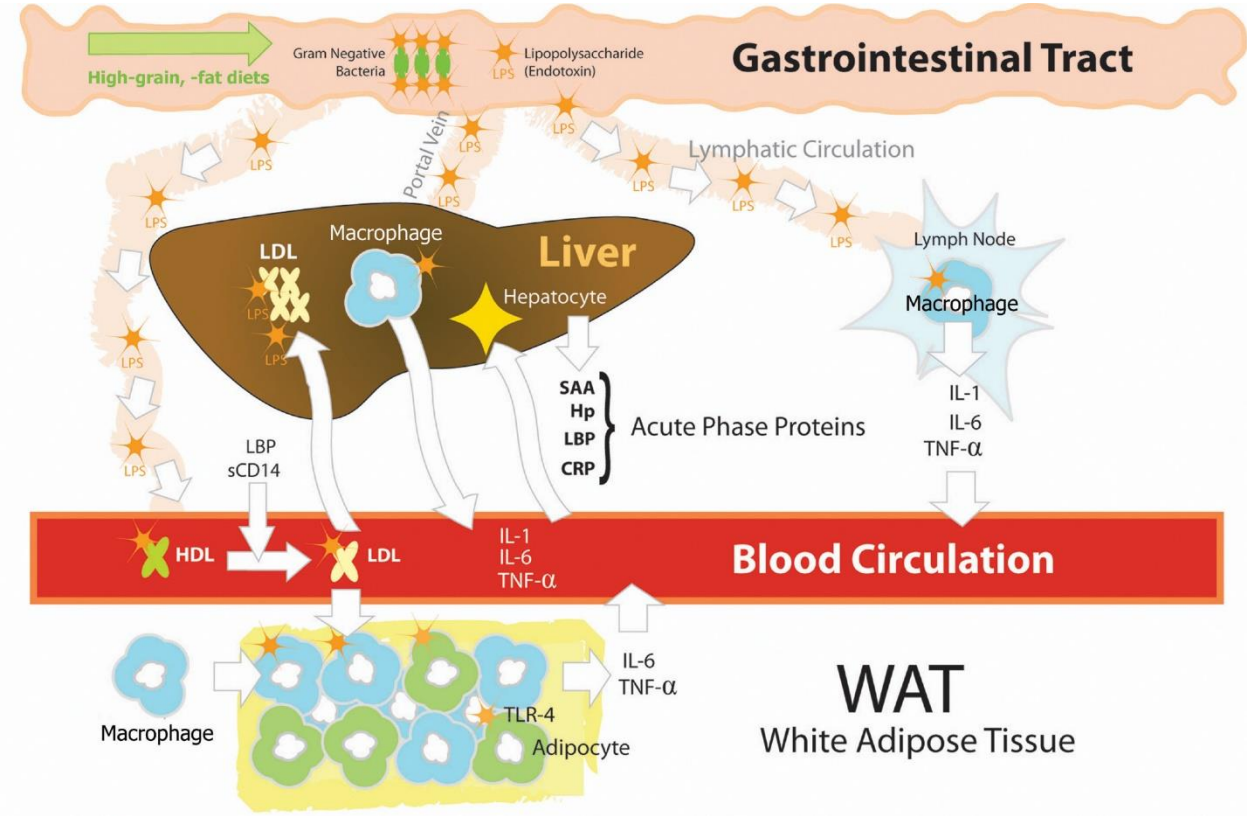

Figure 1.3. Three potential ways lipopolysaccharide (LPS) can enter into the host (Eckel and Ametaj, 2016).

CHAPTER 2. DETERMINATION AND EVALUATION OF OPTIMAL ASPIRIN DOSAGE TO INDUCE GASTROINTESTINAL TRACT BARRIER DYSFUNCTION IN FEEDLOT BEEF CATTLE

2.1 Abstract

Stress negatively affects gastrointestinal tract (GIT) barrier function, resulting in compromised animal health. Aspirin could be used as a model for GIT barrier dysfunction research. The objective of this study was to evaluate the effectiveness of aspirin to intentionally induce GIT barrier dysfunction in beef cattle using Cr appearance in urine as a measure of GIT permeability. In experiment 1, sixteen crossbred heifers (425.0 ± 8.6 kg) were enrolled in 2 experimental periods and allotted by BW to 0, 50, 100, or 200 mg/kg BW aspirin. Four heifers per treatment received the same aspirin dose during each period, which were separated by 4 wks. Heifers were fed a 49.4% corn silage, 50.6% concentrate diet. Aspirin was delivered to animals as an oral bolus. The 200 mg/kg BW aspirin treatment was dosed as 100 mg/kg BW aspirin 36 and 24 h prior to Cr-EDTA dosing (1 L; 180 mM). The 50 and 100 mg/kg BW aspirin treatments were dosed 24 h prior to Cr-EDTA dosing. Urine was collected every 3 h for 48 h and analyzed for Cr using atomic absorption spectrometry. Serum was collected at 0, 24, and 48 h and analyzed for lipopolysaccharide binding protein (LBP), interleukin-6 (IL-6), serum amyloid A (SAA), haptoglobin, and aspartate amino transferase (AST). In experiment 2, sixteen crossbred steers (576.0 ± 14.2 kg) fed a similar diet were allotted by BW to the 0 and 200 mg/kg BW aspirin treatments (8 steers/treatment) and were slaughtered 24 h after the last dose. Jejunal tissues were collected and tight junction mRNA expression was determined. Data were analyzed using the MIXED procedure of SAS. Aspirin linearly increased Cr absorption ($P = 0.02$) and elimination ($P = 0.04$) rate and linearly decreased mean retention time of Cr ($P = 0.02$). Aspirin tended to increase serum LBP ($P = 0.09$), but did not affect any other serum inflammatory markers ($P \geq 0.16$). Aspirin tended to increase jejunal claudin-1 mRNA expression ($P = 0.10$), but did not affect expression of other jejunal tight junction mRNA ($P \geq 0.20$). Results from this study indicate that aspirin disrupts GIT barrier function in beef cattle and has potential as a model in GIT permeability research.

2.2 Introduction

Gastrointestinal tract (GIT) barrier function can be negatively impacted by stress from weaning (Boudry et al., 2004; Moeser et al., 2007), feed restriction (Zhang et al., 2013; Wood et al., 2015; Pederzolli et al., 2018), heat (Baumgard and Rhoads, 2013; Pearce et al., 2013), acidosis (Emmanuel et al., 2007; Khafipour et al., 2009; Minuti et al., 2014), and transportation (Wan et al., 2014). Compromised GIT integrity, in turn, can lead to numerous diseases, such as laminitis (Vermunt, 1992; Nocek, 1997), liver abscesses (Lechtenberg et al., 1988; Nagaraja and Chengappa, 1998), acute interstitial pneumonia (Loneragan et al., 2001), and inflammation (Kvidera et al., 2017b). These diseases have been linked to bacteria and their components found in the digestive tract that leak through a compromised GIT, weakening the immune system, and potentially entering into circulation (Lambert, 2009). Some of the negative effects of stress can be mitigated with proper management; however, many stressors are challenging to mitigate with management alone. An understanding of how dietary and management decisions can affect GIT barrier function of ruminants is needed.

Currently, GIT barrier function is studied through is to inducing leaky gut by imposing a stress, such as transport, feed restriction, or heat, dose with various markers, and then measure marker concentration in urine as an indicator of gut permeability. However, imposing a stress is resource intensive, causes unwanted side effects for the animal (sickness, decreased growth), and there is large variation among animals in their response to stress. Indomethacin, an NSAID, is commonly used to induce leaky gut in laboratory animals (Anthony et al., 1993; Fang et al., 1977), humans (Bjarnason et al., 1986), and has been used in calves (Klein et al., 2007) in order to study leaky gut independent of other stressors. In addition, Kvidera et al., (2017b) intentionally induced leaky gut in dairy cattle with gamma secretase inhibitor (GSI), a drug that has the side effect of inducing goblet cell metaplasia (Milano et al., 2004) and disrupting crypt cell differentiation. However, because GSI and indomethacin are not FDA approved for animal use (FDA, 2020), animals studied cannot enter the food chain, which can increase research costs.

Aspirin (acetylsalicylic acid) is known to cause mucosal injury leading to increased gut permeability and tight junction damage (Oshima et al., 2008) and has already been used as a model to induce leaky gut in laboratory animals and humans (Lambert et al., 2012). Aspirin is commonly used as an analgesic in cattle; the maximum recommended dosage is 100 mg/kg every 12 h (Gingerich et al., 1975) with a 24-h meat and milk withdrawal period (Smith et al., 2008). Aspirin

is a non-steroidal anti-inflammatory drug (NSAID) that covalently binds the active site of cyclooxygenase (COX)-1 and COX-2 isoenzymes (Simmons et al., 2004). Cyclooxygenase is used for the production of prostaglandin from arachidonic acid, and prostaglandins are involved with mucosal protection and repair (Takeuchi and Amagase, 2018). Based on the known mucosal injury in non-ruminants, the objective of this study was to determine the optimal dosage of aspirin that causes GIT barrier dysfunction. We hypothesized that aspirin would cause mucosal damage, inflammation, and GIT dysfunction in beef cattle, resulting in increased GIT permeability.

2.3 Materials and Methods

All procedures performed in this study were approved by the Purdue University Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010). The experiment was conducted at the Purdue University Animal Sciences Research and Education Center (ASREC) in West Lafayette, IN.

2.3.1 Experiment 1

2.3.1.1 Animals and Sampling

Sixteen Angus x Simmental heifers were enrolled into 2 experimental periods to determine the effect of 0, 50, 100, and 200 mg/kg BW aspirin on Cr appearance in urine as a measure of GIT leakiness. Four heifers were allotted by BW and breed composition to each treatment and each heifer received the same aspirin dose during each period, which were separated by 4 wk. The mean BW at the start of each period was 401.0 ± 37.1 kg and 448.0 ± 33.7 kg, respectively and heifers were approximately 10 mo. old. The basal diet (Table 1) was formulated on a DM basis to meet or exceed NASEM (2016) requirements for CP, vitamins, and minerals and consisted of corn silage (49.4%), corn (24.7%), DDGS (24.7%), and vitamin/mineral supplement (1.2%). Diets were fed for 6 wks before the first experimental period in an open-sided barn with straw bedded pens (3.4 x 9.1 m) over a concrete floor. Feed was offered once daily at 0900 h, and heifers were allowed *ad libitum* access to feed and water. Daily feed deliveries were adjusted using a 4-point bunk scoring system (Pritchard, 1993) to allow for *ad libitum* feed intake with little or no accumulation of unconsumed feed. Scales (480 Legend, Rice Lake Weighing Systems, Rice Lake, WI) weighed to

the nearest 0.5 kg and were checked for accuracy at each weigh date. Aspirin (acetylsalicylic acid; 480 grain boluses; Agri Laboratories, Ltd. St. Joseph, MO) was dosed orally using a balling gun. Heifers receiving the 50 and 100 mg/kg treatments were dosed with aspirin 24 h prior to administering Cr-EDTA and collecting urine. Because 100 mg/kg BW is the maximum dosage for cattle (Gingerich et al., 1975), heifers receiving the 200 mg/kg treatment were dosed with 100 mg/kg aspirin, 36 and 24 h prior to the urine collection procedure.

Twenty-four h after aspirin dosing, the 16 heifers were restrained in a working chute and given 1 L of a 180 mM Cr-EDTA solution using an esophageal tube. Chromium-EDTA was prepared according to Binnerts et al. (1968). A Foley urinary catheter (C. R. Bard, Inc., Covington, GA) was inserted into the bladder and closed. Heifers were moved to individual stalls (1.1 x 2.1 m), the urinary catheter line was opened, attached to 4 L drainage bags (Medline, Northfield, IL), and fixed to the stall using string. Urine weight was recorded, and a 45 mL subsample was collected every 3 h and frozen at -20°C for subsequent analysis of Cr. During the sampling period, grass hay and water were provided *ad libitum* for all animals.

2.3.1.2 Cr Analysis

Approximately 2.5 mL of urine was digested in duplicate with 5 mL of nitric and 3 mL of perchloric acid at a temperature of 175°C (Sandell, 1959). Each sample was allowed to cool to room temperature, diluted to 30 mL with nanopure water and analyzed for chromium by atomic absorption spectroscopy at 425.4 nm (SpectrAA 220 FS, Varian, Inc., Palo Alto, CA) according to the procedures of Vicente et al. (2004). Absorption and elimination rates were determined using a one-compartment model as described by Atkins (1969). Elimination rate (k_1, h^{-1}) of Cr was calculated by fitting a linear regression to the natural logarithm of Cr concentration in the urine (mg/L) against sampling time (h) during the terminal phase, after maximum concentration was achieved. Absorption rate (k_2, h^{-1}) of Cr was calculated by fitting a linear regression to the natural logarithm of the residual of Cr concentration in urine against sampling time (h) prior to the terminal phase. Mean retention time was estimated according to Grovum and Phillips (1973).

2.3.1.3 Blood Analysis

Approximately 10 mL of blood was collected from the jugular vein at 0, 24, and 48 h after Cr-EDTA dosing into tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Blood samples were centrifuged at 1,250 x g for 20 min and serum was collected in three aliquots within 2 h of collection. Serum was stored in 5 mL polystyrene tubes and frozen at -20°C until analysis. Serum concentrations of interleukin-6 (Abcam, ab205280, Cambridge, UK), lipopolysaccharide binding protein (LSBio, LS-F7412, Seattle, WA), serum amyloid A (Tridelta Development Ltd., TP 802, Maynooth, County Kildare, IE), and haptoglobin (ICL, E-10HPT, Inc., Portland, OR) were analyzed using enzyme-linked immunosorbent assays (ELISA) kits at 450 nm on a Spark 10M plate reader (Tecan Life Sciences, Männedorf , Zürich, Switzerland). Bovine aspartate aminotransferase concentrations in serum were determined using a colorimetric kit (Sigma-Aldrich, MAK055, St. Louis, MO) at 450 nm with the previously mentioned plate reader. Intra-assay CVs for LBP, IL-6, haptoglobin, SAA, and AST are 5.70, 6.04, 3.47, 4.66, and 5.24, respectively, and inter-assay CVs for LBP, IL-6, haptoglobin, SAA, and AST are 18.97, 12.31, 12.81, 8.97, and 3.42, respectively.

2.3.1.4 Statistical Analysis

Data were analyzed as a completely randomized design using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with individual animal considered the experimental unit. Serum metabolite concentrations and urine Cr concentration were analyzed as repeated measures over time. The model included the random effect of individual and the fixed effect of treatment, time, and the treatment × time interaction. Four covariance structures were compared for each variable and the structure that yielded the lowest Bayesian Information Criterion was used for all results. The SLICE function of SAS was used to determine the simple effect of aspirin within time. Urine Cr kinetics were analyzed using the MIXED procedure of SAS without repeated measures. The model included the random effect of individual and the fixed effect of treatment. Linear and quadratic coefficients were generated with the IML procedure of SAS and used to determine the dose-dependent effect of aspirin inclusion. Treatment comparisons were made using Fisher's protected least significant difference, and the least square means statement was used to calculate

adjusted means. Differences were considered significant when $P \leq 0.05$ and $0.05 < P \leq 0.10$ was considered a tendency.

2.3.2 Experiment 2

2.3.2.1 Animals and Diets

Sixteen crossbred steers (566 ± 29.7 kg) purchased from a commercial auction market were used to determine the effect of aspirin on tight junction gene expression in the jejunum. Eight steers per treatment were allotted by BW to either 0 or 200 mg/kg of BW aspirin. Body weight was determined the day prior to dosing aspirin. Steers were housed in a slatted floor finishing barn in 6.1 x 3.7 m pens with 0.55 m of bunk space and adjustable curtains. Cattle were fed a basal diet (DM basis) that consisted of 50% corn silage, 21% corn, 23% dried distillers grains with solubles, and 6% vitamin/mineral premix for 6 wks before slaughter. The basal diet met or exceeded NASEM (2016) requirements for protein, vitamins, and minerals. Feed was offered once daily at 0900 h, and steers were allowed *ad libitum* access to feed and water. Daily feed deliveries were adjusted using a 4-point bunk scoring system (Pritchard, 1993) to allow for *ad libitum* feed intake with little or no accumulation of unconsumed feed. Scales (480 Legend, Rice Lake Weighing Systems, Rice Lake, WI) weighed to the nearest 0.5 kg and were checked for accuracy at each weigh date. Because 100 mg/kg BW is the prescribed maximum dosage for cattle (Gingerich et al., 1975), steers given aspirin received two doses of 100 mg/kg of BW at 36 and 24 h prior to slaughter. Aspirin boluses (Agri Laboratories, Ltd. St. Joseph, MO) were 480 grain and were dosed orally using a balling gun.

2.3.2.2 Sample Collection

Cattle were slaughtered at the Purdue Animal Science meat laboratory, and samples were collected from the jejunum within 30 min after exsanguination according to the procedures described by Lindholm-Perry et al. (2016). Approximately 0.5 g of mucosa layer was scraped and flash frozen in liquid nitrogen, then stored at -80°C for evaluation of tight junction mRNA expression. Genes evaluated were occludin, zonula occludens, claudin 1, claudin 2, and claudin 3. Total mRNA was isolated using the RNeasy Mini kit (Qiagen; Valencia, CA) according to the manufacturer's protocol. Quantity was determined using a Nanodrop Spectrophotometer and a

RNA integrity number (RIN) was obtained using Bioanalyzer RNA chips. Primers and Taqman assay kits were purchased from Thermo Fisher Scientific (Waltham, MA). A total of 300 ng of RNA was used to synthesize cDNA and each sample and was diluted 1:10 for RT-PCR analysis. Each reaction contained 5 ul of diluted cDNA, 4 ul of water, 1 ul primer, and 10 ul Taqman Fast Advanced Master Mix. Samples were run in triplicate on a Fast 7500 real-time PCR system (Applied Biosystems), according to manufacturer's instructions. Gene expression was calculated relative to GAPDH as a housekeeping gene. Fold expression was calculated using the delta delta Ct method.

2.3.2.3 Statistical Analysis

Data were analyzed as a completely randomized design, using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with individual animal considered the experimental unit. The model included the random effect of individual and the fixed effect of treatment. Body weight was used as a covariate in the statistical analysis. Treatment comparisons were made using Fisher's protected least significant difference, and the least square means statement was used to calculate adjusted means. Differences were considered significant when $P \leq 0.05$ and $0.05 < P \leq 0.10$ was considered a tendency.

2.4 Results and Discussion

2.4.1 Chromium appearance in urine

Urinary Cr excretion increased linearly at h 3, 6, and 12 ($P < 0.01$) as aspirin dose increased from 0 to 200 mg/kg (Figure 2.1) and tended to increase linearly at h 9 ($P = 0.06$). No differences were detected at any other time point ($P \geq 0.20$). Absorption rate (%/hr) and elimination rate (%/hr) increased linearly ($P = 0.02$), and mean retention time decreased linearly ($P = 0.02$) as aspirin dosage increased (Table 2.2). Dosing with chromium-EDTA is a well-established technique for measuring GIT barrier function *in vivo* in non-ruminants (Bjarnason, 1986, Hollander, 1992, Eutamene et al., 2007) and ruminants (Schweigel et al., 2005, Zhang et al., 2013a). Other markers that are commonly used in non-ruminants, such as dextran or mannitol (Wang et al., 2015), cannot be used *in vivo* in ruminants, because rumen microbes ferment and metabolize these carbohydrates. Chromium-EDTA is not digested or metabolized, has a high renal clearance rate (Bjarnason et al.,

1986), permeates the GIT through the paracellular pathway, and appears in the urine (Schweigel et al., 2005, García-Lafuente et al., 2001, Ten-Bruggencate, 2006). In healthy ruminants, approximately 2.5 percent of ruminally dosed Cr-EDTA is absorbed and excreted through urine (Shingfield et al., 2008). The mass of an endotoxin is 2-70 kDa (Magalhães et al., 2007); whereas, Cr-EDTA has the mass of 340 Da and the size of approximately 10 Å (García-Lafuente et al., 2001). Thus, Cr-EDTA is an indicator of gut barrier dysfunction, not necessarily of bacteria or endotoxin translocation. Increased absorption and elimination rates of Cr-EDTA caused by aspirin, as shown in the present study, indicate that aspirin causes GIT leakiness (and potentially damage).

2.4.2 Blood Assays

Lipopolysaccharide binding protein tended to increase ($P = 0.09$) in cattle dosed with 200 mg/kg aspirin treatment compared to cattle dosed with no aspirin (Table 2.3), indicating that lipopolysaccharide (LPS) may have entered into circulation due to compromised gut integrity. However, interleukin-6 (a pro- and anti-inflammatory cytokine), haptoglobin and SAA (acute phase proteins), and AST (a marker of liver stress), did not differ between the 0 and 200 mg/kg treatments ($P \geq 0.25$). The role of LBP is to directly bind with LPS or lipoteichoic acid (LTA) to further signal for opsonization by phagocytes (Ceciliani et al., 2012; Lu et al., 2008). Both LPS and LTA are components of bacterial cell walls that are recognized as endotoxins, meaning they have toxic effects to the host after they are shed from lysed bacteria. The phospholipid membranes in gram negative bacteria are glucosamine-based LPS (Raetz and Whitfield, 2002), whereas the phospholipid membranes of gram positive bacteria are composed of LTA. Endotoxins are normally present in a large amount in the rumen and in the hindgut of ruminants. The amounts of LPS will rise when ruminants are fed with diets rich in easily digestible carbohydrates, because Gram-negative bacteria experience growth benefits from such diets and thereby increased LPS shedding (Plaizier et al., 2012). High-grain feeding has been connected to increased levels of free LPS in rumen fluid and acute phase proteins in peripheral circulation (Khafipour et al., 2009a). The presence of LPS in the portal vein initiates a cascade of immune signaling, which results in inflammation and the production of acute phase proteins (Ceciliani et al., 2012; Lu et al., 2008). Kvidera et al. (2017b) connected increased circulating LBP to decreased jejunum villus height-to-crypt depth ratio in lactating dairy cows, suggesting that an increase in LBP is correlated with greater intestinal damage and decreased barrier function. When Kvidera et al. (2017b) intentionally

induced GIT barrier dysfunction with GSI, they did not see a difference in SAA or haptoglobin, which aligns with the results from this study. In the liver, acute phase protein production is a secondary (non-local) response to toxic stimuli, and this production of acute phase proteins can be an indicator of systemic inflammation (Ceciliani et al., 2012). The roles of SAA and haptoglobin are broader than LBP when combatting infection (Ceciliani et al., 2012), which could explain why a tendency was observed in LBP, but not for SAA or haptoglobin in the current study.

Although IL-6 was not affected by aspirin administration in the current study, its nearly 50% decrease in heifers fed aspirin suggests less inflammation. Zhang et al. (2016) observed an increase in mRNA expression of IL-1β, IL-2, IL-6, and IL-8 and TNF-α in rumen epithelial tissue isolated from the stratum basale and spinosum when LPS was introduced to these tissues. Aspirin is an NSAID that is known to decrease inflammation; thus, a decrease in IL-6 concentrations are not surprising. Non-steroidal anti-inflammatory drugs have anti-inflammatory effects, because they decrease prostaglandin (PG) E_2 and I_2 production by inhibiting COX 1 and COX 2 isozymes (Simmons et al., 2004). These PGs are associated with producing pain and inflammation; however, they also induce protective mucus production and provide mucosal repair (Takeuchi and Amagase, 2018). Anti-inflammatory drugs inhibit the negative effects associated with PG production, but also the beneficial effects of PGs. However, frequent or heavy use of aspirin in humans is known to cause GIT barrier dysfunction (Bjarnason et al., 1986; Lambert et al., 2012) and ulceration (Bjarnason et al., 1987). Bjarnason et al. (1987) discovered that in humans, inflammation from NSAID use can persist for up to 16 mo. after discontinuation. Gastrointestinal tract dysfunction and ulceration causes an immune response, which is signaled by inflammation. Thus, the use of NSAIDs can eventually cause small intestinal inflammation and associated blood and protein loss (Bjarnason et al., 1993).

2.4.3 Tight Junction mRNA Expression

In the present study, claudin 1 expression tended ($P = 0.10$) to increase in the jejunum in cattle dosed with 200 mg/kg aspirin compared to cattle not dosed with aspirin (Table 2.4). However, occludin, zonula occludens, claudin 1, and claudin 2 expression in the jejunum did not change ($P \geq 0.51$). Regulation of paracellular movement and protection against luminal bacteria and endotoxins are the functional roles of tight junction proteins (Lee et al., 2018). Each tight junction protein has a specific role in the intestinal tract. Claudins 1, 3, 4, 5, 8, 9, 11, and 14

decrease paracellular permeability; whereas, claudins 2, 7, 12, and 15 increase paracellular permeability (Robinson et al., 2015). In a human gastric epithelial cell line, aspirin increased permeability and downregulated claudin 7 (Oshima et al., 2008). In contrast, aspirin downregulated ZO-1 and occludin expression in mice (Lai et al., 2015). Increased claudin-1 mRNA expression in the current study indicates that tight junction proteins may have been damaged and were being replaced. In dairy cattle, ruminal claudin 1 and 4 gene expression were upregulated 24 h after acidosis was induced (McCann et al., 2016). Pederzolli et al. (2018) restricted feed intake to 25 percent of DMI for 4 d in Holstein steers and observed that claudin 1, occludin, ZO-1, ZO-2, and toll-like receptor 4 expression in the rumen and jejunum increased. Acidosis and feed restriction cause intestinal lining deterioration, which is indicated by upregulation in tight junction proteins (McCann et al., 2016, Pederzolli et al., 2018). Although the deterioration of the intestinal lining during acidosis and feed restriction seem to be caused by acidic conditions (Emmanuel et al., 2007) or the presence of LPS (Chin et al., 2006), similar GIT damage in the current study when dosing feedlot cattle with aspirin, indicates that aspirin may be a good model to study diet induced leaky gut.

2.5 Conclusion

Administration of 200 mg/kg BW of aspirin is the optimal dosage to induce leaky gut based on the amount of Cr excreted in urine and the absorption and elimination kinetics of Cr. Tendencies for increased serum concentration of LBP and upregulation of claudin 1 in the jejunum further supports the idea that aspirin can induce leaky gut and be used as a model to further study leaky gut in healthy beef cattle. With the ability of the aspirin model to induce leaky gut and still allow animals to enter into human consumption, research costs are lessened which may allow for further research to be conducted. Additional research is needed to determine the effects of leaky gut on feedlot cattle production, and the aspirin model could be used to further this area of study.

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2.7 Tables and Figures

Table 2.1 Basal diet for heifers

	Experiment 1	Experiment 2
Corn silage	49.4	50.0
Dried distillers grains with solubles	24.7	23.0
Corn	24.7	21.0
Vitamin/mineral supplement	1.2[1]	6.0[2]
Diet composition, DM basis[2]		
Crude protein, %	12.9	12.9
NEm, Mcal/kg[3]	2.12	2.07
NEg, Mcal/kg[3]	1.26	1.21
Calcium, %	1.00	1.19
Phosphorus, %	0.48	0.39
Sulfur, %	0.26	0.24

[1]Vitamin/mineral supplement contained: 19.94% salt, 20.05% Ca, 11.97% Cl, 8.04% Na, 5.03% P, 3.00% Fe, 2.004% Mn, 2.003% Zn, 1.26% Mg, 0.803% Cu, 0.70% S, 0.26% K, 0.05% Co, 0.05% I, 0.01% Se, 678,000 IU vitamin A/kg, and 1547 IU of vitamin E/kg of premix.

[2]Vitamin/mineral supplement contained (DM basis): 18.25% Ca, 1.32% K, 0.44% Mg, 0.18% S, 563.91 ppm Zn, 522.90 ppm Fe, 440.41 ppm Mn, 183.33 ppm Cu, 9.66 ppm I, 4.48 ppm Se, 3.43 ppm Co, 42.19 IU/g vitamin A, 4.98 IU/g vitamin D, 0.155 IU/g vitamin E, 413.6 ppm monensin (176.4 g/kg, Elanco Animal Health, Indianapolis, IN).

[3]Analyzed at Cumberland Valley Analytical Services (Waynesboro, PA)

[4]Calculated based on NASEM (2016)

Table 2.2. Kinetics of chromium appearance in urine, 24 h after an aspirin dose

	Aspirin dose, mg/kg of BW[1]					P-value	
	0	50	100	200	SE	Linear	Quadratic
Absorption rate, K_2 (%/hr)	0.227	0.256	0.264	0.289	0.0161	0.02	0.62
Elimination rate, K_1 (%/hr)	0.063	0.064	0.077	0.081	0.0082	0.02	0.67
Mean retention time (hr)	24.71	21.16	18.73	17.33	2.02	0.02	0.34

[1]0 = no aspirin; 50 = 50 mg/kg of BW of aspirin; 100 = 100 mg/kg of BW of aspirin; 200 = 200 mg/kg of BW of aspirin

Table 2.3. The effect of aspirin on serum inflammatory markers

	Treatment[1]			P-value	
	0 mg/kg	200 mg/kg	SEM	TRT[2]	TRT[2] x time
LPS[3] binding protein, ug/mL	22.7	37.3	4.22	0.09	0.49
Interleukin-6, pg/mL	83.0	42.4	50.77	0.65	0.16
Haptoglobin, ug/mL	7.69	9.87	5.044	0.80	0.20
Serum amyloid A, ug/mL	58.8	78.7	9.08	0.25	0.51
Aspartate aminotransferase, mU/mL	15.6	15.2	1.26	0.86	0.96

[1]0 = no aspirin; 50 = 50 mg/kg of BW of aspirin; 100 = 100 mg/kg of BW of aspirin; 200 = 200 mg/kg of BW of aspirin

[2]TRT = treatment

[3]LPS = Lipopolysaccharide

Table 2.4. Effect of aspirin on fold-change mRNA expression of tight junction proteins in the jejunum

	Treatment[1]			
Item	0 mg/kg	200 mg/kg	SEM	P-value
Occludin	1.00	0.82	0.274	0.64
Zonula Occludens	0.99	0.85	0.173	0.97
Claudin 1	0.98	1.47	0.270	0.10
Claudin 2	0.97	1.29	0.342	0.51
Claudin 3	1.00	0.76	0.270	0.56

[1]0 = no aspirin; 50 = 50 mg/kg of BW of aspirin; 100 = 100 mg/kg of BW of aspirin; 200 = 200 mg/kg of BW of aspirin

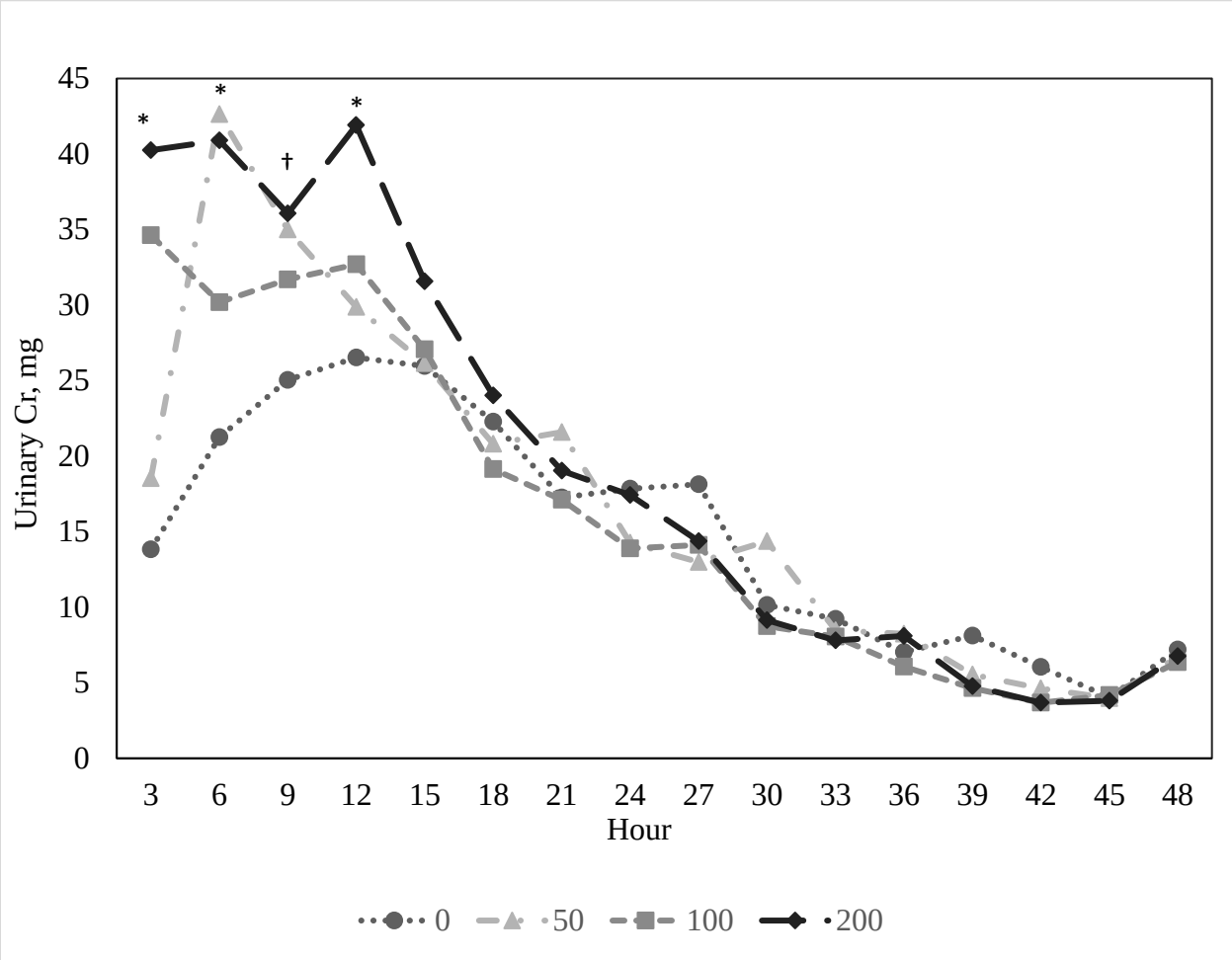

Figure 2.1. The effect of aspirin on chromium (mg) appearance in urine. * Indicates a linear effect as aspirin dose increased ($P \leq 0.05$). † Signifies a tendency for a linear increase as aspirin dose increased ($0.05 < P \leq 0.10$). 0 = no aspirin; 50 = 50 mg/kg of BW of aspirin; 100 = 100 mg/kg of BW of aspirin; 200 = 200 mg/kg of BW of aspirin. SE = ± 3.314

CHAPTER 3. EFFECT OF INTENTIONALLY INDUCED LEAKY GUT USING ASPIRIN ON PERFORMANCE AND CARCASS QUALITY OF FEEDLOT BEEF CATTLE

3.1 Abstract

The negative impacts of stress on the gastrointestinal (GIT) barrier function can result in compromised animal health. The objective of this study was to determine the long-term impact of leaky gut on animal physiology. In this experiment, 96 Simmental x Angus steers (355.0 ± 14.8 kg) were allotted by body weight and breed into two treatments: control (no aspirin); and aspirin fed at 50 mg/kg/d. Steers were housed in 16 pens (8 pens/treatment) with 6 steers in each pen. Weight was recorded monthly and blood serum was collected on d 159 to be analyzed for lipopolysaccharide binding protein (LBP), interleukin-6 (IL-6), serum amyloid A (SAA), haptoglobin, and aspartate aminotransferase (AST). Data were analyzed using the MIXED procedure of SAS. Average daily gain (ADG) tended to decrease in cattle fed aspirin ($P = 0.10$). Aspirin decreased hot carcass weight ($P = 0.05$) and rib-eye area ($P = 0.01$) and increased fat thickness ($P = 0.02$), marbling score ($P = 0.003$), and yield grade ($P = 0.01$). Percent KPH tended to increase ($P = 0.10$) for steers fed aspirin. Aspirin had no effect on body weight, dry matter intake, gain:feed, days on feed, dressing percentage, liver abscess score, or percent liver abscesses. Aspirin tended to increase serum LBP ($P = 0.07$), but had no effect on serum concentrations of IL-6, haptoglobin, SAA, and AST ($P \geq 0.30$). This study indicates that aspirin induced leaky gut has negative impacts on feedlot performance and carcass leanness. The negative impact of aspirin induced leaky gut on animal performance suggests that leaky gut caused by other factors (subacute acidosis, stress) may be a significant problem for the feedlot industry.

3.2 Introduction

Stress, such as what can occur from weaning, feed restriction, transportation, and acidosis can negatively impact gastrointestinal tract (GIT) barrier function. Diseases that arise from GIT dysfunction decrease efficiency and potentially increase mortality, thus negatively impacting producer profitability. Liver abscesses occur in 12-32 percent of feedlot cattle (Nagaraja and Lechtenberg, 2007), which cause carcass losses of about $38 to $52/hd (Brown and Lawrence,

2010; Reinhardt and Hubbert, 2015). Heat stress alone can cause a financial burden of about $300 M/yr for the beef industry (St. Pierre et al., 2003). Acute interstitial pneumonia has been reported to have a mortality rate in feedlots of 0.03 to 0.15 percent of received cattle (Vogel et al., 2015). In addition, the GIT and the lung are connected through the mucosal immune system (mucosal associated lymphatic tissue), thus pulmonary inflammation can be driven by gut microbiota (He et al., 2012). Bovine respiratory disease (BRD) accounts for 70-80 percent and 40-50 percent of all feedlot morbidity and mortality, respectively (Hilton, 2014). Costs associated with BRD lessens the value of cattle from $23.23 to $151.18 when compared to healthy cattle (Smith, 2009).

The activated immune system is energy demanding; more than a kilogram of glucose was reported to be used in 720 min in a Holstein cow (Kvidera et al., 2017a). The immune system's energy demand adds to the maintenance requirement for cattle, thus, pulling energy away from growth and production (NASEM, 2016). The exact repercussions of long-term leaky gut and maintenance of an activated immune system on beef cattle performance are currently unknown. Aspirin (acetylsalicylic acid) is known to cause mucosal injury leading to increased gut permeability and tight junction damage (Oshima et al., 2008). Aspirin is commonly used as an analgesic in cattle; the maximum recommended dosage is 100 mg/kg every 12 h (Gingerich et al., 1975) with a 24 h meat and milk withdrawal period (Smith et al., 2008). Dietary administration of 50 to 100 mg/kg BW aspirin is a safe and effective method to intentionally induce leaky gut in cattle (Chapter 2). Thus, our objective was to determine the impact of leaky gut on animal physiology, growth, and production. We hypothesized that long-term administration of 50 mg/kg BW aspirin will compromise GIT barrier function, leading to inflammation and immune system activation that will negatively impact animal performance and carcass characteristics.

3.3 Materials and Methods

All procedures performed in this study were approved by the Purdue University Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010). The experiment was conducted at the Purdue University Animal Sciences Research and Education Center (ASREC) in West Lafayette, IN.

3.3.1 Animals and Diets

Ninety-six Angus x Simmental steers (355.0 ± 14.8 kg) were used to determine the effect of intentionally induced leaky gut on cattle performance and carcass characteristics. Leaky gut was induced by daily administration of 50 mg/kg BW of aspirin (chapter 2) over a 159-d period and was compared to a control diet of no aspirin inclusion. Steers were allotted to 16 pens (8 pens/treatment; 6 animals/pen; 48 animals/treatment) based on breed (% Simmental), body weight, and source (Animal Science Research and Education Center or Feldun Purdue Agricultural Center). Pens (6.1 x 3.7 m) were located in a slatted floor, curtain-sided finishing barn and provided 0.55 m of bunk space. Control diets (Table 1) contained 50% corn, 24% dried distiller's grains with solubles (DDGS), 20% corn silage, and 6% vitamin/mineral supplement (DM basis). Aspirin was delivered to steers in a corn/DDGS premix (38% corn, 57% DDGS, 5% aspirin) that replaced a portion of DDGS and corn in the diet. Diets were formulated to meet or exceed NASEM (2016) requirements for CP, vitamins, and minerals. Feed was offered once daily at 0900 h, and steers were allowed *ad libitum* access to feed and water. Daily feed deliveries were adjusted using a 4-point bunk scoring system to allow for *ad libitum* feed intake with little or no accumulation of feed (Pritchard, 1993). Feed delivery was recorded daily, and feed samples were collected every two wks for DM analysis. A subsample was taken, composited, and analyzed by wet chemistry for CP, NDF, P, and Ca (Cumberland Valley Analytical Services, Waynesboro, PA).

Upon entry into the feedlot, steers were vaccinated against Infectious Bovine Rhinotracheitis, Bovine Viral Diarrhea Types I and II, Parainfluenza-3, Bovine Respiratory Syncytial Virus, *Mannheimia haemolytica,* and *Pasturella multocida* (Vista Once, Merck Animal Health, Summit, NJ), against *Clostridia* and *Haemophilus somnus* (Vision-7 Somnus; Merck Animal Health), treated with a pour-on (Cydectin, Bayer, Shawnee Mission, KS) for external parasites, and drenched with a de-wormer (Safeguard, Merck Animal Health, Madison, NJ) for internal parasites. Steers were given booster vaccines 4 wks later. Steers were weighed twice on consecutive days at the initiation and termination of the study and weighed once monthly to monitor growth and health. Steers were weighed prior to feeding with scales (480 Legend, Rice Lake® Weighing Systems, Rice Lake, WI) that weighed to the nearest 0.5 kg. Scales were checked for accuracy at each weigh date. Steers were implanted with Synovex-ONE Feedlot (200 mg of testosterone and 28 mg of estradiol benzoate; provided courtesy of Zoetis Animal Health, Kalamazoo, MI) at feedlot entry. Heavier pens of steers were weighed every other wk as pen body

weights approached 548 kg. Pens of steers that achieved an average body weight of approximately 548 kg were fed 300 mg of Optaflexx (ractopamine hydrochloride; provided courtesy of Elanco, Greenfield, IN) daily during the last 42 d before slaughter. Performance data was analyzed for the first half (d 0 to 85), second half (d 86 to 159), and overall (d 0 to 159).

Steers were transported 400 km to a USDA inspected commercial abattoir (Tyson Foods Inc., Joslin, IL) and slaughtered at 3 different time points (147, 161 and 175 d) according to when 42 d of Optaflexx feeding was achieved (average pen BW of 617 ± 35.2 kg). Final body weights were not pencil shrunk. One steer was selected based on pen average BW for slaughter at Purdue University. Hot carcass weight was recorded immediately after evisceration. Carcasses were chilled for 24 h, and qualified University personnel measured subcutaneous fat thickness between the 12th and 13th rib, longissimus dorsi area via direct grid reading between the 12th and 13th rib, kidney-pelvic-heart fat as a percent of HCW, marbling score, and USDA quality and yield grades (USDA, 1997).

3.3.2 Blood Analysis

Approximately 10 mL of blood was collected from the jugular vein into tubes (Becton Dickinson, Franklin Lakes, NJ) one d prior to shipping to slaughter. Within 2 h of collection, blood samples were centrifuged at 1,250 x g for 20 min. Serum was harvested, separated into three aliquots, and frozen at -20°C until analysis of lipopolysaccharide binding protein (LBP), interleukin-6 (IL-6), haptoglobin, serum amyloid A (SAA), and aspartate aminotransferase (AST). Serum concentrations of LBP (LSBio, LS-F7412, Seattle, WA), IL-6 (abcam, ab205280, Cambridge, UK), SAA (Tridelta Development Ltd., TP 802, Maynooth, County Kildare, IE), and haptoglobin (ICL, E-10HPT, Inc., Portland, OR) were extracted using commercial ELISA kits according to manufacturer instructions and measured at 450 nm on a Spark 10M plate reader (Tecan Life Sciences, Männedorf , Zürich, Switzerland). Concentrations of bovine AST (Sigma-Aldrich, MAK055, St. Louis, MO) were determined using a colorimetric assay kit at 450 nm with the previously mentioned plate reader. Lipopolysaccharide binding protein, IL-6, haptoglobin, SAA, and AST inter-assay CVs are 18.97, 12.31, 12.81, 8.97, and 3.42, respectively. In addition, LBP, IL-6, haptoglobin, SAA, and AST intra-assay CVs are 5.70, 6.04, 3.47, 4.66, and 5.24, respectively.

3.3.3 Statistics

Data were analyzed as a completely randomized design using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with pen considered the experimental unit. Performance and serum data were analyzed as repeated measures over time. The model included the random effect of pen and the fixed effect of treatment, time, and the interaction of treatment and time. Four covariance structures were compared for each variable and the structure that yielded the lowest Bayesian Information Criteria was used for all results. The SLICE function of SAS was used to determine the simple effects of aspirin within time and the simple effects of aspirin within time are presented in the results section. Overall performance data and carcass data were analyzed as a completely randomized design using the MIXED procedures of SAS without repeated measures. The model included the random effect of pen and the fixed effect of treatment. Treatment comparisons were made using Fisher's protected least significant difference, and the least square means statement was used to calculate adjusted means. Differences were considered significant when $P \leq 0.05$ and $0.05 < P \leq 0.10$ was considered a tendency.

3.4 Results and Discussion

Aspirin inhibits the COX-1 and COX-2 isoenzymes by covalently modifying their active sites (Simmons et al., 2004). This modification leads to decreased production of prostaglandin (PG) E_2 and PGI_2 (Simmons et al., 2004). These PGs are responsible for stimulating mucus production that protects and heals gastric ulcers and small intestinal lesions (Takeuchi and Amagase, 2018). Our results from chapter 2 indicate that short term administration of aspirin increased turnover of jejunal claudin-1, increased the appearance of Cr in the urine, and increased serum LBP indicating increased GIT leakiness. Results from the performance study are presented in Table 3.2. First half average daily gain did not differ between treatments ($P = 0.61$). Second half and overall average daily gain (ADG) tended to decrease for steers fed 50 g/kg BW aspirin compared to steers fed 0 mg/kg ($P \leq 0.10$), but no difference in BW occurred ($P \geq 0.21$). There were no differences between treatments for DMI, gain:feed, or days on feed ($P \geq 0.15$). The tendency for a decrease in second half and overall ADG indicates that long-term administration of aspirin had a negative effect on growth, which could be explained by leaky gut. The production losses could be accounted for by an activated immune system, which is energetically costly.

Kvidera et al. (2017a) determined that the immune system will use more than a kilogram of glucose in 720 min when acutely activated in Holstein cows.

There were no differences between treatments for serum concentrations of interleukin-6, haptoglobin, serum amyloid A, or aspartate aminotransferase ($P \geq 0.30$) as depicted in Table 3.3. Aspirin tended to increase lipopolysaccharide binding protein in serum ($P = 0.07$), indicating that aspirin induced leaky gut and caused increased endotoxin to reach circulation. The increase in serum concentrations of LBP in cattle fed aspirin suggest that greater amounts of lipopolysaccharide (LPS) crossed the GIT barrier compared to cattle not fed aspirin. Lipopolysaccharide binding protein's role is to signal for opsonization by phagocytes after binding with lipoteichoic acid (LTA) or LPS (Ceciliani et al., 2012; Lu et al., 2008). Both LPS and LTA are components of bacterial cell walls that are recognized as endotoxins, meaning they have toxic effects to the host after they are shed from lysed bacteria. The phospholipid membranes in gram negative bacteria are glucosamine-based LPS (Raetz and Whitfield, 2002), whereas the phospholipid membranes of gram positive bacteria are composed of LTA. Increases in circulating LBP have been connected to a decrease in jejunum villus height-to-crypt depth ratio in lactating dairy cows, suggesting greater intestinal damage and decreased barrier function (Kvidera et al., 2017b). A similar increase in LBP as a result of aspirin supplementation in feedlot heifers was noted in chapter 2. The presence of LPS in the portal vein initiates a cascade of immune signaling, which results in production of inflammatory cytokines and acute phase proteins, including LBP (Ceciliani et al., 2012; Lu et al., 2008). Thus, increased total (bound and unbound) LBP could indicate an increase in LPS. Zhang et al. (2016) observed that rumen epithelial tissues isolated from the stratum basale and spinosum respond with increased inflammatory cytokines, IL-1β, IL-2, IL-6, and IL-8 and TNF-α, when LPS was introduced. Aspirin is a non-steroidal anti-inflammatory drug (NSAID) that is known to decrease inflammation and a nearly 50% numerical decrease in serum IL-6 concentrations in heifers fed aspirin in chapter 2 suggests that short-term aspirin administration lessened inflammation. The fact that IL-6 concentrations were not affected by aspirin administration in the present study indicates that aspirin's anti-inflammatory effects may have been diminished with longer term administration or that inflammation caused by leaky gut offset any anti-inflammatory effect that aspirin may have had. When Kvidera et al. (2017b) intentionally induced GIT barrier dysfunction with GSI, they did not see a difference in SAA or

haptoglobin, which aligns with the current study. In addition, short term administration of aspirin in chapter 2 did not affect SAA or haptoglobin.

Carcass data are presented in Table 3.4. There were no differences in dressing percentage, liver abscess score, or percent of livers abscessed ($P \geq 0.62$). Cattle fed 50 mg/kg of aspirin produced carcasses with decreased hot carcass weight ($P = 0.05$) and rib-eye area ($P = 0.01$), increased fat thickness ($P = 0.02$), marbling score ($P = 0.003$), and yield grade ($P = 0.01$), and a tendency for increased %KPH ($P = 0.10$). The increase in fat deposition in the current study is likely a result of an inflammatory response to LPS that leaked into circulation. Adipocytes are used for intermediate storage of LPS, which can later be neutralized by macrophages (Hersoug, 2012). After binding with LPS, LBP mediates the association between LPS and cluster of differentiation 14 (CD14; Lu et al., 2008; Ceciliani et al., 2012). In circulation, LPS that is bound to LBP and CD14 will bind with high-density lipoproteins (HDL) and low-density lipoproteins (LDL) and migrate to hepatocytes or white adipose tissue (Eckel and Ametaj, 2016). An increase in circulating LPS may lead to increased binding with HDL and LDL, which would deliver more triglycerides to the liver, potentially causing a greater incidence of fatty liver, or more free fatty acids and triglycerides being deposited in adipose tissue (Eckel and Ametaj, 2016). No difference in serum AST concentrations in the current study indicate that the liver was not inflamed. However, increased fat thickness and marbling and a tendency for increased KPH may be explained by the immune system trying to rid the body of LPS and minimizing liver inflammation by depositing more adipose tissue for LPS storage. A switch towards fat production is consistent with data from Farney et al. (2013) who demonstrated that 1.95 g/L of sodium salicylate (also an NSAID) supplementation in a molasses carrier for 7 d in early post-partum dairy cows increased fat content in milk.

3.5 Conclusion

Long-term supplementation of aspirin to feedlot cattle increased serum LBP concentrations, and increased fat deposition while decreasing muscle deposition and daily gains. These results indicate that aspirin induced leaky gut and that leaky gut has a negative impact on feedlot cattle performance and carcass composition. Long-term use of aspirin can be used as a model to study

the effects of leaky gut in feedlot cattle on growth and carcass characteristics. Strategies that decrease leaky gut may be able to improve feedlot cattle performance.

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3.7 Tables and Figures

Table 3.1. Composition of diets (DM basis)

	Control[1]	Aspirin[1]
Corn	50	48
Dried distillers grains with solubles	24	21
Corn silage	20	20
Vitamin/mineral supplement[2]	6	6
Aspirin supplement[3]	0	5
Diet composition, DM basis[4]		
Crude protein, %	13.07	13.07
NEm, Mcal/kg[5]	1.95	1.95
NEg, Mcal/kg[5]	1.33	1.33
NDF, %	22.85	22.85
Calcium, %	1.15	1.15
Phosphorus, %	0.49	0.49
Sulfur, %	0.25	0.25

[1]Control = 0 mg/kg of aspirin/d; aspirin = 50 mg/kg of BW of aspirin/d

[2]Vitamin/mineral supplement contained (DM basis): 18.25% Ca, 1.32% K, 0.44% Mg, 0.18% S, 563.91 ppm Zn, 522.90 ppm Fe, 440.41 ppm Mn, 183.33 ppm Cu, 9.66 ppm I, 4.48 ppm Se, 3.43 ppm Co, 42.19 IU/g vitamin A, 4.98 IU/g vitamin D, 0.155 IU/g vitamin E, 413.6 ppm Rumensin (176.4 g/kg, Elanco Animal Health, Indianapolis, IN).

[3]Aspirin supplement contained 57% dried distillers grains with solubles, 38% corn, and 5% aspirin (DM basis)

[4]Analyzed at Cumberland Valley Analytical Services (Waynesboro, PA)

[5]Calculated based on NASEM (2016)

Table 3.2. Effect of 50 mg/kg of BW of aspirin/d on performance of steers

	Treatment[1]			
Item	Control	Aspirin	SEM	*P* value
BW, kg				
d 0	355.2	354.6	4.88	0.93
d 85	520.4	516.7	4.88	0.59
d 159	621.3	612.6	4.88	0.21
ADG, kg/d				
First half (d 0 to 85)	1.94	1.91	0.050	0.61
Second half (d 86 to 159)	1.39	1.27	0.050	0.09
Overall (d 0 to 159	1.69	1.61	0.035	0.10
DMI, kg/d				
First half (d 0 to 85)	9.4	9.5	0.21	0.71
Second half (d 86 to 159)	10.3	10.1	0.21	0.39
Overall (d 0 to 159	9.8	9.8	0.18	0.84
Gain:feed				
First half (d 0 to 85)	0.206	0.200	0.0048	0.35
Second half (d 86 to 159)	0.135	0.126	0.0048	0.19
Overall (d 0 to 159	0.173	0.164	0.0038	0.15
Days on feed	158	161	4.49	0.59

[1]Control = 0 mg/kg of aspirin/d; aspirin = 50 mg/kg of BW of aspirin/d

Table 3.3 The effect of long-term aspirin dosage on serum inflammatory markers

	Treatment[1]			
	Control	Aspirin	SEM	P-value
LPS[2] binding protein, ug/mL	31.1	41.1	3.69	0.07
Interleukin-6, pg/mL	45.7	39.4	8.04	0.58
Haptoglobin, ug/mL	2.98	2.47	0.522	0.50
Serum amyloid A, ug/mL	46.8	37.9	6.02	0.30
Aspartate aminotransferase, mU/mL	33.7	34.4	1.07	0.68

[1]Control = 0 mg/kg of aspirin/d; aspirin = 50 mg/kg of BW of aspirin/d

[2] LPS = lipopolysaccharide

Table 3.4. Effect of aspirin on carcass characteristics of steers

	Treatment[1]			
Item	Control	Aspirin	SEM	*P* value
Hot carcass weight, kg	389.0	382.7	2.11	0.05
Dressing percent, %	62.6	62.5	0.19	0.62
Fat thickness, cm	1.35	1.53	0.049	0.02
Rib-eye area, cm	88.4	84.7	0.91	0.01
Kidney, pelvis, heart fat, %	1.99	2.09	0.040	0.10
Yield grade	3.11	3.43	0.080	0.01
Marbling score[2]	418.5	448.6	5.76	< 0.01
Quality grade distribution				
Select, %	4.3	2.1	2.48	0.55
Choice $^{-}$, %	39.5	33.3	4.40	0.33
Choice ^{0}, %	35.4	33.4	6.07	0.82
Choice $^{+}$, %	16.5	23.0	4.87	0.36
Prime, %	4.1	8.5	3.70	0.42
Liver abscess score[3]	0.21	0.19	0.071	0.85
Liver abscess, %	18.8	16.8	5.62	0.80

[1]Control = 0 mg/kg of aspirin/d; aspirin = 50 mg/kg of BW of aspirin/d.

[2]Practically devoid = 100 to 199; slight = 200 to 299; small = 300 to 399; modest = 400 to 499; moderate = 500 to 599.

[3]Elanco Animal Health (Indianapolis, IN) liver abscess scores converted to numeric: 0 (no abscesses present) = 0; −A (one or two minor abscesses) = 1; A (two to four well-established abscesses) = 2; and +A (large, active abscesses, may contain inflammation on the abscess periphery) = 3

CHAPTER 4. CONCLUSION AND IMPLICATIONS

Cattle are susceptible to many diseases and disorders that stem from the segmentation in the beef industry. Stress from transitioning cattle from cow/calf operations to the feedlot is thought to cause GIT barrier dysfunction and lead to the development of many diseases. The resulting diseases and disorders can negatively affect animal performance, thus resulting in economic losses for producers. As a result, further research to understand the effect of leaky gut and its effect on disease susceptibility in cattle is needed.

Different models have been created and evaluated, and each have positives and negatives. Stress inducing models are resource intensive and have unwanted side effects. Alternatively, the GSI or indomethacin model induces leaky gut without these stressors, but the animals cannot enter into the food chain, which increases the cost of research. Aspirin induced leaky gut in lab animals and humans, while food animals dosed with aspirin could enter into the food chain, which lessens the expense to conduct leaky gut research. However, there is no research to determine if aspirin causes leaky gut in cattle.

In the first experiment of chapter 2, the results show that the optimal dosage of aspirin is 200 mg/kg of BW of aspirin causes leaky gut based on the Cr- EDTA kinetics and increase in LBP in serum. As aspirin dosage increased, Cr-EDTA retention time decreased, which indicated that more GIT permeability occurred in these animals. The increase in serum LBP indicated that LPS is able to pass through the intestinal wall and enter into the circulatory system. In addition, the second experiment in chapter 2 indicated that the 200 mg/kg of BW of aspirin disrupted tight junction mRNA expression in the jejunum. Aspirin potentially caused damage to claudin 1, which led to an increase in repair of claudin 1 based on the tendency for upregulation in claudin 1. Overall, aspirin dosed at 200 mg/kg of BW induced GIT dysfunction in beef cattle.

With ruminants, the question arises, how much GIT dysfunction occurs in the reticulorumen compared to the rest of the digestive tract? Narrowing down the location of where GIT dysfunction predominately occurs in ruminants could aid in the development of products that help mitigate the negative effects of GIT dysfunction. The aspirin model will assist leaky gut research independent of other stressors; but, aspirin is an NSAID, so it could interact negatively with feed additives.

In chapter 3, the results indicate that long-term aspirin use (leaky gut) negatively impacts feedlot performance and carcass characteristics. Endotoxin appearance in the blood increased between treatments based on the increase in LBP. The increase in LPS in blood could have shifted carcass characteristics towards increased fat deposition. Fat weighs less than muscle, which explains why live BW and hot carcass weight decreases.

Additional long-term effects of leaky gut are currently unknown. Studying cattle that have developed Johnne's disease may provide some clues. The aspirin model allows researchers to gain a better understanding of the mechanisms underlying leaky gut, such as LPS detoxification or intestinal barrier repair. Currently researchers known that when LPS exits the gastrointestinal tract, there are three known pathways that LPS can follow (Eckel and Ametaj, 2016). A pathway is into lymphatic circulation, where macrophages in lymph nodes neutralize the LPS (Eckel and Ametaj, 2016). Another pathway is through the portal vein to the liver where macrophages neutralize the LPS (Eckel and Ametaj, 2016). The final pathway is into blood circulation where LPS binds with LBP and bind with HDLs and LDLs, which are then deposited into white adipose tissue (Eckel and Ametaj, 2016). Macrophages in adipose tissue will neutralize the deposited LPS. However, research is not clear about which pathway LPS prefers to take (Eckel and Ametaj, 2016). *In vivo* research on LPS deposits in fat could help conclude when, how, and why animals change LPS clearance methods. Carcass data could conclude how much LPS deposited in fat is detoxified or still toxic. More importantly, what kind of effects could consuming fat deposited LPS have on human health? The addition of the aspirin model could help bring clarity to GIT dysfunction.

Future studies could measure the amount of LPS from different adipose tissue (intermuscular, intramuscular, and visceral) to determine if LPS storage is preferential to certain tissue. In addition, blood could be analyzed for additional indicators of immune activation, such LBP, Ca concentration, glucose and insulin levels in the blood. The increase in LBP could indicate that more LPS is in the blood as shown by the present studies. Plasma Ca binds with LPS in the blood (Khiaosa-ard and Zebeli, 2018), so if total plasma Ca increases for additional binding, a rise of Ca in the blood could indicate LPS in the blood. In addition, the immune system uses glucose when it is activated, which is why animals become hyperinsulemic during an immune challenge (Rodrigues et al., 2015). These measures could help further show when an animal has LPS in the blood. Further blood indicators, such as LBP, glucose and insulin, and Ca concentration could be measured to determine if LPS enters into the blood.

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