Understanding intestinal lipopolysaccharide permeability and associated inflammation

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Understanding intestinal lipopolysaccharide permeability and associated inflammation

by

Venkatesh Mani

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Toxicology

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Iowa State University
Ames, Iowa
2012

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DEDICATION

To all the animals who we have sacrificed their innocent lives for the betterment of human beings and to my family who sacrificed so many things for my research endeavors.
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Lipopolysaccharide (LPS) and the inflammation associated with its stimulation of the innate immune responses can have major implications for human and animal health and production. This dissertation research goal was to further understand dietary modulation of intestinal LPS permeability and LPS associated inflammation. Additionally, we sort to examine LPS detoxification and the relationship LPS has with swine health and feed efficiency.

High caloric and high dietary fat increases the risk of endotoxemia which can result in a low grade inflammation, a predisposing factor for common metabolic diseases such as Type II diabetes and atherosclerosis. However, little is known about the effect of dietary oil fatty acid composition on intestinal LPS permeability and postprandial endotoxemia. Therefore, we examined whether dietary oil composition differentially modulated intestinal LPS permeability and postprandial endotoxemia. Our in vivo and ex vivo research using pigs and isolated pig intestinal tissues indicated that a single administration of oils rich in long chain n-3 polyunsaturated fatty acids (PUFA), such as fish oil and cod liver oil, decreases LPS permeability and postprandial circulating LPS levels (P<0.05). Furthermore, oils rich in saturated fatty acids, such as coconut oil, augmented LPS permeability and postprandial endotoxemia (P<0.05). Mechanistically, this may be associated with the structure and function of cell membrane lipid raft microdomain structures.

Dietary long chain n-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to antagonize LPS signaling. Therefore, we
examined the ability of dietary EPA and DHA to attenuate intestinal LPS permeability and lipid raft localization of key LPS signaling proteins. Long term dietary EPA and DHA supplementation to pigs enriched intestinal epithelial membrane with EPA and DHA (P<0.05). Phospholipid fatty acid composition of the lipid raft fractions also revealed enrichment of phosphatidyl ethanolamine and phosphatidyl serine with EPA and DHA. Mechanistically, membrane EPA and DHA enrichment decreased localization of LPS signaling proteins, TLR4 and CD14, into ileum and colon lipid raft microdomains. Collectively, this decreased ex vivo LPS permeability and circulating LPS concentrations (P<0.05). Interestingly, an acute systemic inflammatory challenge resulted in a decreased localization of TLR4 and CD14 into lipid rafts, which has the potential to desensitize the pigs to a subsequent immune challenge otherwise known as LPS tolerance.

The ability of the maternal diet and prenatal nutrition to impact postnatal growth, development and health has received much attention in recent years. Knowing that DHA and EPA can regulate the innate immune response to an LPS challenge, we wanted to study if maternal n-3 PUFA supplementation of n-3 PUFA could modulate an acute inflammatory challenge in the offspring later in life. Sows and piglets received nutrition devoid or enriched with EPA and DHA during gestation and lactation or throughout life from gestation to ten weeks of age. The offspring was then challenged with LPS or saline to initiate an inflammatory response and buffy coats isolated 4 h post challenge. Interestingly, maternal n-3 PUFA supplementation attenuated the LPS induced inflammatory response in the offspring late in the nursery phase of growth (P<0.05). This was comparable to that of continuous n-3 PUFA supplementation. Both treatment groups exposed to DHA and EPA had a decreased
febrile and serum TNF-α cytokine response to LPS, buffy coat mRNA abundance of TNF-α, IL-1β and IL-10 and the mRNA abundance of the LPS signaling proteins, TLR4, CD14 and Myd88, compared to control group (P<0.05).

Lastly, we used pig lines divergently selected for residual feed intake (RFI, with low RFI being more efficient compared to high RFI) to understand the relationship between intestinal barrier integrity, LPS and associated inflammation with pig feed efficiency. Our research indicates that HRFI pigs seem to be undergoing a greater level of basal inflammation contrary to pigs selected for LRFI. The LRFI pigs had a lower circulating endotoxin concentration, more robust intestinal and liver LPS detoxification and higher active anti-microbial enzymes including alkaline phosphatase and lysozyme (P<0.05). Furthermore, LRFI pigs had a reduced activity of the inflammatory biomarker enzyme myeloperoxidase (P<0.05). Altogether, LPS and low grade inflammation may partially explain the divergence in feed efficiency and RFI in grow-finisher pigs.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Gram negative bacteria such as *Salmonella* and *Escherichia* contain lipopolysaccharide (LPS) in their cell wall outer membrane which is a potential stimulator of the innate immune system of humans and livestock species. Over the recent years, LPS has received much attention due to its ability to stimulate a low grade inflammation. Lipopolysaccharide is a glycolipid composed of a hydrophobic domain lipid A through which it is attached to the outer membrane of the cell wall, a core and distal oligosaccharide and two phosphate molecules. Even though the terms LPS and endotoxin are interchangeably used in the literature, there are biochemical and molecular differences between these two compounds. To reflect our original experimental nature we will be using lipopolysaccharide more commonly throughout this dissertation. Lipopolysaccharide recognition and the associated inflammation have been widely studied. It is first recognized by the innate immune system, which is usually non-specific towards the foreign molecules. The innate immune system depends on pattern recognition receptors (PRR) which recognize specific patterns of molecular structures present in the microbes. Lipopolysaccharide is recognized by Toll-like receptor 4 (TLR4), present on the cell membrane of immune cells, adipocytes, myocytes, and epithelial cells (Abreu, 2010; Beutler, 2004; Gabler et al., 2008; Lenardo and Baltimore, 1989). After recognition, the host cells initiate an inflammatory response via the activation of the master transcription factor, nuclear factor-κB (NF-κB). The resulting increase in pro-inflammatory cytokines helps in recruiting other immune cells, inducing
febrile response to remove the foreign agent, and communicating with the adaptive immune system (McGettrick and O’Neill, 2010).

Gram-negative bacteria are present ubiquitously in the environment and also in the respiratory and gastrointestinal tract. Thus, most mammals are constantly exposed to LPS. In humans, low grade endotoxemia induced by persistent presence of LPS in the blood has been shown to be a predisposing factor for the development of metabolic diseases including Type II diabetes, obesity, and atherosclerosis (Delzenne and Cani, 2011; Vaarala et al., 2008). Moreover, high fat, high caloric diet or high carbohydrate diets have been shown to increase the serum lipopolysaccharide levels (Erridge et al., 2007; Ghanim et al., 2009). In production animals such as pigs, LPS exposure antagonizes appetite, digestion, and skeletal muscle protein synthesis that ultimately leads to diversion of nutrients and energy away from important production orientated pathways and systems (skeletal muscle, reproductive tract etc…) to support the immune system (Johnson, 1997). Although this is important for immune system function and health of the animal, long term exposure to LPS and subsequent activation of immune system and the development of metabolic syndrome affects growth and production efficiencies.

Considering the significance of LPS and its effect on human and animal health and metabolism, little is known or studied on the pathways through which LPS enters circulation via the intestinal tract and also its detoxification. This is surprising considering the gastrointestinal tract is a major source of gram negative bacteria and LPS. The mode of LPS permeability across the intestinal epithelium and into systemic circulation may be through a transcellular route or paracellular route between two epithelial cells through tight junctions.
Additionally, micelle facilitated LPS permeability also happens when the dietary fat digestion occurs in the intestine (Kelly et al., 2012; Tomlinson and Blikslager, 2004).

Therefore, the overall goal of this dissertation research presented herein is to further understand and characterize intestinal LPS permeability and LPS signaling. Furthermore, this new knowledge gained could be used to develop dietary, genetic, or pharmacological mitigation strategies to promote health, growth, and metabolic efficiencies in humans and livestock. To address this goal, the specific objectives of this dissertation were:

1. To evaluate the composition of dietary fat to differentially modulate intestinal LPS permeability and postprandial endotoxemia.
2. To identify whether dietary n-3 PUFA alter intestinal membrane composition and function resulting in attenuated LPS permeability.
3. To examine whether maternal exposure to dietary n-3 PUFA would attenuate the febrile response and inflammatory response to an exogenous LPS challenge later in the life.
4. To evaluate the contribution of intestinal endogenous LPS and its associated uptake and detoxification to feed efficiency using pigs divergently selected for residual feed intake.

Dissertation organization

Based on the work to achieve the objectives of this dissertation, five manuscripts were written for submission. Each of the manuscript is presented as a separate chapter in the format prepared for the journal we submitted then for publication. A review of literature as background for this research is outlined in Chapter 2. This chapter has, in part, been published in the Journal of animal science as a symposium review paper pertaining to LPS,
gastrointestinal tract LPS permeability and LPS associated inflammation and detoxification. In Chapter 3, the effect of dietary oil composition on intestinal LPS permeability and postprandial endotoxemia was studied. This article was submitted to Nutrition & Metabolism journal and is under review. Chapter 4 examines the effects of dietary n-3 PUFA eicosapentaenoic acid and docosahexaenoic acid supplementation on intestinal epithelial cell membrane composition and lipid raft associated LPS permeability in pigs. This research will be submitted to the Journal of Lipid Research for review and publication. The evaluation of maternal n-3 PUFA supplementation on LPS induced febrile response and inflammation is presented in Chapter 5. This research will be submitted to the Journal of Nutrition. The final research chapter uses pigs divergently selected for feed efficiency to examine the role of endogenous LPS on finisher pig growth and performance (Chapter 6). This research is submitted to the Journal of Animal Science and is under review. The final chapter (#7) includes general conclusions and an overall discussion of the research.

**Literature Cited**


CHAPTER 2. REVIEW OF LITERATURE: LIPOPOLYSACCHARIDE, INFLAMMATION AND INTESTINAL FUNCTION

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Abstract: Lipopolysaccharide (LPS) often referred to as endotoxin, can stimulate localized or systemic inflammation via the activation of pattern recognition receptors. Additionally, LPS and associated inflammation can regulate intestinal epithelial function by altering epithelial barrier integrity as well as nutrient transport and utilization. The gastrointestinal tract is a large reservoir of both Gram positive and negative bacteria, of which the Gram negative bacteria serve as a source of LPS. Luminal LPS can enter circulation via three major routes: 1) nonspecific paracellular permeation through epithelial cell tight junctions and 2) transcellular permeation through lipid raft membrane domains involving receptor mediated

1 Based on a presentation at the Growth and Development Symposium titled “Understanding and Mitigating the Impacts of Inflammation on Animal Growth and Development” at the Joint Annual animal science meeting, July 10-14, New Orleans, LA”. A modified version of this Chapter was published in the Journal of Animal Science 90: 1452-65 (2012).
endoctysis, 3) through micellar assisted permeation while fat is consumed through diet.

Paracellular permeability of LPS occurs through dissociation of tight junction protein complexes resulting in reduced intestinal barrier integrity, which can be a result of enteric disease, inflammation, or environmental and metabolic stress. Transcellular permeability, via specialized membrane regions rich in glycolipids, sphingolipids, cholesterol, and saturated fatty acids, is a result of raft recruitment of LPS-related signaling proteins leading to signaling and endocytosis. All permeability routes and sensitivity to LPS may be altered by diet, environmental and metabolic stresses. Intestinal derived LPS and inflammation result in suppressed appetite, activation of the immune system, and partitioning of energy and nutrients away from growth towards supporting the immune system requirements. In livestock, this leads to the suppression of growth, particularly, suppression of lean tissue accretion. In this chapter, we summarize the evidence that intestinal permeability of LPS and the subsequent inflammation leads to decrease in the production performance of agricultural animals, and we present an overview about LPS detoxification mechanisms in livestock.

**Key words:** Lipopolysaccharide, Endotoxin, Intestine, Inflammation

**Introduction**

Growth performance of agricultural animals in commercial settings and human health is affected by various physical, social, and microbial factors that may predispose animals to physiological or immunological stresses (Holck et al., 1998). Among the stressors that can attenuate the growth performance and alter metabolism are viruses, live bacteria, and dead bacteria that contain cell wall compounds such as lipopolysaccharide (LPS) and
peptidoglycans (Schinckel et al., 1995; Smith, 1998). Additionally, recent biomedical evidence suggests that low grade inflammation caused by intestinal derived LPS is linked to metabolic diseases like Type II diabetes, atherosclerosis and cardiovascular diseases (Cani and Delzenne, 2010). Importantly, the mucosal epithelium of the gastrointestinal tract serves as a major barrier to the LPS whereas the bacteria present in the intestinal lumen acts as a major source of LPS (Ravin et al., 1960; Schweinburg and Fine, 1960; Wiznitzer et al., 1960). Specific to the focus of this review, we will discuss LPS, otherwise referred to as endotoxin in the literature, the cell wall component of Gram negative bacteria which is a potent immune stimulator in the context of livestock production and human health.

Lipopolysaccharide in mammals is recognized by various cells expressing the pattern recognition receptor, Toll-like receptor (TLR)4, and other proteins including LPS binding protein (LBP), cluster of differentiation 14 (CD14), and MD-2. These proteins and receptors are also shown to be present in intestinal epithelial cells and have been associated with the permeability of luminal LPS into circulation (Hornef et al., 2003; Neal et al., 2006). Once in the systemic circulation, LPS can be deactivated or detoxified by immune cells, such as macrophages, Kupffer cells present in the liver or splenic cells, or by binding with plasma proteins (Buttenschoen et al., 2010; Rutenberg et al., 1967; Satoh et al., 2008). However, if there is failure of systemic detection and deactivation because there is more permeability of LPS from the intestinal tract the resulting increased circulating LPS can lead to systemic inflammation which can lead to endotoxemia, multi organ failure and even death (Rice et al., 2003; Zweifach and Janoff, 1965). The importance of LPS to livestock production is that chronic activation of the immune system has been shown to antagonize the growth and performance of animals, as nutrients are being partitioned towards production of cytokines,
acute phase proteins, and other immune modulators rather than towards the anabolic processes that support milk and muscle synthesis (Johnson, 1997; Spurlock, 1997). Further, LPS can lead to various diseases including laminitis in equines and also endotoxemia is the leading cause of death in equine species (Sykes BW, 2005; Werners et al., 2005). Lipopolysaccharide has also been shown to activate the heterophils and up-regulate the pro-inflammatory cytokine and chemokine expression in poultry (Kogut et al., 2005). In human health, presence of LPS in the circulation have been shown to contribute to the development of chronic inflammatory processes which eventually promote the development of dysregulated metabolism which results in many metabolic diseases like type II diabetes and non-alcoholic fatty liver disease through the stimulation of TLR4 (Erridge, 2011).

Interestingly, permeability of LPS from the intestine has been shown to be modulated by dietary factors, as well as by stressors including heat stress, systemic disease, and also by feed restriction or malnutrition (Cani and Delzenne, 2010; Hall et al., 2001). The major dietary factor that appears to modulate the permeability of luminal LPS is dietary fat. As indicated in the biomedical and human health literature, as the percentage of dietary fat increases, so does the concentration of circulating LPS (Amar et al., 2008; Erridge et al., 2007). Further, the form of the lipid ingested may modulate the LPS permeability with emulsified lipids increasing LPS permeability (Laugerette et al., 2011). In ruminants, feeding easily-digestible carbohydrates and grains have been shown to increase the permeability of LPS to the peripheral circulation, indicating that carbohydrates could also influence LPS permeability (Khafipour et al., 2009; Zebeli et al., 2011). In addition to dietary nutrients, systemic increases in intestinal-derived LPS can also be attributed to environmental and immunological stressors. Hyperthermia increases intestinal permeability and, presumably,
intestinal LPS permeability too (Lambert, 2004, 2008; Pearce et al., 2011). Plasma
antibodies to LPS are inversely related to growth in malnourished young children and are
associated with increased intestinal permeability and systemic immune system activation
(Campbell et al., 2003). Further studies are warranted to investigate the relationship between
LPS, growth, and metabolic changes.

**Lipopolysaccharide**

Lipopolysaccharide is a glycolipid present in the outer membrane of Gram negative
bacterial cell wall. It consists of a hydrophobic domain, lipid A, through which it is inserted
into the outer leaflet of the outer membrane of the bacterial cell wall, a core oligosaccharide
and a distal oligosaccharide (Elin and Wolff, 1976; Raetz and Whitfield, 2002a). The
hydrophobic lipid A domain is the most biologically active portion of the LPS molecule and
it is synonymously known as ‘endotoxin’ because of its ability to stimulate the innate
immune cells (Erridge et al., 2002). In a wild type *Escherichia coli*, lipid A contains the
following structural properties: 1) the backbone of the lipid A contains di-glucosamine,
which is phosphorylated at positions 1 and 4'; 2) two 3-hydroxymyristate molecules are
directly attached to each glucosamine, and 3) at positions 2' and 3', the hydroxyl groups of
the fatty acids are substituted by laurate and myristate, and they form an acyloxyacyl bond
with the primary fatty acid chains (Figure 1). Diphosphorylated hexaacyl lipid A molecules
have been shown to be effective stimulators of the immune system because they have been
optimally recognized by the mammalian immune system. Mono-phosphorylated or
deposphorylated LPS molecules have been shown to substantially lose their potency and
immune reactivity (Holst et al., 1996; Munford, 2005). However, monophosphorylated lipid A is a potent adjuvant and is being formulated into vaccines being used for humans. Another important characteristic of lipid A is that mostly all the fatty acyl chains are made up of saturated fatty acids. If the saturated fatty acids are replaced with unsaturated fatty acids, the resultant LPS molecule induces an attenuated immune response (Kitchens et al., 1992; Munford and Hall, 1986).

Lipopolysaccharide can enter systemic circulation from live bacteria, or as cell wall components of dead bacteria. Either way, if the amounts are too great, they can ultimately antagonize anabolic growth (Kimball et al., 2003; Orellana et al., 2007) or lead to septic shock and death (Moore and Morris, 1992). Lipopolysaccharide is released not only during bacterial death but also during growth and division, making it a ubiquitous contaminant (Petsch and Anspach, 2000; Yaron et al., 2000). The biological activity of the LPS is measured in endotoxin units (EU). For example, 100 pg of LPS is considered to have 1 EU of activity, or 10 EU is equivalent to 1 ng of LPS. A single Gram negative bacteria contains approximately $10^{-15}$ g of LPS and $10^5$ bacteria can generate 1 EU. It has been shown that a single *E. coli* contains approximately $10^6$ lipid A residues (Raetz et al., 1991). Furthermore, the size of the individual LPS molecules varies between 10 to 20 kDa in monomeric form and because of the amphiphilic nature; they can arrange themselves into large micellar structure achieving 1,000 kDa.

**Innate Immune Response**

The innate immune response is the first line of defense against infectious diseases and foreign particles. Compared to adaptive immune system, innate immune response is
instantaneous, usually activated within minutes to hours of a stimulus, whereas the adaptive immune response takes hours to days (Janeway and Medzhitov, 2002). The innate immune response contains five key elements for protection: 1) physical barriers like skin, epithelial layers, and mucus to prevent the entry of pathogens; 2) enzymes with antimicrobial properties like lysozyme and muramidase in body fluids to kill the invading agents; 3) a recognition system like germ line encoded pattern recognition receptors (PRRs) such as TLRs) and NOD-like receptors allow for immediate detection and response; 4) anti-microbial responses including the complement system; and 5) the recruitment of other immune cells for an enhanced response (Aderem and Ulevitch, 2000; Beutler, 2004; Heeg, 2007; Hoffmann et al., 1999). Like the adaptive response, the innate immune system can also be classified into cellular and humoral components. Hematopoietic cells like macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer cells and non-hematopoietic cells such as epithelial cells make up the cellular component. Complement proteins, LPS binding proteins, C-reactive protein, collectins, and anti-microbial peptides like defensin’s make up the humoral component (Turvey and Broide, 2010).

The innate immune system was thought to be non-specific; however, a series of discoveries in the late-1990s proved that this theory was only partially true and that innate receptors recognized a narrow range of compounds from particular organisms, e.g. TLR4 can recognize LPS from many organisms (Medzhitov et al., 1997). There are specific receptors present in immune cells, such as macrophages, dendritic cells, B cells, and certain types of T cells, that could recognize a particular pattern in the invading microbes which came to be known as PRRs (Janeway and Medzhitov, 2002; Medzhitov, 2001). Moreover, myocytes and adipocytes also express these same PRRs (Gabler and Spurlock, 2008). These ‘patterns’
present in the different microbial species are essential for their survival and have come to be known as pathogen associated molecular patterns (PAMP), later to be renamed microbe associated molecular patterns (MAMP) to include all the microbes including pathogens and non-pathogens (Akira et al., 2006; Ausubel, 2005). Collectively, the family of PRRs senses the presence of a variety of molecules from the invading pathogens, as well as commensals, and regulates the immune response by stimulating the secretion of various immune mediators (Brikos and O'Neill, 2008). More recently, PRRs have been shown to recognize not only the pathogenic patterns, but also commensals, as well as cellular degradation products from the same organism, which are known as damage associated molecular patterns (Chen and Nuñez, 2010; Rosin and Okusa, 2011). The first Toll-like pattern recognition receptor to be identified was TLR4, which recognizes bacterial LPS and other proteins including heat shock proteins (Poltorak et al., 1998). At present, there are 11 human and 13 murine TLRs, which recognize different pathogen components including flagella, peptidoglycan, double-stranded RNA, and DNA (McGettrick and O'Neill, 2010; Moresco et al., 2011).

Recognition of inflammatory compounds by PRRs leads to the activation of the master transcription factor nuclear factor-κB (NF-κB). After NF-κB activation, increased transcription and secretion of a class of pleiotropic molecules, known as cytokines occurs. Cytokines then act on other cells to induce specific cellular immune response (Lenardo and Baltimore, 1989). Cytokines can exert autocrine, paracrine, and even endocrine functions. The physiological actions of cytokines include development of cellular and humoral immune response, induction of the inflammatory response, regulation of hematopoiesis, control of cellular proliferation and differentiation, and healing of wounds (Arai et al., 1990; Kindt et al., 2007). Cytokines can be pro- and anti-inflammatory and lead to the increase or decrease
in the magnitude of the inflammatory response. Tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-8 are considered pro-inflammatory, whereas IL-10 and transforming growth factor-β are considered anti-inflammatory. These classes of cytokines are secreted by immune cells and many other cell types (Ashley et al., 2012). For example, IL-1β secreted by monocytes, endothelial cells and epithelial cells, TNF-α secreted by macrophages, IL-12 secreted by macrophages and dendritic cells, interferon-β secreted by fibroblasts are some of the examples of cytokines of innate immune cells. Interleukin-2 secreted by T cells, interferon γ secreted by TH1 cells, and IL-4 secreted by TH2 cells and mast cells, are some of the examples of cytokines of adaptive immunity. Overall, cytokines exert their function through acting on five classes of receptors: 1) immunoglobulin superfamily receptors, 2) class I and 3) class II cytokine receptor family, 4) TNF receptor family and 5) chemokine receptor family (Borish and Steinke, 2003; Dinarello, 2000; Miyajima et al., 1992). Chemokines are a sub-family of cytokines with approximately 90-130 amino acids and mainly helps in the leukocyte recruitment (Allen et al., 2007).

Classically, innate immune response is characterized by inflammation. Inflammation is associated with clinical signs such as redness, pain, heat, swelling, and loss of function. Inflammation can be acute or chronic depending on the duration it takes to remove the immune stimuli. The inflammatory process is usually compartmentalized to the affected tissue (Kindt et al., 2007). During acute inflammation, when the tissue damage occurs by the immune stimulant, innate immune cells which encounter the invading agent secrete pro-inflammatory mediators. This results in the classical signs of inflammation as well as bringing together of leukocytes, antimicrobial mediators like defensins, cathelicidins and interferons, complement system, kinins, clotting and fibrinolytic proteins, lipid mediators like
prostaglandins, leukotrienes, platelet-activating factor, peptides and amines like histamine, serotonin, neuropeptides and pro-inflammatory peptides and cytokines such as IL-1α, IL-1β, TNF-α and IL-6 which act to destroy the invading agent. Neutrophils are the first immune cells to be recruited followed by macrophages, eosinophils, and platelets, and followed by lymphocytes. If the invading agent is eliminated, the anti-inflammatory process mediated by cytokines such as IL-10 and transforming growth factor-β takes over to limit the damage to the surrounding tissues (Ballou, 2012; Dolgachev and Lukacs, 2010; Kindt et al., 2007; Libby, 2002).

If the local inflammatory process is not contained within the affected tissue, it may lead to a systemic response, otherwise known as acute-phase response. The cytokines secreted during the initial response then enters systemic circulation and act on bone marrow, hypothalamus and liver. Stimulation of bone marrow results in the increased production of leukocytes needed to fight the infection. Activation of hypothalamus by the cytokines, directly through cytokine receptors or indirectly through vagus nerve, stimulates the secretion of prostaglandins which results in a fever response. This febrile response helps in preventing the growth of the pathogen and increases the overall immune response (Johnson, 1997; Karin et al., 2006; Wright et al., 2000). Fever is a regulated change in homeostasis of the body and a complex acute phase response in which a temporary resetting of the body’s thermostatic set point causing an increase in core body temperature (Hasday et al., 2000; Kluger et al., 1996). Apart from preventing the pathogen growth and inducing immune response, fever also results in uncoupling of electrons during oxidative phosphorylation resulting in decreased ATP synthesis and decreased feed efficiency. Fever also reduces the appetite and, thus, acquisition of iron from the diet which is required by most pathogens. Also, the acute phase response
results in an increase in the production of transferrin and lactoferrin which further restricts the availability of iron for the pathogen (Kozak et al., 2000).

Further, hypothalamus activates the pituitary gland resulting in the release of adrenocorticotropic hormone (ACTH) which stimulates the secretion of corticosteroids from adrenal cortex. Together, the cytokines and hypothalamus-pituitary axis results in the secretion of acute phase proteins (APP) primarily from the liver. C-reactive protein, serum amyloid A, fibrinogen, haptoglobin, mannose-binding protein, and complement components are the major APP secreted (Steel and Whitehead, 1994). Their concentration increases dramatically, particularly C-reactive protein and serum amyloid A, after an acute phase response. Acute phase proteins usually bind to the inflammatory agent which in turn helps facilitates its neutralization through the complement system (Balaji et al., 2000; Eckersall and Bell, 2010; Gabay and Kushner, 1999).

**Lipopolysaccharide Signaling and Permeability**

Lipopolysaccharide is recognized and signaled by the PRR, TLR4 (Poltorak et al., 1998). However, the presence of LPS is not sensed by TLR4 alone. Lipopolysaccharide is usually present as an aggregate bound to other LPS molecules on which LBP acts and separates a monomer which is then presented to CD14 receptor. The CD14 receptor is present in two forms, membrane-bound (mCD14) or soluble (sCD14). The CD14 protein doesn’t have an intracellular domain so it associates with TLR4, which has a Toll-interleukin 1 receptor (TIR) intracellular domain through which it can transmit the intracellular signal (Beutler, 2000; Triantafilou and Triantafilou, 2002). Toll-like receptor 4 then dimerizes and
binds with MD-2 which transmits the signal through the TIR intracellular domain through two different pathways. One is a myeloid differentiation factor 88 (MyD88) dependent and the other one is a MyD88 independent pathway. The first pathway leads to translocation of nuclear factor kappa beta (NFκB) to the nucleus and the initiation of transcription of inflammatory mediators. Alternately, the independent pathway leads to the activation of interferon regulatory factor 3 (IRF3) as well as NFκB (Coll and O'Neill, 2010; Verstrepen et al., 2008). Basically, both pathways lead to the secretion and stimulation of pro-inflammatory cytokines and other immune mediators. The signaling is quenched by endocytosis of TLR4, along with LPS, to an endosome where it is then degraded (Saitoh, 2009). Current research indicates that apart from the signaling proteins, lipid rafts are essential for the TLR4 signaling (Figure 2) and permeability through the membrane to occur (Olsson and Sundler, 2006; Pfeiffer et al., 2001; Triantafilou et al., 2002; Triantafilou et al., 2004).

Lipid rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol-and sphingolipid-enriched domains that compartmentalize cellular signaling processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interaction (Pike, 2006). They are specialized membrane domains, which are rich in saturated fatty acids, cholesterol, and glycosphingolipids (Brown and London, 1998; Pike, 2003). Some proteins are thought to be preferentially localized in the raft region, particularly GPI anchored proteins like CD14 (Brown and Rose, 1992). Lipid rafts have been shown to act as a membrane signaling hub for many receptors and have been implicated in forming the signaling complex in T cell signaling (He and Marguet, 2008; Janes et al., 2000; Yaqoob, 2009). Lipid rafts have also been implicated in endocytosis of pathogens (Manes et al., 2003). Interestingly, TLR4 has been shown to localize to these membrane raft
domains upon LPS stimulation and disruption of LPS signaling occurs if the lipid raft is
dissociated (Triantafilou et al., 2002). Further saturated and unsaturated fats have been
shown to reciprocally modulate the TLR4 localization into lipid raft and its signaling.
Saturated fatty acids stimulate the TLR4 to localize into rafts and start the inflammatory
signaling cascade whereas n-3 PUFA’s prevent the stimulation and localization into lipid raft
(Wong et al., 2009).

**MicroRNA Regulation of LPS Signaling**

MicroRNA (miRNA) are small non-coding RNA molecules of endogenous origin
measuring approximately 22-25 nucleotides that regulate gene expression at the post-
transcriptional level (Figure 3) (He and Hannon, 2004). They are encoded in the genome and
can be found in both introns and exons, and RNA polymerase II is the main enzyme
responsible for their transcription (Wahid et al., 2010). The majority of miRNA genes are
located in intergenic regions and oriented in either sense or antisense fashion to already
annotated genes (Zhou et al., 2011). MiRNA genes are transcribed into poly-cistronic
primary miRNAs (Pri-miRNA) which are processed into approximately 70 nucleotide
imperfect stem-loop structure precursors (Pre-miRNA) by the nuclear localized RNase-III
enzyme, Drosha. Pre-miRNA is exported into the cytoplasm through the Ran-GTP dependent
nucleo/cytoplasmic cargo transporter, exportin 5 (Lund et al., 2004). Here they are cleaved
by another RNase-III enzyme, Dicer, into a dsRNA duplex of approximately 21-25
nucleotide strands with only two complementary strands without the stem loop structure. One
strand of the duplex is loaded into argonaute proteins to produce the effector RNA induced
silencing complex which helps the miRNAs reach their target (Khvorova et al., 2003).

miRNA bind to the 3' untranslated region of the mRNA through imperfect complementarity at multiple sites which leads to either decreased translational efficiency or decreased mRNA expression. Of these two processes, decreased mRNA expression has been shown to account for approximately 84% of the decreased protein production (Guo et al., 2010). Further, very highly conserved miRNA have been shown to bind to several hundred distinct mRNA indicating the promiscuous nature of miRNA. Additionally, they have the potential to regulate most of the transcribed mRNA in a mammalian cell (Baek et al., 2008). It is predicted that approximately 30-50% of all mammalian protein-coding genes are targeted by miRNA and almost all the cellular processes have been shown to be miRNA regulated (Filipowicz et al., 2008). Because miRNA cannot completely knockdown the function of a target mRNA, it is assumed that miRNA fine tune gene expression rather than exerting complete control over any specific target (Contreras and Rao, 2012).

With regard to the innate immune response, recent research indicates that miRNA are expressed in immune cells and evidences have been published showing their ability to regulate an inflammatory reaction (O'Neill et al., 2011). An inflammatory stimulant such as LPS increases the expression of proteins which interact with miRNA and cytokines decrease the expression of Dicer, which is necessary for processing miRNA (Ma et al., 2011). miRNA expression could be affected through TLR signaling and could also be dependent on the transcription factor NF-κB (O'Neill et al., 2011; Zhou et al., 2011). The major miRNAs which are under the exclusive regulation of NF-κB and thus play significant role in the inflammatory process include miR-146a, mir-155, and mir-21 (Boldin and Baltimore, 2012).
These act as both positive and negative regulators of inflammatory pathways resulting in enhancing or decreasing an immune response (Lindsay, 2008). Further, the adaptive immune response is also regulated by miRNA. Extensive miRNA regulation has been shown during the selection and differentiation of T and B lymphocytes (O'Connell et al., 2012).

Recent evidences also indicate that diet could modify the expression of miRNA associated with cancer (Saini et al., 2010). High saturated fats have been shown to up-regulate mir-143, which plays a major role in the pathophysiology of obesity (Takanabe et al., 2008). Nutritional amounts of polyphenols have also been shown to affect miRNA expression in liver (Milenkovic et al., 2012). Further, n-3 PUFA supplementation in a rat model of colon cancer can suppress the expression of miRNA (Davidson et al., 2009). Therefore, dietary factors can be used to regulate miRNA expression which could modify subsequent expression of genes involving various physiological pathways depending on the nature of the dietary supplement.

**Gastrointestinal Tract Function**

The lumen of the gastrointestinal tract (GIT) is considered a space outside the body because of its continuity with the external environment. Gastrointestinal track has the arduous task of absorbing the nutrients that are essential for the organism while preventing the absorption of substances that are not needed and harmful to the system. The GIT primarily serves two important functions, absorbing nutrients from the lumen and forming a barrier between the luminal contents and systemic circulation. Primarily, the intestinal tract aids in the digestion and absorption of proteins, carbohydrates, lipids, vitamins, minerals, and water. A single layer of intestinal epithelial cells (IECs), which line the intestine selectively
absorbs most of the nutrients needed through active and passive processes with the help of specific transporters or carrier proteins. For example, glucose and fructose are absorbed through Na-dependent glucose transporter 1 and glucose transporter 5, respectively. Water is absorbed through aquaporin receptors, and amino acids and di- and tri-peptides are absorbed through numerous transporter proteins located on the apical and basolateral membranes. Additionally, the GIT serves as a major excretory organ which helps in waste products including excessive nutrients and toxic substances secreted by the biliary system. All these functions becomes more difficult given the fact that only a single layer of IEC acts as a selective permeability barrier throughout the intestinal tract.

The epithelial or intestinal integrity is critical for maintaining a physical barrier between the intestinal lumen and the body. This is dependent largely on the junctional complexes connecting enterocytes together and is achieved via a well-organized intercellular array of tight junctions, adhesion junctions, and desmosomes surrounding the apical region of epithelial cells. Cell to cell adhesion and tight junctions are regulated by the membrane spanning proteins claudin, occludin, zonula occudens (ZO) 1 and 2, and cingulin (Oswald, 2006; Turner, 2006). Additionally, adhesion junction proteins, such as E-cadherin, also contribute to gut integrity. Tight junctions are the most apical junctions between two epithelial cells that are formed by claudin and occludin family protein strands along with other protein complexes (Chiba et al., 2008; Denker and Nigam, 1998). It is becoming clear that there are different claudin isoforms that participate in intestinal barrier function. Together, claudin and occludin proteins are attached to actin cytoskeleton through other proteins, such as ZO-1 and junction adhesion molecules (Nusrat et al., 2000; Turner, 2006). It was initially thought that tight junctions form a physical barrier without any cellular
regulation, but recent research indicates that tight junction proteins are dynamic and very well regulated; intracellular translocation of tight junction proteins from the cell membrane and back occurs regularly during normal cellular processes (Shen et al., 2011).

**Intestinal LPS Permeability**

Circulating LPS can be derived from the environment where the bacteria can be ingested along with feed and water or through respiration (Spaan et al., 2006). The other major source is the commensal bacteria in the GIT particularly members of the Enterobacteriaceae such as *E. coli* and *Salmonella*, which is a rich source of Gram negative organisms (Ley et al., 2006; Wiznitzer et al., 1960). The bacterial population is very scarce in the stomach because of the acidic environment, but the numbers increase exponentially the more distal the location along the intestinal tract from duodenum to colon (Magalhaes et al., 2007; Tlaskalová-Hogenová et al., 2004). The main entry point for pathogenic bacteria, LPS, mycotoxin, and other pathogens is via the digestive tract. Thus, the intestine forms a major physical barrier to prevent pathogens and toxic compounds from entering the mucosa and circulation and then activating the immune system.

The permeability of LPS from the GIT lumen to the systemic circulation is not fully understood, but two primary routes exist. The first mode is through the paracellular route where the LPS permeability occurs through tight junctions formed between two intestinal epithelial cells (Drewe et al., 2001; Hietbrink et al., 2009). Various factors have been shown to regulate the permeability properties of the intestinal tight junction barrier (Shen et al., 2011). When animals are under stress or have intestinal inflammation, small quantities of
luminal contents including LPS, commensals, and pathogens may enter the epithelium and circulation through the tight junctions. These pathogens and MAMPs can stimulate the localized secretion of pro-inflammatory cytokines, including TNF-α and IL-1β from immune and intestinal epithelial cells. Consequently, these inflammatory and stress responses may cause the phosphorylation of myosin light chain by myosin light chain kinase, which results in the contraction and opening of the intestinal epithelial tight junctions and increases intestinal permeability (Chen et al., 2006; Moriez et al., 2005; Turner et al., 1997; Turner, 2009, 2011).

Disruption of tight junctions and increased paracellular permeability by oxidative stress has also been demonstrated in IEC. Treating Caco-2 intestinal epithelial-like cells with the oxidant hydrogen peroxide, increases barrier permeability and leads to a redistribution of ZO-1 and occludin (Sheth et al., 2009). Interestingly, Caco-2 cell monolayers treated with LPS increases lipid peroxidation and paracellular permeability (Courtois et al., 2003). The increased permeability can be reversed by treatment with the antioxidant butylated hydroxytoluene. This indicates that LPS itself can decrease intestinal barrier function by a mechanism that is mediated by oxidative stress. Further indicating a link between redox status and intestinal barrier function is the finding that treating Caco-2 cells with bile salts (e.g., Cholic acid) increases paracellular permeability by increasing reactive oxygen species (Araki et al., 2005). Blocking the increase in reactive oxygen species with the antioxidant, N-acetyl cysteine, prevents the decrease in trans-epithelial electrical resistance (TER) in bile acid treated IEC. Bile acid treatment leads to ZO-1 and occludin redistribution and the increased permeability can be reversed by 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride, a myosin light chain kinase inhibitor, indicating a linkage
between cellular redox status and tight junctions.

Under normal physiological conditions, tight junction barrier integrity remains intact, and luminal contents and transport of molecules across the tight junctions is very well regulated (Edelblum and Turner, 2009). Nevertheless, metabolic stress and environmental stresses, such as heat stress, have been reported to cause increased intestinal permeability or ‘leaky gut’ (Lambert et al., 2002; Lambert, 2004; Singleton and Wischmeyer, 2006). However, the pathways through which tight junction proteins are regulated by these conditions are not fully characterized.

Intestinal and systemic diseases are associated with leaky epithelial barrier and increased intestinal permeability to LPS. The TER of cell monolayers or intestinal epithelial membranes is a good indicator of the degree of tight junction organization and gut integrity. Pigs challenged with LPS showed altered intestinal TER compared to their controls, indicating changes have occurred in intestinal integrity and junctional organization (Albin et al., 2007). Furthermore, treatment with the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has effectively been shown to prevent reduced TER induced by the pro-inflammatory cytokines, interferon-γ and TNF-α, and prevent the redistribution of occludin and ZO-1 (Li et al., 2008). Also, DHA treatment of Caco-2 monolayers has been shown to increase paracellular permeability via the intracellular redistribution of the tight junction proteins (Roig-Perez et al., 2004).

The second mode of intestinal LPS and bacteria permeability is via transcellular permeability occurring through epithelial cells (Neal et al., 2006; Tomita et al., 2004). Further, evidence indicates that lipid rafts are required for the recruitment of TLR4 and that receptor-mediated endocytosis is a key mechanism of transcellular permeability of bacteria
and LPS in in many cell types (Ancuta et al., 2008; Chassin et al., 2008; Triantafilou et al., 2002). Initially, it was thought that IEC did not have the necessary receptors to recognize the innate immune ligands like MAMPs and damage associated molecular patterns. However, research over the past 10 years has discovered that IEC do play a major role in the recognition of pathogens and endotoxin, and IEC express specific receptors including TLRs, nucleotide oligomerization domain receptors, and RIG-I-like receptors (Cario et al., 2000; Cario, 2005; Santaolalla et al., 2011). The expression of mRNA for all of the TLRs, which are expressed by immune cells, have been described in human IEC (Abreu, 2010b). The confounding issue regarding the presence of TLR in IEC is that they are expressed on the apical side of the membrane; however, they are not believed to be continually activated by the presence of luminal LPS. Research with IEC has shown that TLR4 is present on the apical and basolateral membranes, as well as within the golgi apparatus (Abreu, 2010a; Cario and Podolsky, 2006; Hornef et al., 2003). However, the overall consensus regarding the TLR4 location and expression indicates that IEC have a hypo-responsiveness towards LPS and that the location of TLR4 within the cell may be a major contributing factor for the hypo-responsiveness (Vamadevan, 2010).

Both paracellular and transcellular mode of LPS permeability are two important ways through which most of the LPS enters systemic circulation from the gut. The gut is the first line of defense against LPS and if compromised via nutrition, stress or metabolic state, LPS permeability can increase (Clark et al., 2009; Liu et al., 2009; Suganuma et al., 2002). A greater understanding of gut LPS permeability will allow for the development of nutritional and pharmacological mitigation strategies to avert the negative effects of LPS.
Lipopolysaccharide Detoxification

After crossing the intestinal barrier, LPS is transported by both lymph and blood; however, most of the LPS is transported to the liver through the portal vein where a major portion of the LPS detoxification process occurs (Lemaire et al., 1999; Olofsson et al., 1986; Van Leeuwen et al., 1994). If the amount of LPS entering the GIT overwhelms the barrier function of IECs and the detoxification capacity of liver, endotoxemia ensues (Olofsson et al., 1985). Mammals have developed an elaborate system to tolerate and detoxify LPS at either the mucosal surface or in systemic circulation. Lipopolysaccharide tolerance can also occur by the down-regulation of proteins that participate in LPS signaling and the innate immune response (Fan and Cook, 2004). Bile plays an important role in detoxifying LPS because of the detergent action of bile salts in the intestinal lumen. Furthermore, after LPS detection by hepatocytes and Kupffer cells in liver, active and inactive forms of LPS may be transferred to the bile and excreted into the lumen (Lóránd, 2004; Maitra et al., 1981). Approximately 7% of the absorbed LPS is excreted through bile. Munford (2005) describes four mechanisms through which LPS may be neutralized. First, there are molecules that bind LPS and prevent it from engaging TLR4. Second, there are enzymes that degrade lipid A to decrease its activity. Third, LPS can be deactivated following its uptake by the liver. Lastly, there are target cell adaptations that modify the response to LPS. Moreover, reports show that incubation of LPS with plasma makes it less pyrogenic and less inflammatory (Rall et al., 1957; Rudbach and Johnson, 1964; Ulevitch and Johnston, 1978). Specific plasma proteins are able to bind LPS and this is speculated to aid in the inactivation and detoxification of LPS (Brade and Brade, 1985; Johnson et al., 1977; Rudbach and Johnson, 1966). Serum amyloid A has been shown to increase during the acute phase response, and
also binds to LPS monomers and eliminates this toxin via the liver (Coetzee et al., 1986; Emmanuel et al., 2008). Additionally, proteins such as collectins, along with bactericidal permeability increasing protein and neutrophil granules, are also plasma proteins that bind and neutralize LPS (Chaby, 2004; Munford, 2005).

Intestinal chylomicrons, which are involved in transporting the absorbed fatty acids, have been shown to promote the absorption of LPS (Ghoshal et al., 2009). However, chylomicrons have been reported to mitigate the toxic effects of LPS by binding the LPS and promoting its inactivation via contact and the action of bile (Harris et al., 1993; Read et al., 1993). Further, LBP can bind to the chylomicrons and enhance the binding of LPS to the chylomicrons, which helps in reducing its bioactivity (Vreugdenhil et al., 2003). Binding of LPS to the chylomicron helps in its recognition by low density lipoproteins (LDL) and LDL-associated receptors present in hepatocytes, which promote the endocytosis of LPS into the cell and its rapid clearance from circulation (Harris et al., 2002). Presence of apolipoprotein E in the chylomicrons is also protective against LPS because it delivers the LPS directly to hepatocytes, bypassing Kupffer cells and their pro-inflammatory cytokine production (Van Oosten et al., 2001). Lipopolysaccharide is also found to bind with high density lipoprotein (HDL) (Ulevitch et al., 1979). The role of HDL in detoxifying LPS seems to be controversial. It is suggested that HDL aids in sequestering and detoxifying LPS, but also makes it more difficult to clear from circulation (Birjmohun et al., 2007; Vreugdenhil et al., 2003). Further, LPS may be transferred from HDL to LDL with the help of LBP and phospholipid transfer proteins. The transfer of LPS to LDL results in dyslipidemia and the loss of HDL’s capacity to bind cholesterol leading to metabolic diseases (Levels et al., 2005).

A major detoxification mechanism for LPS is by enzyme modification via
acyloxyacyl hydrolase (AOAH). This hydrolase enzyme is classified as a lipase and is present in macrophages, dendritic cells, neutrophils, Kupffer cells in liver and renal cortical tubule cells (Erwin and Munford, 1991). Interestingly, AOAH can be produced by the renal cortical tubule cells where it is secreted into the urine and can deacylate and neutralize LPS (Feulner et al., 2004). Acyloxyacyl hydrolase selectively removes the secondary fatty acyl chains attached to the primary chains in the lipid A moiety producing a LPS structure that is capable of binding MD2/TLR4 but doesn’t initiate the signal or only can be a partial agonist (Lu et al., 2005). It is believed that AOAH plays a role in mediating macrophage tolerance to LPS because AOAH mRNA levels are increased in LPS -primed and -tolerant macrophages versus LPS-naïve macrophages (Mages et al., 2007). When compared with wild-type mice, mice that lack AOAH and are challenged with LPS have enlarged livers and sustained hepatic cytokine production, indicating that this enzyme prevents prolonged inflammatory reaction to LPS (Shao et al., 2011). Regarding agriculturally relevant species, AOAH activity is increased during localized inflammation in cattle, and its activity has been localized to neutrophils (McDermott and Fenwick, 1992). The regulation of AOAH by stressors and diet, together with its direct role in intestinal detoxification warrants further investigation in livestock.

Further evidence that enzyme modification of LPS plays a role in LPS neutralization and detoxification is supported by recent reports that intestinal alkaline phosphatase (AP) directly deactivates LPS (Bates et al., 2007; Goldberg et al., 2008). Mechanistically, AP deactivates LPS by dephosphorylating the diphosphoryl moiety of lipid A, rendering it inactive (Koyama et al., 2002; Munford et al., 2009; Poelstra et al., 1997). Alkaline phosphatase had been shown to inactivate LPS in zebrafish (Bates et al., 2007) and its
activity is increased in inflamed intestinal tissue (Sanchez de Medina et al., 2004). Also, there is debate regarding how AP dephosphorylates LPS and evidence is limited in livestock as to its role in detoxification. The expression and activity of intestinal AP can be modulated by stress and dietary factors (Lalles, 2010). Dietary lipids regulate the activity of intestinal AP. For example, jejunal AP activity was greater in pigs fed a diet high in saturated fat (i.e., 15% beef tallow) versus pigs fed a diet high in unsaturated fat (i.e., 15% corn oil) (Dudley et al., 1994). Another example indicating that AP is regulated by dietary lipids is that n-3 fatty acid rich cod liver oil has been shown to increase the secretion of intestinal AP (Kaur et al., 2007). Interestingly, this may be explained by the increased expression of resolvin-E1, an anti-inflammatory n-3 fatty acid lipid mediator, which induces AP activity (Campbell et al., 2010). Furthermore, high dietary fat consumption reduces intestinal AP activity in obesity-prone rodents (de La Serre et al., 2010). Interestingly, the decrease in ileal AP activity is associated with an increase in plasma LPS and increased inflammation as assessed by myeloperoxidase activity (de La Serre et al., 2010).

Mechanistically, the alteration of intestinal AP by dietary lipids may be mediated by pro-inflammatory cytokines, such as IL-1β and TNF-α, which inhibit the induction of AP (Malo et al., 2006). Stress and disease in livestock may decrease intestinal AP via reductions in feed intake (Goldberg et al., 2008; Lalles and David, 2011). It has been observed that weaning pigs at a young age (i.e., 10 d) decrease both the expression and activity of AP in the jejunum compared to pigs weaned at 28 days of age (Lackeyram et al., 2010). This same age period near weaning is also associated with decreased feed intake and increased intestinal pro-inflammatory cytokine expression (Pie et al., 2004), perhaps both of which are responsible for decreased intestinal AP expression and activity that occurs with weaning in
pigs. Altogether, dietary factors and stressors likely impact intestinal and systemic inflammation and LPS concentrations via alterations in mechanisms of detoxification and neutralization.

**Dietary Fat and Inflammation**

Fatty acids (FA) are monocarboxylic acids usually having an aliphatic chain length between C_4 and C_{22} and consumed as individual fatty acids or part of larger lipid molecules such as triacylglycerol, phospholipids, and sphingolipids. Fatty acids are concentrated in energy (Mangold, 1995). Apart from their main function as energy storing molecules, FA are an integral part of phospholipids which are part of the cell membrane and essential for structural integrity, used as lubricants, and also used as signaling molecules (Mangold, 1995; Tvrzicka et al., 2011). Based on the chain length FAs can be classified as short chain (6 or less carbons), medium chain (6-12 carbons), long chain (13-21 carbons), and very long chain (greater than 22 carbons) FA. Further, whether they have double bonds in their structure will determine if they are classified as saturated, monounsaturated or polyunsaturated FA (*Table 1*). Polyunsaturated fatty acids (PUFA) are classified into n-3 and n-6 (more commonly referred to as omega-3 and omega-6) FA depending on the carbon number of the first double bond from the methyl end (Calder, 2008; Tvrzicka et al., 2011). Linoleic and α-linolenic acid are the simplest members of n-6 and n-3 family, respectively. These two fatty acids are considered essential fatty acids as they cannot be synthesized by mammals because they lack the Δ5, Δ6, and Δ15 desaturase enzymes necessary for synthesizing these fatty acids (Hornstra et al., 1995). So, both fatty acids need to be supplemented through dietary sources
either through plant foods or marine sources. After ingestion, mammals can metabolize linoleic acid into long chain n-6 PUFA like arachidonic acid (AA; 20:4n-6) and α-linolenic acid into n-3 PUFA like EPA (20:4n-3) and DHA (22:6n-3) (Calder, 2012).

Recent research indicates that short chain FA, particularly lauric (C:12) myristic (C:14) and palmitic (C:16) are the ones which form the fatty acyl chains of lipid A or the endotoxic component of LPS. Saturated FAs have been shown to activate the LPS receptor TLR4 and consumption of high saturated fat diets have been postulated to lead to a chronic inflammatory state and associated metabolic diseases (Erridge et al., 2002; Raetz and Whitfield, 2002b; Schaeffler et al., 2009; Vaarala et al., 2008).

Eicosanoids are inflammatory mediators synthesized from n-3 and n-6 PUFA cleaved from cell membrane phospholipids where these fatty acids play a structural role (Calder, 2012). Arachidonic acid is the major n-6 PUFA in the membrane phospholipids and is cleaved by phospholipase A2, resulting in the release of free AA. Arachidonic acid acts as a substrate for inflammatory mediator enzymes lipoxygenase (LOX) and cyclooxygenase (COX) I and II. This results in the secretion of prostaglandins, thorombaxanes and leukotrienes which play important roles in the inflammation signaling (Fetterman and Zdanowicz, 2009; Kremmyda et al., 2011). Prolonged consumption of n-3 PUFA, such as EPA and DHA, also leads to enrichment of cell membranes at the expense of arachidonic acid which will have positive health consequences. Further, EPA and DHA also compete with AA as substrates for both COX and LOX enzymes (Figure 4). This result in the secretion of either eicosanoids which are 10-100 fold less pro-inflammatory properties or anti-inflammatory mediators resolvins, ultimately resulting in reduced pro-inflammatory reactions (Chapkin et al., 2008a; Kremmyda et al., 2011).
Interestingly, n-3 PUFA also have many other beneficial effects including antagonizing the activation of pro-inflammatory transcription factor, NF-κB, and decreasing the secretion of inflammatory cytokines such as TNF-α and IL-6 (McMurray et al., 2011). Additionally, n-3 PUFA enhances the anti-inflammatory transcription factor PPAR-γ leading to attenuated immune response (Sampath and Ntambi, 2005). Further, n-3 PUFA incorporation into the cell membrane causes increased fluidity which will have positive health consequences. Recent evidences indicate that n-3 PUFA supplementation leads to its incorporation into cell membrane microdomains called lipid rafts which act as a signaling hub for numerous important cellular activities (Ma et al., 2004). Incorporation of n-3 PUFA in lipid rafts occurs at the cost of AA and saturated fatty acids, which has the potential to reduce inflammation. The unsaturated fatty acids, particularly long chain PUFAs have a “kink” in their structure because of the unsaturated bonds, which prevents the compact structure formation needed for lipid raft. The intercalation of PUFAs into the cell membrane results in the dissociation of lipid rafts and decreases the LPS signaling which might be beneficial to the animals (Chapkin et al., 2008b; Shaikh et al., 2012; Wassall and Stillwell, 2008).

Implications of Intestinal LPS and Inflammation

The GIT is the major site of nutrient uptake. The nutrient transport function of the GIT decreases when the intestine is under prolonged inflammatory or metabolic stress. A study looking at small intestinal absorptive function during endotoxemia showed that Na and Cl ions, as well as glucose absorption, was decreased even 24 h after the administration of
LPS (Kanno et al., 1996). Further, marked epithelial inflammation occurs around 6 h after challenge and villous atrophy occurs at 24 h but there are signs of recovery after 7 d. It has also been shown that LPS challenge results in decreased absorption of various sugars and amino acids (Albin et al., 2007; Flinn et al., 2010; Meng et al., 2005). One mechanism for this decreased transport might be through the inhibition of Na-dependent system of transport, as well as a decrease in the Na\(^+/\)K\(^+\) ATPase activity (Abad et al., 2001; Amador et al., 2007a; García-Herrera et al., 2003). Further, the pro-inflammatory cytokine, TNF-\(\alpha\), has been shown to decrease the absorption of galactose (Amador et al., 2007b).

Overall, LPS mediated inhibition of nutrient absorption seems to be manifested by several interrelated signaling cascades including those involving protein kinase C, protein kinase A, and mitogen activated protein kinases, as well as proteasomal degradation (Amador et al., 2008; García-Herrera et al., 2008). While the animal itself is trying to fight the cause of the stress, the intestine develops a reduced ability to transport nutrients and carry out other functions. The end result is an increased catabolic cascade and degradation of muscle proteins (Daiwen et al., 2008; Webel et al., 1998) to support gluconeogenesis and increased whole body metabolic energy demands.

Because of its strategic position between the luminal microbes and essentially sterile systemic circulation, the intestine needs to possess excellent immune capabilities to defend against any pathogenic attack. Thus, evolutionarily, the intestine developed an extensive immune system network. The GIT can be classified as the largest immune organ in the body (Collins et al., 1998; Targan et al., 2003). There are specific lymph nodes that are part of gut-associated lymphoid tissue placed in the submucosal layer to defend against any invading pathogens. A variety of mononuclear phagocytes, such as monocytes, macrophages, and
dendritic cells, are present in the gut associated lymphoid tissue, as well as dispersed throughout the sub-epithelial connective tissue, the lamina propria. Interestingly, these immune cells, when isolated from inflamed tissue, display pro-inflammatory profiles and secrete cytokines such as TNF-α (Bar-On et al., 2011). A typical intestinal inflammatory response progresses through the following steps. A leaky intestinal epithelial barrier allows luminal commensal organism components to enter the sub-mucosa and stimulate local immune cells. Dendritic cells, through their PRRs, recognize commensal constituents as pathogenic components and initiate the activation of T cells and natural killer cells. The immune cells can also be activated by their own PRRs. This leads to the secretion of regulatory cytokines by T cells, which in turn, stimulate the secretion of TNF-α, IL-1β, and IL-6 by macrophages. Natural killer cells also play an active role by secreting cytokines, as well as causing tissue damage (Baumgart and Carding, 2007). The interplay between immune cells, LPS, and cytokines can augment the inflammatory state of the intestine.

During an inflammatory response, nutrient partitioning is redirected towards meeting the metabolic requirements of the immune system. Inflammation is associated with increase in body temperature and an increase in 1 °C equates to a 13 % increase in basal metabolism (Kluger, 1978). The rise in the levels of pro-inflammatory cytokines, such as TNF-α and IL-1β, have been shown to decrease feed consumption (Plata-Salamán et al., 1996), rates of weight gain, and efficiency of feed utilization (Evock-Clover et al., 1997; Steiger et al., 1999). Thus, LPS-associated inflammation results in an estimated 30 % increase in energetic costs and leads to a significant negative nitrogen balance because of protein breakdown and decreased weight gain (Lochmiller and Deerenberg, 2000). Further, multiple immune challenges occurring simultaneously lead to a cumulative reduction in growth performance
too (Hanssen et al., 2004).

All the evidence indicates that a significant decrease in feed intake occurs during an inflammatory challenge. Appetite regulation is a complex process and it occurs mainly through the neuronal control through the vagus nerve or through hormonal control via the secretion of leptin, ghrelin, cholecystokinin, and glucagon-like peptide 1. The hypothalamus receives and integrates these signals and brings about the desired effect of altered appetite control (Cummings, 2006; Sartin et al., 2011). The appetite regulation under an immune challenge might occur through either one or both of these mechanisms. During most disease conditions in livestock, there is a reduction in feed intake accompanied by an increase in metabolic rate, which is significantly different than fasting because during fasting the decrease in feed intake is accompanied by decreased metabolic rate (Sartin et al., 2011). The inflammatory cytokines secreted upon an LPS challenge decrease feed intake and nutrient transport by acting on the somatotrophic axis (Johnson, 1997, 1998). Tumor necrosis factor-α has been shown to be present in the central nervous system after an immune challenge with LPS which indicates that it could act on the appetite regulatory center directly (Sakumoto et al., 2003). The appetite-stimulating neurotransmitters in the hypothalamus, such as neuropeptide Y and Agouti-related protein, maybe reduced or unchanged, whereas appetite-inhibiting neurotransmitters including proopiomelanocortin and cocaine- and amphetamine-regulated transcript are increased during immune challenges. During disease stress, the latter may promote α-melanocyte stimulating hormone suppression of appetite via the MC4 receptors to decrease appetite (Sartin et al., 2008; Sartin et al., 2011). This mechanism of action results in typical sickness behavior such as decreased appetite and increased energy expenditure (Grossberg et al., 2010).
The other plausible mechanism by which appetite is regulated under an LPS challenge and inflammation is that LPS and other TLR4 ligands, such as saturated fatty acids, have been shown to activate enteroendocrine cells that act as nutrient sensors in the intestine. This leads to the secretion of appetite regulating peptides, such as cholecystokinin and glucagon-like peptide 1, from the enteroendocrine cells which act on the satiety centers in the hypothalamus and ultimately results in reduced feed intake and nutrient absorption from the intestine (Bogunovic et al., 2007; de Lartigue et al., 2011). While this has been shown in the cell lines, further research is needed to prove this theory in vivo.

**Summary and Conclusion**

The literature reviewed herein describes how luminal LPS enters the circulation and its effects on gastrointestinal function and animal performance (Figure 5). Additionally, we briefly describe plausible mechanisms of LPS detoxification and neutralization. Even at low concentration, LPS is a potent stimulator of pro-inflammatory cytokine production from various cell types within the body, not just immune competent cells. The resulting immune activation and associated inflammation makes LPS an important factor that is commonly overlooked in livestock production. However, more research is needed to understand how LPS enters the circulation and its effect on metabolism and energetics. Additionally, research describing how stress and nutrition modulate LPS permeability and clearance in agricultural relevant species is warranted.
**Literature Cited**


Figure 1. Simplified structure of lipopolysaccharide (LPS) from Gram negative bacteria such as *Escherichia coli*. Lipopolysaccharide contains a distal ‘O’ polysaccharide region, a core polysaccharide region divided into outer and inner core and an interior lipid A component through which LPS is inserted into the cell membrane. ‘O’ polysaccharide region is highly variable and contains approximately 10 to 25 repeated units and made up of common hexose (Hex) sugars. Outer core polysaccharide contains common hexose sugars such as glucose (Glc) and galactose (Gal) whereas inner core polysaccharide contains unusual sugar such as 3-deoxy-D-manno-octulosonic acid (Kdo). Lipid A structure is explained in the text. Arrows with acronyms AP (alkaline phosphatase) and acyloxyacyl hydrolase (AOAH) indicate the cleavage points where these enzymes cleave the phosphate and secondary fatty acyl chains respectively. GlcN - N-acetyl glucosamine; Hep - Heptose.
Figure 2. Pathways of TLR 4 signaling. Lipopolysaccharide is recognized by TLR4 which causes its localization to lipid raft. From lipid raft, TLR4 signals either through a MyD88 – dependent pathway or TRIF-dependent pathway which results in the translocation of NF-κβ or IRF3 to the nucleus and transcription of inflammatory cytokines. Adapted from (McGettrick and O’Neill, 2010; Uematsu and Akira, 2007)
Figure 3. MicroRNA biogenesis and function in animal cells. MicroRNA is transcribed as primary miRNA and then cleaved in the nucleus by RNase enzyme Drosha to a pre-miRNA which is exported to the cytoplasm. In cytoplasm Dicer cleaves the Pre-miRNA to a 21-25 nucleotide dimer. This dimer is loaded into RNA induced silencing complex and one strand is destroyed and the other strand binds to the target mRNA either by repressing the translation or by destabilizing the mRNA. Adapted from (Lodish et al., 2008)
Figure 4. Production of eicosanoids by n-3 and n-6 PUFA. Even though the pathway and the enzymes remain the same, n-3 PUFA produces less potent or anti-inflammatory eicosanoids. Dotted arrows indicate a decrease in synthesis. AA, arachidonic acid; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; LOX, Lipoxygenase; COX, Cyclooxygenase; CYP, Cytochrome 450 enzymes. (Adapted from (Stipanuk, 2006))
Figure 5. A summary of intestinal endotoxin/LPS permeability and inflammation on gut integrity and function. Gram negative bacteria in the intestine releases LPS during growth, division, and death (1). Endotoxin/LPS may be free or bound to proteins such as LPS binding protein (LBP) in the lumen. Recruitment of toll-like receptor 4 (TLR4), and associated proteins to membrane lipid raft regions allow receptor mediated endocytosis of bacteria and LPS in cells (2). Intracellular endotoxin/LPS may be transported bound to organelles (i.e., golgi) or albumin proteins in the cytosol (3). Opening of tight junctions (TJ) and increased paracellular permeability of LPS (4) can occur due to intestinal inflammation or stress. Increased proinflammatory cytokine secretion and activation of innate and adaptive immune cells and intestinal inflammation occurs from LPS transported across the intestinal barrier (5). Secreted cytokines may enter the IEC through the basolateral side, resulting in increased inflammation and the activation of myosin light chain kinase (MLCK) and phosphorylate-myosin light chain (P-MLC). Together, this causes the disruption of tight junction (TJ) complexes (6) and increased paracellular LPS permeability. After sensing of LPS via TLR4, suppression of nutrient transport and enteroendocrine cell signaling (7) can reduce appetite via the depolarization and secretion of appetite regulating neuropeptides such as cholecystokinin (CCK) and glucagon like peptide-1 (GLP-1).
### Table 1. General classification and major sources and functions of common fatty acids

<table>
<thead>
<tr>
<th>Fatty acid class</th>
<th>Nomenclature</th>
<th>Major sources</th>
<th>Important functions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Lauric</td>
<td>C12:0</td>
<td>Coconut oil</td>
<td>Energy storing molecule</td>
</tr>
<tr>
<td>- Myristic</td>
<td>C14:0</td>
<td>Coconut oil, butterfat, cod liver oil</td>
<td>Structural integrity</td>
</tr>
<tr>
<td>- Palmitic</td>
<td>C16:0</td>
<td>Palm oil, lard, butterfat, beef tallow, cotton seed oil, cod liver oil</td>
<td>Signaling molecules</td>
</tr>
<tr>
<td>- Stearic</td>
<td>C18:0</td>
<td>Beef tallow, lard, butterfat, flax seed oil</td>
<td>Structural integrity</td>
</tr>
<tr>
<td>- Arachidic</td>
<td>C18:0, C20:0</td>
<td>Peanut oil, corn oil</td>
<td>Energy storing molecule</td>
</tr>
<tr>
<td><strong>Monounsaturated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Oleic</td>
<td>C18:1n-9</td>
<td>Olive oil, almond oil, canola oil, peanut oil, lard, beef tallow</td>
<td>Energy storing molecule</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Structural integrity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Signaling molecules</td>
</tr>
<tr>
<td><strong>n-3 Polyunsaturated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- α-Linolenic (ALA)</td>
<td>C18:3n-3</td>
<td>Flaxseed oil, canola oil, soybean oil, walnut</td>
<td>Structural integrity</td>
</tr>
<tr>
<td>- Eicosapentaenoic (EPA)</td>
<td>C20:5n-3</td>
<td>Sardines, salmon, certain algae</td>
<td>Production of anti-inflammatory eicosanoids such as prostaglandins, leukotriene’s and resolving’s.</td>
</tr>
<tr>
<td>- Docosahexaenoic (DHA)</td>
<td>C22:6n-3</td>
<td>Sardines, salmon, certain algae</td>
<td>Structural integrity</td>
</tr>
<tr>
<td><strong>n-6 Polyunsaturated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Linoleic (LA)</td>
<td>C18:2n-6</td>
<td>Sunflower oil, corn oil, soybean oil, cottonseed oil, sesame oil, peanut oil, canola oil</td>
<td>Structural integrity</td>
</tr>
<tr>
<td>- Arachidonic (ARA)</td>
<td>C20:4n-6</td>
<td>Chicken, egg, beef tallow, lard</td>
<td>Production of pro-inflammatory eicosanoids such as prostaglandins and leukotriene’s.</td>
</tr>
</tbody>
</table>
CHAPTER 3: DIETARY OIL COMPOSITION DIFFERENTIALLY MODULATES INTESTINAL ENDOTOXIN TRANSPORT AND POSTPRANDIAL ENDOTOXEMIA

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Abstract

Background: Uptake of intestinal derived endotoxin and the subsequent endotoxemia can be considered major predisposing factors for diseases such as atherosclerosis, sepsis, obesity and diabetes. Dietary fat has been shown to increase postprandial endotoxemia. Therefore, the aim of this study was to assess the effects of different dietary oils on intestinal endotoxin transport and postprandial endotoxemia using swine as a model. We hypothesized that oils rich in saturated fatty acids (SFA) would augment, while oils rich in n-3 polyunsaturated fatty acids (PUFA) would attenuate endotoxin transport from the intestine and reduce circulating concentrations.

Methods: Postprandial endotoxemia was measured in twenty four pigs following a porridge meal made with either water (Control), fish oil (FO), vegetable oil (VO) or coconut oil (CO). Blood was collected at 0, 1, 2, 3 and 5 hours postprandial and measured for endotoxin. Furthermore, ex vivo ileum endotoxin transport was assessed using modified Ussing chambers and intestines treated with either no oil or 12.5% (v/v) VO, FO, cod liver oil...
Ex vivo mucosal to serosal endotoxin transport permeability (Papp) was then measured by the addition of fluorescent labeled-lipopolysaccharide.

**Results:** Postprandial serum endotoxin concentrations were increased after a meal rich in saturated fatty acids and decreased with higher n-3 PUFA intake. Compared to the no oil control, fish oil and CLO which are rich in n-3 fatty acids reduced ex vivo endotoxin Papp by 50% (P<0.05). Contrarily, saturated fatty acids increased the Papp by 60% (P=0.008). Olive and vegetable oils did not alter intestinal endotoxin Papp.

**Conclusion:** Overall, these results indicate that saturated and n-3 PUFA differentially regulate intestinal epithelial endotoxin transport. This may be associated with fatty acid regulation of intestinal membrane lipid raft mediated permeability.

**Key words:** Dietary fat, Endotoxin, Intestine

**Background**

The link between dietary fat and endogenous blood endotoxin has attracted increased medical and biomedical interest over the last few years. Furthermore, hyperphagia, increased adiposity and metabolic changes associated with high fat feeding can be recapitulated in mice chronically infused with LPS for four weeks [1]. It has been reported that the structure of fat consumed (emulsion vs. free oil) changes the extent of endotoxemia and that altering the composition, structure and quality of dietary fats could improve health [2]. In healthy humans, postprandial plasma endotoxin concentrations increase on average 18% after a high fat meal (approximately 380 kcal from fat, 42 % of total energy) compared to the fasted state [3]. These authors concluded that increased postprandial LPS may contribute to the
development of postprandial inflammation and disease. Ghanim et al. [4, 5] also showed that in healthy adults, high fat, high carbohydrate meal (~900 kcal) increased postprandial plasma LPS concentrations by 70%. However, Laugerette et al. [6] recently reported that dietary oil composition differentially modulated murine inflammation and endotoxin permeability. These authors also showed that fat composition, not quantity in the diet (22 vs. 3%) was critical in modulating plasma endotoxemia. Collectively, these data show that dietary fat intake and composition is able to modulate blood endotoxin and that this is associated with acute inflammation and the metabolic diseases of obesity and diabetes.

Both Gram positive and Gram negative bacteria are present in large quantities in the intestine. Interestingly, the total quantity of endotoxin, which is the gram negative bacterial outer cell wall component, in the intestine alone could be up to one gram [7]. Even very small quantities of endotoxin, pico-gram scale, in the systemic circulation have the potential to elicit an inflammatory response in humans and animals [8]. Endotoxin is also synonymously referred to as lipopolysaccharide (LPS), and both of these compounds are major immunogens that elicit an inflammatory response in numerous tissues and cell types via their recognition through pathogen-associated molecular patterns (PAMPs) and Toll-like receptors in the innate immune system [9]. Lipopolysaccharide is thought to enter circulation by permeability across the intestinal epithelium either via paracellular pathways through the openings of intestinal tight junctions between two epithelial cells or by a transcellular pathway [7]. Transcellular permeability and the associated endocytosis of intestinal derived endotoxin may be facilitated by intracellular signaling processes mediated by the innate immune receptor complex CD14/Toll like receptor 4 (TLR4)/MD-2, in association with the cell membrane micro domain lipid raft [10]. Furthermore, circulating LPS concentrations
may also be augmented by permeability coupled to dietary lipids and chylomicrons [11].

In recent years accumulating research has investigated the link between dietary fat and endogenous LPS in relation to metabolic inflammation [12, 13]. Current evidence suggests that dietary fat augments circulating endotoxin concentrations and the resultant postprandial endotoxemia leads to low-grade systemic inflammation which has been implicated in the development of several metabolic diseases [1, 3, 14]. Intestinal derived endotoxin and the subsequent acute endotoxemia are considered major predisposing factors for inflammation associated diseases such as atherosclerosis, sepsis, obesity, type 2 diabetes and Alzheimer's [15-17]. However, the ability of different types of oil and fatty acids to facilitate uptake of intestinal endotoxin has been poorly characterized. Interestingly, saturated and n-3 polyunsaturated fatty acids (PUFA) have been shown to reciprocally modulate the LPS receptor, TLR4 signaling and its redistribution to cell membrane micro domains lipid rafts [18]. This is postulated to be due to saturated fatty acids (SFA) such as lauric and myristic acid being part of the fatty acyl side chain composition of lipid-A component of LPS and the ability of n-3 PUFA to reduce the potency of LPS when substituted in place of saturated fatty acids in lipid-A [19, 20]. Thus, there is clear linkage between fatty acids (saturated, n-3 polyunsaturated, monounsaturated) and LPS signaling.

Therefore, the aim of this study was to assess the effects of various dietary fats on in vivo and ex vivo intestinal LPS permeability and circulating concentrations using the pig as a biomedical model. We hypothesize that oils rich in saturated fatty acids (SFA) would augment, while the oils containing the n-3 PUFA (docosahexaenoic acid [DHA] and eicosapentaenoic acid [EPA]) would attenuate, intestinal endotoxin permeability and postprandial endotoxemia.
Methods

Materials and Animals

All the chemicals used for this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. All animal use and procedures were approved by the Iowa State University Institutional Animal Care and Use Committee.

Effect of dietary oil on postprandial serum endotoxin concentration

Twenty four pigs (49 ± 7 kg BW) were raised on a typical corn-soybean diet that met or exceeded their nutrient requirements [21] and randomly allocated to one of four treatments. The treatments consisted of 500 g ground corn-soybean meal dough made up with either 1) 50 ml water (Control); 2) 50 ml FO (Spring Valley Inc., UT); 3) 50 ml VO (Hy-Vee Inc., IA); or 4) 50 ml CO (Spectrum Naturals Inc., NY). After an overnight fast, six pigs were fed one of each porridge meal. Pigs voluntarily consumed the porridge meal with in ten minutes after the feed was offered in front of them. Blood was collected at 0, 1, 2, 3, and 5 hours postprandially by jugular venipuncture into pyrogen-free vaccutainer tubes using a sterile needle. Proper precautionary measures were taken to prevent external contamination of blood. Serum was separated by centrifuging at 2000 × g and was stored at -80 °C until further analysis in pyrogen-free tubes.

Serum endotoxin concentration was measured using the end point fluorescent assay using the recombinant factor C (rFC) system (Lonza™, Switzerland). Briefly, the serum samples were diluted 1000 times and 100 µl of the samples or standards were added to a 96 well plate and incubated at 37 °C for 10 min. Thereafter, 100 µL of rFC enzyme, rFC assay buffer and rFC substrate were added at a ratio of 1:4:5 to the plate and an initial reading were
taken followed by 1 h incubation at 37 °C. The relative fluorescence unit (RFU) for each well was determined (excitation 380 nm and emission 440 nm). A positive control from the assay kit was used to ascertain the validity of the assay and the concentration of the endotoxin was interpolated from the standard curve constructed from the standards and corrected for sample dilution.

**Ex vivo intestinal integrity and LPS permeability**

Freshly isolated ileum segments from eleven pigs (21-28 days old) were placed in chilled Krebs-Henseleit buffer (consisting of, in mmol/L: 25 NaHCO₃, 120 NaCl, 1 MgSO₄, 6.3 KCl, 2 CaCl₂, 0.32 NaH₂PO₄; pH 7.4) for transport to the laboratory while under constant aeration. Intestinal tissues were then stripped of their outer serosal layer and immediately mounted into modified Ussing chambers (Physiologic Instruments Inc., San Diego, CA and World Precision Instruments Inc. New Haven, CT). Each chamber and intestinal segment (0.71 cm²) was bathed on its mucosal and serosal sides with 5 ml of Krebs-Henseleit buffer and constantly gassed with 95% O₂-5% CO₂ mixture. Chambers were connected to a pair of dual channel current and voltage electrodes containing 3% noble agar bridges and filled with 3 M potassium chloride to measure electrophysiological parameters of the intestinal membranes or to measure the mucosal to serosal permeability of endotoxin. Transepithelial resistance (TER) was not different across pigs, indicating no differences in paracellular permeability or leaky gut (data not shown).

To rule out any influence that bile acids may have on intestinal integrity, TER and macromolecule permeability was first tested on isolated ileum samples that were incubated with porcine bile acid (0, 3, 6 and 9 mg/ml) for thirty minutes. Thereafter, FITC-labeled
dextran (FITC-Dextran, 4.4 KDa) mucosal to serosal transport was measured as described previously [22]. Briefly, the mucosal chambers were challenged with 2.2 mg/mL FITC-Dextran and chamber samples from both sides were collected every 10-15 min for eighty minutes. The relative fluorescence was then determined using a fluorescent plate reader (Bio-Tek, USA) with the excitation and emission wavelengths of 485 and 520 nm, respectively. Thereafter, an apparent permeability coefficient (Papp) was calculated for each treatment: 

\[ P_{\text{app}} = \frac{dQ}{dt \times A \times C_0} \]

Where: \( dQ/dt \) = transport rate (µg/min); \( C_0 \) = initial concentration in the donor chamber (µg/ml); \( A \) = area of the membrane (cm²).

The effect of dietary fat on endotoxin permeability was studied using ex vivo permeability of fluorescein isothiocyanate (FITC) labeled-LPS (Escherichia coli 055:B5) mounted into modified Ussing chambers. Briefly, segments of swine intestinal tissues were treated with either 12.5 % (v/v) buffered saline control (CON), FO or CLO manufactured by Spring Valley Inc., UT), VO, CO and OO purchased from Hy-Vee Supermarkets Inc., IA). All oils were commercial retail available and then mixed with 20 mM sodium taurodeoxycholate (bile acid) for micelle formation to simulate the intestinal milieu. Each mucosal chamber was then challenged with 20 µg/mL FITC-LPS and chamber samples were collected every 10-15 min for eighty minutes. The relative fluorescence of each sample was then determined using a fluorescent plate reader (Bio-Tek, USA) with the excitation and emission wavelengths of 485 and 520 nm, respectively. The apparent permeability coefficient was then calculated similar to that described above for FITC-Dextran.
Lipid rafts, dietary oil and ex vivo intestinal endotoxin permeability

To examine the role of lipid rafts in intestinal endotoxin permeability, ileum segments from 16 pigs (56±4 days of age) were mounted in Ussing chambers as described above. Segments were pre-treated with or without 25 mM Methyl-β-cyclo dextrin (MβCD, a synthetic lipid raft modifier) for 30 min. Thereafter, the mucosal chamber was spiked with either saline-bile acid (CON) or Coconut oil-bile acid (12.5% v/v) and the FITC-LPS apparent permeability coefficient was calculated.

Fatty acid analysis

Fatty acid profiles of the dietary oils used to make the porridge were determined and analyzed by GC-MS [23, 24]. One ml oil was mixed with 0.5 mL of 4:1 hexane and 125 µg/L heptadecanoic acid was added to each sample as an internal standard. FAME were analyzed by GC on a Hewlett-Packard model 6890 fitted with an Omegawax 320 (30-m × 0.32-mm i.id. 0.25 um) capillary column. Hydrogen was the carrier gas. The temperature program ranged from 80 to 250°C with a temperature rise of 5°C/min. The injector and detector temperatures were 250°C and 1 µL of sample was injected and run split. Fatty acids methyl esters were identified by their relative retention times on the column with respect to appropriate standards and heptadecanoic acid.

Data analysis

Results are presented as means ± S.E.M and were analyzed with the Proc Mixed procedure of SAS (Cary, NC). In the model, repetition or day of Ussing chamber run was used as a random effect. Statistical significance of difference was analyzed by analysis of variance
(ANOVA) followed by Tukey’s range test for pair wise comparison of all treatment means. Differences were considered significant at P ≤ 0.05 and a tendency at P ≤ 0.10.

Results

Dietary oil fatty acid profiles

The fatty acid composition of the oils used to make the porridge meal and/or in the ex vivo transport study are reported in Table 1. The coconut oil contained high concentrations of saturated fatty acids (89 %), particularly lauric, myristic, and palmitic acids. Olive oil contained a very high content of monounsaturated oleic acid and a moderate amount of palmitic acid, with a saturated fat content of 29 %. Vegetable oil used in this study contained a high quantity (50 %) of arachidonic acid (20:4n6), 32 % oleic acid and 13 % palmitic acid. The fish oil used consisted of 35 % docosahexaenoic acid (DHA) and 19 % eicosapentaenoic acid (EPA), while the cod liver oil contained 32 % palmitic acid, 25 % arachidonic acid, 8.6% EPA and 4.3% DHA. The n6:n3 ratio was highest in the olive oil > vegetable oil > cod liver oil > fish oil > coconut oil.

Effect of dietary oil on postprandial serum endotoxin concentration

To assess the effect of dietary lipids on postprandial serum endotoxin concentrations, pigs received a porridge meal containing either 50 mL of saline, CO, VO or FO. The endotoxin concentration of the various oils used did not differ (data not shown). Change in postprandial serum endotoxin concentration due to different meal treatments are presented in Figure 1A. The overall postprandial serum endotoxin concentrations were significantly lower in the meals constituting saline or FO, with the mean overall serum endotoxin concentration
increasing two-fold over the saturated coconut oil meal treatment (P<0.05, Figure 1B). However, meals made up with VO were not different from the saline, CO or FO treatments (P<0.05). Interestingly, the CO meal significantly elevated serum endotoxin concentrations after 2 hours versus the saline and FO, and these remained elevated at 3 and 5 hour postprandial (P<0.05, Figure 1A).

**Effect of exogenous porcine bile acid on ex vivo intestinal integrity**

Bile acids have been shown to increase the intestinal permeability in cultured Caco-2 cell lines [25]. To rule out the effect that exogenous bile acid may reduce intestinal integrity, freshly isolated pig ileum segments were used to measure TER (Figure 2A) and FITC-Dextran permeability (Figure 2B). As these segments were exposed to increasing concentrations of porcine bile ex vivo, no differences in intestinal integrity were observed (P>0.10, Figure 2). This might be due to the tolerance of intestinal tissues towards bile acid because of previous exposure in vivo contrary to cell cultures where the cells are not exposed to the bile acids previously.

**Effect of dietary oil on ex vivo intestinal endotoxin permeability**

The ex vivo mucosal to serosal ileum endotoxin permeability was assessed using modified Ussing chambers and FITC-LPS permeability assay (Figure 3). Compared to the saline no oil control treatment, the endotoxin Papp was significantly lower in both the FO and CLO treatments (P<0.05). As hypothesized, the higher saturated fat content of CO significantly increased the endotoxin Papp compared to the saline, FO and CLO (P<0.05). However, mucosal treatment with VO and OO did not differ from the saline or n-3 treatments (P>0.05), but still attenuated endotoxin Papp versus the coconut oil treatment (P<0.05, Figure 3). Transepithelial resistance was not different due to ex vivo oil treatment (data not shown).
Effect of lipid raft modification of saturated fat induced endotoxin permeability

To test the hypothesis that destabilization of intestinal lipid rafts would decrease saturated fat induced endotoxin permeability, colon samples were pretreated with the lipid raft modifier methyl-β-cyclodextrin (MβCD) and coconut oil ex vivo. FITC-LPS permeability was then measured (Figure 4A). As expected, the CO treatment significantly augmented the colon endotoxin Papp compared to the saline control (P<0.05). However, the endotoxin Papp was significantly reduced with the MβCD treatment compared to the saline control (1.54 vs. 0.07, P=0.04). In the presence of MβCD and CO, the colon Papp was attenuated three fold from the CO alone treatment (P<0.05). Importantly, colon integrity and permeability as measured by transepithelial resistance was not altered by either short term CO, MβCD and the combination of these two treatments compared to the saline control (P=0.98, Figure 4B).

Discussion

In Western diets, vegetable, canola and palm oils are common components of the diet and to a lesser extent, long chain n-3 PUFA (DHA and EPA) oils from algal or marine sources [26, 27]. In recent years, the development of obesity, inflammation, atherosclerosis and other metabolic diseases has been linked to low grade endotoxemia associated with high dietary fat and energy intake [3, 14, 28-30]. Further, these studies and others have raised questions on whether this diet induced endotoxemia reflects changes in energy and fat content of the diet, intestinal permeability or diet induced changes in gut microbiota. In the current study, we used ex vivo and in vivo methods to examine intestinal permeability to endotoxin as it relates to dietary oil composition. All pigs were clinically healthy and raised
on typical commercial swine corn-soybean diets. We observed no differences in intestinal integrity due to our in vivo or ex vivo treatments [31]. Importantly, we only examined the acute actions of a meal or oil bolus treatment and did not conduct a prolonged feeding trial in an attempt to change the pig microbiota populations or the fatty acid profiles of tissues.

We hypothesized that dietary intake of oils rich in DHA and EPA would attenuate intestinal LPS permeability and postprandial circulating endotoxin. We found that dietary cod liver and fish oils attenuated serum endotoxin concentrations compared to the coconut oil and the endotoxin levels in these pigs were similar to the control group (Figure 1). To the best of our knowledge, there are no other studies that have shown this effect of DHA and EPA on endotoxin transport and blood endotoxemia.

Interestingly, only one paper has examined the effects of dietary oil composition on endotoxin uptake and related inflammation [6]. However, contrary to our results, the report by [6] Laugerette et al states that rape seed (canola) and sunflower oil, with high unsaturated fatty acid content, augmented plasma endotoxemia by 50-75%. Cani et al. [1], also observed a similar increase in endotoxemia in mice orally administered corn oil with or without LPS compared to water alone. However, we observed no change in serum postprandial endotoxin concentration or intestinal endotoxin transport due to dietary vegetable oil compared to the saline control (Figures 1 & 3). Further work is needed to explain these discrepancies between the two studies. Additionally, we also observed a significant increase in postprandial endotoxemia after a porridge meal mixed with coconut oil (Figure 1). Again, this contradicts data presented by Laugerette et al. [6] in which palm oil, high in saturated fatty acids, had no effect on plasma endotoxin concentrations in mice. However, these authors did report an increase in plasma LPS binding protein and argued that this protein is a better marker of
endotoxemia due to the short half-life of circulating endotoxin. One issue is that LPS binding protein can be up regulated by inflammation and acute stress as well as both gram positive and negative infections. As the magnitude of LBP response goes down with multiple episodes of infection [32], this could be a result of the agonistic effects of saturated fatty acid on pro-inflammatory signaling and not circulating endotoxin.

Gram negative bacteria, particularly those found in the distal ileum and colon might be one of the major sources for circulating endotoxin [33]. It has been estimated that a single cell of Escherichia coli contains approximately $10^6$ Lipid A or endotoxin molecules and a typical human intestinal tract could harbor approximately one gram of endotoxin [33-35]. Interestingly, the bacterial population in the intestine is not static. Multiple studies have shown that bacterial composition shifts to either gram positive majority or gram negative majority based on the composition of the diet consumed [36-38]. A majority of these studies show that consuming high saturated fat diet for longer period results in higher gram negative bacterial populations and high fiber diets results in gram positive bacterial populations. Even though there are no known techniques available to identify which bacterial species the LPS molecules originated from, it is believed that this endotoxemia is due to a raise in Enterobacteriaceae [37, 39]. Laugerette et al. [6], reaffirmed this and showed that fatty acid composition of different dietary oils can alter intestinal microbiota populations. Moreover, these authors demonstrated that feeding a diet high in palm oil which is rich in SFAs significantly increased the gram negative bacteria Escherichia coli groups, which can be significant source of endotoxin in the cecal content of mice compared to milk fat, rape seed and sunflower oil fed diets.
During intestinal stress, ischemia, inflammation and diseases, paracellular permeability occurs through the tight junction, as known as “leaky gut” [40]. Alternatively, transcellular or intracellular permeability can occur, particularly in healthy individuals [41]. Transcellular endotoxin transported across a cell membrane has been shown to occur via TLR4 and soluble GPI anchored receptor CD14 in a lipid raft mediated mechanism [42, 43]. Additionally, chylomicron associated LPS permeability has also been suggested to play a key role in intestinal LPS transport from the intestinal epithelial cell [11, 44, 45]. Importantly, we observed no decrease in intestinal integrity which might enhance paracellular permeability as assessed by transepithelial resistance or FITC-dextran permeability due to treatment or short term raft destabilization (Figure 4B). These data suggest that under healthy intestinal epithelial conditions, endotoxin is most likely transported via lipid raft mediated endocytosis.

The signaling and transport process for endotoxin is initiated in specialized membrane micro domains called lipid rafts [43]. Lipid rafts are membrane regions rich in cholesterol, glycolipids, sphingolipids and saturated fatty acids, which result in a ‘rigid’ membrane structure compared to the adjacent ‘fluid’ regions [46]. In immune cells, LPS triggers the recruitment of TLR4 into the lipid raft, where it interacts with CD14 and other associated proteins such as MD-2 resulting in an inflammatory signaling cascade [47, 48]. Thus, the two major consequences of preventing endotoxin recognition by dissociating the lipid raft to attenuate TLR4 recruitment include reduced inflammatory signaling and attenuated LPS permeability. We observed that if intestinal lipid rafts are dissociated ex vivo with MβCD, then endotoxin permeability is attenuated in the ileum (Figure 4). Interestingly, saturated fat induced endotoxin permeability is also significantly reduced. Stimulation of
TLR4 receptor has been shown to result in the endotoxin permeability across the intestinal epithelial cells [41]. TLR4 is not only implicated in the transcellular permeability of LPS but also for live bacteria [49]. Since saturated fatty acids and n-3 PUFA can reciprocally modulate TLR4 signaling [50], the fatty acid composition of oil in the diet has the potential to increase or decrease endotoxin transport. Altogether, these data suggest that apical endotoxin transport in the intestines is arguably raft mediated in healthy individuals.

In vitro experiments show clearly that n-3 PUFA disrupt TLR4 signalling and the activation of NFκB by LPS in a murine monocytic cell line [51]. Moreover, DHA modulates TLR4 signaling in vitro in RAW 264.7 macrophages and 293T cells [50], human monocytes and dendritic cells [52] and adipose tissue. We have previously shown in pigs that dietary EPA and DHA are effective means of influencing the inflammatory status and pathways influenced by TLR4 signaling induced by LPS [53] and in altering intestinal function [24, 54]. Therefore, one could postulate that antagonizing TLR4 recruitment to lipid rafts and it’s signaling by DHA and EPA, or stimulating these processes with saturated fatty acids, would lead to increased endotoxin transport and circulating postprandial endotoxin.

Another mechanism through which LPS can enter the circulation is through micelles. Since the LPS side chains are made up of fatty acids, LPS can be incorporated into the micelles and transported into the intestinal epithelial cell [55]. In intestinal epithelial cells, chylomicrons transport the absorbed lipids into various parts of the body. High fat administration has been shown to proportionately increase the endotoxin content of the chylomicron indicating that high fat consumption indeed enhances higher endotoxin permeability into the intestinal epithelial cell and incorporation into chylomicron [11, 56]. Furthermore, even though the mechanism is not clear, high intake of fat has been shown to
cause internalization of tight junction proteins and increase in the paracellular permeability to macro molecules including endotoxin [37]. Even though, this mode of endotoxin permeability cannot be ruled out, we speculate that the rate of incorporation of fatty acids into micelles would not vary due to oil composition. Therefore, we propose that the difference in intestinal endotoxin permeability we observed is primarily transcellular permeability that involves lipid rafts and receptor mediated endocytosis [43].

In conclusion, these data suggest that dietary oils can differentially alter intestinal LPS permeability. Oils rich in DHA and EPA seem to attenuate LPS permeability, while oils high in saturate fatty acids seem to augment LPS permeability. Furthermore, intestinal LPS permeability in healthy subjects may be regulated through a lipid raft mediated mechanism. Saturated fatty acids may be stabilizing the lipid rafts allowing for greater LPS permeability. Even though a transient increase in the permeability level may not cause an immediate significant physiological effect, this has been shown to result in transient increase in the pro-inflammatory cytokines. Additionally, the biological relevance of the transient increase in serum endotoxins and their associated impact on health need to be studied further.

Acknowledgements

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List of abbreviations used

CON: Control; VO: Vegetable oil; CLO: Cod liver oil; CO: Coconut oil; OO: Olive oil; FITC: fluorescein isothiocyanate; TLR4: Toll like receptor 4; TER: Transepithelial resistance; LPS: Lipopolysaccharide; FA: Fatty acids; PAMPs; Pattern associated molecular patterns; PUFA: Polyunsaturated fatty acids; SFA: Saturated fatty acids; Papp: Apparent permeability co-efficient; FAME: Fatty acid methyl esters; MβCD: Methyl-beta-cyclo dextrin; rFC: Recombinant factor C; RFU: Relative fluorescent unit; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; IEC: Intestinal epithelial cell.

Competing Interests

The authors declare that they have no competing interests.

Author’s contributions

VM, JH and NKG designed and conducted research presented. NKG was the principle investigator and the corresponding author and both NKG and JH obtained funding for this work. VM was the graduate student supervised by NKG whom conducted most of the animal
and laboratory work and wrote the manuscript. JH and NKG supervised and revised the manuscript. All authors read and approved the final manuscript.

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Figure 1. Dietary oil alters postprandial serum endotoxin concentrations in pigs fed a single dietary oil-based meal. A) Delta change in serum endotoxin concentrations. B) Mean postprandial serum endotoxin concentration. Different letters (a,b) represent significant difference at P<0.05. Treatments are a porridge meal made with either no oil (saline), fish oil (FO), vegetable oil (VO) and coconut oil (CO). n=6 pigs/treatment. Data are means ± S.E.M.
Figure 2. The effect of increasing porcine bile acid concentration on ex vivo intestinal integrity and permeability. A) Transepithelial resistance (TER) and B) FITC-Dextran transport (4.4 kDa). Freshly isolated ileum samples were mounted into modified Ussing chambers and incubated with the indicated concentration of bile acid for 30 minutes and then FITC-Dextran was added to mucosal side. Permeation coefficient was calculated by taking samples from chambers every 10-15 minutes and measuring the amount of fluorescence. Different letters represent significant difference at P < 0.05. n = 11 pigs. Data are means ± S.E.M.
**Figure 3.** Ex vivo endotoxin transport in pig ileum tissue exposed to different dietary oil treatments. Freshly isolated ileum samples were mounted into modified Ussing chambers and mixed with the indicated oils and 20mM bile acid for 120 minutes and FITC-LPS transport was measured. Different letters represent significant difference at $P < 0.05$. $n = 11$ per treatment. Data are means ± S.E.M.
Figure 4. Lipid raft modifier methyl beta cyclodextrin (MβCD) decreases ex vivo endotoxin transport. A) Endotoxin transport and B) transepithelial resistance was measured using Ussing chambers in ileum tissues treated with either control (water), MβCD, coconut oil, or coconut oil plus MβCD. Tissue (n= 7 /trt) were pretreated with these treatments for 30 min before FITC-LPS transport was assessed. Different letters represent significant difference at P < 0.05. Data are means ± S.E.M.
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¹Source coconut oil was (Spectrum Naturals, NY)
²Source fish oil was (Spring Valley, UT)
³Source olive oil was (Hy-Vee, IA)
⁴Source vegetable oil was (Hy-Vee, IA)
⁵Source cod liver oil was (Spring Valley, UT)
CHAPTER 4: DIETARY N-3 FATTY ACIDS REDUCE INTESTINAL LIPOPOLYSACCHARIDE PERMEABILITY AND ALTER MEMBRANE RAFT LIPID COMPOSITION AND FUNCTION

A manuscript prepared for submission to Journal of Lipid Research

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Abstract

Fish oil and its n-3 polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been shown to antagonize lipopolysaccharide (LPS) or endotoxin signaling and alter membrane lipid raft size and order in immune cells. Therefore, we hypothesized that enrichment of intestinal epithelial membrane phospholipids with DHA and EPA would alter membrane composition and function. This enrichment would also reduce intestinal lipid raft mediated LPS permeability and signaling. Further, we also hypothesized that in the presence of a peak inflammatory challenge, intestinal LPS permeability and signaling would be dramatically altered due to desensitization of Toll like receptor 4 (TLR4) recruitment into lipid rafts. Twenty pigs (22±2.4 kg) were fed two diets: 1) control (CON); 2) CON plus 0.5% Gromega™ (Dn3, JBS
United Inc.), high in DHA and EPA n-3 PUFA. After eight weeks, CON and Dn3 pigs were
challenged (n=5 pigs/trt) with either an intramuscular injection of *Escherichia coli* LPS
(LPS; 10 µg/kg BW) or saline (SAL). Four hours after LPS or SAL, pigs were euthanized
and ileum and colon segments mounted into Ussing chambers to measure ex vivo FITC-LPS
apparent permeability coefficient (Papp) as a marker of LPS permeability. Ileum and colon
mucosa were assessed for n-3 FA enrichment, lipid raft isolated and membrane localization
of TLR4 determined. Compared to the CON, pigs fed Dn3 had increased ileum and colon
EPA, DHA and total n-3 PUFA content (P<0.05; 200, 250, 300%, respectively). Overall,
ileum LPS permeability did not differ due to FA or LPS treatments. However, Dn3-SAL
treated pigs tended to have decreased LPS permeability by 37% compared to the CON-SAL
pigs (P=0.06). Pigs challenged with LPS had attenuated colon LPS permeability (P=0.02).
Pigs fed Dn3 also had reduced colon LPS permeability compared to the CON (P=0.03; 2.0
vs. 7.4 Papp, respectively). Compared to CON-SAL pigs, ileum and colon TLR4 recruitment
into lipid raft micro domains was decreased in the Dn3-SAL pigs. However, LPS reduced
ileum lipid raft TLR4 protein in CON, but not in Dn3 fed pigs. Localization of TLR4 into
lipid raft did not differ in the colon of CON-LPS and Dn3-LPS groups. These data indicate
that DHA and EPA decrease TLR4 recruitment into intestinal lipid raft. This may explain
how n-3 PUFA attenuate receptor mediated LPS permeability and febrile response.
Furthermore, reduced lipid raft localization of TLR4 post LPS challenge, may describe an
LPS tolerance mechanism.

*Supplementary key words:* Lipopolysaccharide • Endotoxin • Intestine • Lipid raft • n-3
polyunsaturated fatty acids • Pigs
Introduction

Lipopolysaccharide (LPS) and endotoxemia is associated with the prevalence and incidence of inflammation, sepsis, Alzheimer’s disease atherosclerosis and metabolic dysfunctions (1-3). Even though LPS is also synonymously known as endotoxin in the scientific literature, there are biochemical and physiological differences between these two compounds. Therefore, for scientific clarity we will refer to LPS in this chapter.

Lipopolysaccharide is an integral component of the gram negative bacterial cell wall outer membrane. Importantly, gram negative bacteria particularly that found in the distal small intestine, cecum and colon, can be major sources of circulating Lipopolysaccharide (4). However, the intestinal epithelium provides a physical barrier that separates luminal bacteria and other inflammatory molecules from entering the systemic circulation. This is critical, as it has been estimated that a single cell of *Escherichia coli* contains approximately $10^6$ LPS molecules (5).

Lipopolysaccharide is recognized by numerous cell types including immune cells, myocytes, adipocytes and intestinal epithelial cells (IEC), to produce pro-inflammatory cytokines that contribute to the efficient control of invading molecule. Structurally, LPS consists of an O-polysaccharide, core polysaccharide and a Lipid A region which possess the most biological endotoxic activity. Its recognition and signaling is mediated via TLR4, a key membrane pattern recognition receptor that in association with LPS-binding protein (LBP), CD14 and MD2, results in the transmission of the signal into the cell to initiate the inflammatory signaling cascade (6). Moreover, recent work has revealed that for this signal to occur TLR4 protein is recruited in membrane microdomains called lipid rafts following
stimulation by LPS and that subsequent lipid raft integrity is crucial for LPS-induced cellular activation (7).

Lipid rafts are membrane regions rich in cholesterol, glycolipids, sphingolipids and saturated fatty acids (SFA), which result in a ‘rigid’ membrane structure compared to the adjacent ‘fluid’ regions (8). However, these dynamic assemblies of cholesterol and sphingolipids are not only important in signal transduction and partitioning of receptors into raft regions, but also bacterial invasion (9). Interestingly, recent work in T cell and B cell models has demonstrated dietary fatty acids to alter lipid raft structure and function. Moreover, when n-3 polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are packed into raft microdomains, changes in the molecular composition and order of lipid rafts may occur (10-12). Consequently, these disordered-dissociated raft environments due to n-3 PUFAs alter protein clustering and cellular function and may explain the immuno-suppressive and anti-inflammatory benefits of n-3 PUFA.

Therefore, in the present study we hypothesized that enrichment of intestinal epithelial membrane phospholipids with DHA and EPA would alter membrane composition and function. This enrichment would reduce intestinal lipid raft mediated LPS permeability and signaling. Further, in the presence of a peak immune challenge, intestinal LPS permeability and signaling would be dramatically altered due to desensitization of TLR4 recruitment to lipid rafts.
Materials and Methods

Materials. All the chemicals used for the experiment were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The dietary source of n-3 PUFA, Gromega™ (JBS United Inc., Sheridan, IN) contained 14% DHA and 14% EPA of total fat. Anti-TLR4, anti-CD14 and anti-galectin4 and their respective secondary antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA).

Animals and Experimental Design. All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee and adhered to the ethical and humane use of animals for research. Twenty pigs (22 ± 2.4 kg BW) were individually penned and fed one of two diets for eight weeks. Ten pigs were fed a standard corn-soybean control diet (CON) and ten pigs were fed the basal diet supplemented with 0.5 % Gromega™ (Dn3, JBS United Inc., Sheridan, IN), which was high in DHA and EPA (Table 1). Gromega™ was mixed in the basal diet in substitution to corn. Both diets were formulated to meet or exceed the swine nutrient requirements (13). Pigs were fed ad libitum and had free access to water all the time. After 8 weeks of tissue n-3 PUFA enrichment, both the CON and Dn3 groups were fasted overnight, equally sub-divided (n=5/trt) and challenged with either an intramuscular injection of saline (SAL) or lipopolysaccharide (LPS, from Escherichia coli serotype 055:B5, 10 µg/kg BW, Sigma, St. Louis, MO). Following the peak febrile response, as measured by rectal temperature, blood was collected for measuring serum endotoxin concentrations and other inflammatory parameters. Blood was collected by venipuncture in pyrogen-free vacutainer tubes using a sterile needle. Proper precautionary measures were taken to prevent external contamination of blood. Pigs were euthanized via captive bolt and
immediate exsanguination. Immediately following euthanasia, fresh segments of ileum and colon were collected for intestinal function assays. A 20 cm segment of ileum 120 cm from the ileal-cecal junction and a 10 cm segment of proximal colon 60 cm from the rectum were isolated. Serosal stripped segments and mucosal scrapings were collected from both the ileum and colon, snap frozen in liquid nitrogen and stored at -80 °C until further analysis. All fresh and frozen segments of ileum and colon were flushed with ice cold Krebs buffer to remove any undigested food material before freezing as well as before mucosal scrapings collection.

Intestinal Function and Permeability. Fresh segments of the ileum and colon were removed and placed in chilled Krebs-Henseleit buffer (consisting of, in mmol/L: NaHCO3, 120 NaCl, 1 MgSO4, 6.3 KCl, 2 CaCl, 0.32 NaH2PO4; pH 7.4) for transport to the laboratory while under constant aeration until clamped in the modified Ussing chambers. Tissues stripped of outer serosal layers were immediately mounted in a modified Ussing Chamber. Each segment (0.71 cm²) was bathed on its mucosal and serosal sides with Krebs buffer and constantly gassed with 95% O2-5% CO2 mixture and the temperature was maintained at 37°C by circulating water. Each chamber was connected to a pair of dual channel current and voltage electrodes submerged in 3% noble agar bridges and filled with 3M potassium chloride for electrical conductance (Physiologic Instruments Inc., San Diego, CA and World Precision Instruments Inc. Sarasota, FL) to measure tight junction integrity and the mucosal to serosal permeability. A short circuit current was established and stabilized for about 10 minutes and basal measurements like potential difference (PD), resistance (R), short circuit current (I_SC) were taken using the included software (Acquire and Analyze, Physiological
instruments). The measurements were observed in a computer monitor in real-time as line graphs and tissue responses were monitored continuously. Resistance of the tissue was determined by passing a current of 100 µA across the tissue using the electrodes and correcting for the PD deflection for fluid resistance. The current required to nullify the PD present on the tissue is termed $I_{SC}$ and it is a measure of net active ion transport in the intestinal tissue. The $I_{SC}$ of the intestinal tissue was calculated from simultaneous PD and R measurements by using ohm’s law, $I = V/R$, where $I$ is the current in amperes, $V$ is the electromotive force in volts, and $R$ is the resistance in ohms.

Additionally, the mucosal to serosal permeability of LPS was assessed as previously described by Tomita et al (14). Briefly, the mucosal chambers were challenged with 20 µg/mL fluorescein isothiocyanate labeled LPS (FITC-LPS) and chamber samples from both sides were collected every 10-15 min. The relative fluorescence was then determined using a fluorescent plate reader (BioTek, Winooski, VT) with the excitation and emission wavelengths of 485 and 520 nm, respectively. An apparent permeability coefficient (Papp) was then calculated (14) using the area of the membrane and rate of FITC-LPS permeability, where $dQ/dt =$ transport rate (µg/min); $C_0 =$ initial concentration in the donor chamber (µg/mL); $A =$ area of the membrane (cm$^2$):

$$Papp = \frac{dQ}{(dt \times A \times C_0)}$$

**Serum Endotoxin Assay.** Serum LPS concentration was measured by an end point fluorescent assay using the recombinant factor C (rFC) system (Lonza™, Basel, Switzerland). Briefly, the serum samples were diluted 1,000 times and 100 µL of the samples and standards were added to a 96 well round bottom plate and incubated at 37 °C for 10 min. After incubation,
100 µL of rFC enzyme, rFC assay buffer and rFC substrate were added at a ratio of 1:4:5 to the plate and an initial reading were taken followed by 1 h incubation at 37 °C. Thereafter the relative fluorescence unit (RFU) for each well was determined (excitation 380 nm and emission 440 nm). A positive control from the assay kit was used to ascertain the validity of the assay and the concentration of the endotoxin was interpolated from the standard curve constructed from the standards and corrected for sample dilution.

Subcellular Fractionation of the Intestinal Epithelium. Apical membranes were isolated from fresh mucosal scrapings based on divalent cation precipitation method as previously described (15, 16). All the procedures were performed on ice unless otherwise stated. Briefly, the mucosal scrapings were homogenized (1:10 w/v) in a homogenization buffer (containing 2 mM Tris-HCL and 50 mM mannitol, pH 7.1 and protease inhibitor cocktail (Complete, Roche, IN)) using a mechanical homogenizer. The resulting homogenate was centrifuged at 500 x g for 10 min to remove all the unbroken cells and nuclear debris. The resulting supernatant was then further centrifuged at 2,700 x g for 10 min to sediment the mitochondria. The supernatant was then adjudged with 10 mM MgCl₂, incubated on ice for 10 min and centrifuged at 1,150 x g. The supernatant was then further centrifuged at 48,000 x g for 60 min. The resulting pellet was saved and contained the apical membranes. Purity of the apical membrane preparation was ascertained by measuring the alkaline phosphatase activity using quantichrom™ alkaline phosphatase assay kit (DALP-250, Gentaur, Kampenhout, Belgium) according to the manufacturer’s instructions.
Lipid Raft Isolation. Lipid raft fractions from the pig intestinal membranes were obtained according to the method described by Danielsen et al (17) and Nguyen et al (18). Briefly, 1-2 mg/mL of apical membrane protein were dissolved in a Hepes/NaCl buffer (pH 7.1, 25 mM and 150 mM, respectively) containing 1% triton-100 for 10 min on ice. The extracts were then laid on top of equal volume of 80% sucrose with in an ultracentrifuge tube resulting in a final concentration of 40% sucrose. This preparation was layered on top with 30% and 5% sucrose with a volume of 4.3 mL and 1.75 mL respectively. This was centrifuged at 217,000 x g for 20 h at 4 °C and 12 fractions were collected per tube. Each fraction was 800 µL starting with fraction one at the top and twelve at the bottom. The protein concentration of each lipid raft fraction was determined using BCA assay (Pierce, Rockford, IL). Lipid raft fractions were confirmed by cholesterol enrichment and the presence of the intestinal lipid raft marker protein galectin 4. Cholesterol content of the lipid raft fractions were measured using a Cholesterol Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions.

Protein Expression. Lipid raft protein marker galectin 4 and proteins of interest, TLR4 and CD14, were measured by dot blot method. Briefly, 10-20 µg protein of the lipid raft fractions were applied directly on a nitrocellulose membrane by vacuum and then blocked with 5% non-fat dry milk. The primary antibody was added at a concentration according to the manufacturer’s instruction and incubated overnight. The secondary antibody was added at a concentration of 1:10,000 to 1:20,000. Blots were developed by a chemiluminescence detection kit according to the protocol supplied by the manufacturer (Pierce, Rockford, IL).
**Phospholipid fatty acid composition.** Total lipids from lipid raft and non-raft regions of the membrane fractions were extracted by the method of Folch et al (19). Individual phospholipids were separated by one dimensional thin layer chromatography as described by Chapkin et al (10) using silica gel 60 G plates. Developing solvent used was Chloroform/methanol/acetic acid/water (50:37.5:3.5:2, v/v). Individual phospholipids were scraped from TLC plate and spiked with 125 µg/L of heptadecanoic acid (17:0) as an internal standard. Trans-esterification was carried out using 6% methanolic-HCl for 16-18 h while heating at 75 °C. The resulting FAME were then analyzed using gas chromatography by the method described below.

**Fatty acid profiles.** Intestinal fatty acid profiles were determined according to Lepage and Roy (20) and analyzed by GC-MS. Briefly, 0.5 g of tissue was homogenized in 2.5 ml of 4:1 Hexane and 125 µg/L of heptadecanoic acid/L methanol was added to each sample as an internal standard. FAME were analyzed by GC on a Hewlett-Packard model 6890 fitted with a Omegawax 320 (30-m × 0.32-mm i.d, 0.25 µm) capillary column (Sigma-Aldrich, St Louis, USA). Hydrogen was the carrier gas. The temperature program ranged from 80 to 250°C with a temperature rise of 5°C/min. The injector and detector temperatures were 250°C and 1 µL of sample was injected and run splitless. Fatty acids methyl esters were identified by their retention times on the column with respect to appropriate standards.

**Statistical Analysis.** All data are expressed as means ± SEM. The main effects of dietary treatment, challenge and their interaction were determined by the Proc Mixed procedure in
SAS (Cary, NC), and differences were established using the least significant difference. Differences were deemed significant at P < 0.05 and tendency at P < 0.10.

**Results**

**EPA and DHA supplementation leads to enrichment of the intestine.** Pigs were fed a regular corn-soybean meal diet (CON, n=10) or a diet supplemented with 0.5% EPA and DHA (Dn3, n=10) for eight weeks from weaning. Although not the objective of this study, no differences in feed intake or body weight gains were observed between the two treatments (data not shown). Both ileum and colon mucosal scrapings fatty acid profiles were analyzed to ascertain the EPA and DHA enrichment of the intestinal epithelial tissue (Table 2). As expected, the Dn3 group had a significantly higher EPA and DHA in both ileum and colon than the CON group indicating that the intestinal epithelium was enriched with n-3 PUFA’s (Table 2). Interestingly, we saw a corresponding decrease in the n-6 fatty acid, arachidonic acid (AA) in ileum, as well as colon. The n6:n3 PUFA ratio was also reduced in the Dn3 treatment. The 4 h LPS challenge did not affect the fatty acid composition of the tissues (data not shown).

**Dietary n-3 PUFA reduces serum LPS concentration.** Circulating LPS concentrations were measured in serum to assess whether dietary EPA and DHA could attenuate blood endotoxemia. Compared to the CON group, serum LPS concentrations were significantly lower in the Dn3 pigs (P<0.05, Fig. 1). This indicates that long term dietary n-3 PUFA supplementation can modulate endogenous blood endotoxin concentrations.
**Anti-inflammatory effect of EPA and DHA supplementation.** CON and Dn3 pigs were subdivided into two groups (n=5) and challenged with either an intramuscular injection of saline or lipopolysaccharide to study the effect of dietary n-3 PUFA on an inflammatory challenge. A gross assessment of this inflammatory challenge was indicated by the changes in the pig’s febrile response to SAL or LPS (Figure 2). Irrespective of diet, LPS challenged pigs had higher rectal temperatures than SAL group (P<0.001). After 1 h, both the LPS challenged groups had significantly higher rectal temperatures compared to the SAL groups. However, the Dn3 pigs had an attenuated febrile response to LPS compared to the CON-LPS pigs (P=0.011). Interestingly, Dn3 challenged group had a numerically lower febrile response at two hours which became significantly lower than the CON challenged group by 4 hour (P<0.05).

**Ileum integrity increases under EPA and DHA supplementation.** Transepithelial electrical resistance (TER) is a measure of intestinal permeability and integrity. Higher TER values indicate a healthy, less macromolecular permeable intestine with well-formed tight junctions. TER was measured in the ileum of CON and Dn3 pigs before the FITC-LPS permeability. Dn3-SAL group had a higher TER than any other treatment groups indicating supplementation with EPA and DHA improves the health of the intestine. Interestingly, LPS challenge did not change the TER integrity in the CON-LPS, but lowered the TER in the Dn3-LPS group (Fig. 3). This indicates that n-3 PUFA helps in maintaining a healthy intestine under normal circumstances, but not under a systemic immune challenge.
We hypothesized that EPA and DHA enrichment of the intestinal epithelium would mitigate mucosal to serosal permeability of LPS from the intestinal lumen. To test this hypothesis, we used the Ussing chamber model to study the ex-vivo permeability of FITC-LPS in the ileum and colon tissues. In ileum, the Dn3-SAL group tended to have a lower ex-vivo LPS permeability compared to the CON-SAL group (4.7 vs. 1.8). Furthermore, LPS challenges tended to reduce the LPS permeability compared to the CON-SAL (P < 0.10). However, colon LPS permeability (Fig. 4B) in the CON-SAL pigs were higher than the Dn3-SAL pigs (11.4 vs. 3.3, P=0.02). Irrespective of diet, LPS challenge lowered LPS permeability compared to SAL challenged pigs in colon. However, colon LPS permeability did not differ between the CON-LPS and Dn3-LPS pigs (P>0.05).

EPA and DHA enrichment alters the fatty acid composition of ileum and colon lipid rafts. The fatty acid composition of phospholipids in the lipid raft fractions from the ileum (Fig. 5) and colon (Fig. 6) which were separated using thin layer chromatography was analyzed by GC-MS. Lipopolysaccharide challenge did not affect the phospholipid composition in the raft and non-raft fractions and therefore only the CON and Dn3 treatment is shown. The sphingomyelin raft fractions were rich in the n-6 fatty acid, arachidonic acid (AA). The CON raft fractions were numerically higher in AA compared to other Dn3 raft and CON and Dn3 diet non-raft fractions. In the colon, again sphingomyelin AA content was higher in the CON raft fraction versus the Dn3 lipid raft, CON and Dn3 non-raft fractions. Interestingly, EPA and DHA were not detected in the sphingomyelin irrespective of dietary treatment (Fig. 5A and 6A).
Phosphatidyl serine had a better distribution of fatty acids across the fractions. AA was approximately three-fold higher in the CON raft and non-raft fractions of ileum, and two-fold higher in CON lipid raft colon fraction compared to Dn3 counterparts. EPA was detectable across all fractions. In ileum, Dn3 non-raft fraction was ten-fold higher in EPA than CON. In colon, Dn3 raft and non-raft fractions were eight- and eighteen-fold higher than their respective raft and non-raft fractions. DHA concentration, in ileum and colon, was four-fold higher in the Dn3 raft fraction compared to the CON raft. In the non-raft fractions, DHA was 1.5-fold higher in ileum compared to 3-fold higher in colon (Fig. 5B and 6B).

Phosphatidyl ethanolamine had a more even distribution of EPA, and DHA, as well as AA between the raft and non-raft fractions (Fig. 5C and 6C). Ileum AA concentration was 1.8-fold higher in CON non-raft fraction compared to raft. Colon concentration of AA was approximately three- and two-fold higher in both CON raft and non-raft fractions, respectively, compared to Dn3 fractions. EPA was undetectable in CON raft from ileum and in the colon, higher EPA was present in CON non-raft ileum, while only trace amounts were present in CON non-raft colon. Both ileum and colon Dn3 raft and non-raft fractions were enriched with EPA and DHA. DHA was detectable in all the fractions and DHA concentration was four-to six-fold higher in the Dn3 raft fractions compared to the CON.

Phosphatidyl inositol (Fig. 5D and 6D) had a higher AA in non-raft fractions of both ileum and colon and there was a corresponding decrease in the AA concentration in the Dn3 fractions. EPA was not present in any of the lipid raft fractions. EPA concentration was six-fold higher in the Dn3 non-raft group in ileum, whereas present in equal concentration in colon non-raft fractions. DHA was not present in any of the lipid raft groups except colon
Dn3. In the non-raft groups, DHA concentration was ten-fold higher in Dn3 than CON in ileum, and five-fold higher in colon (Figure 5D and 6D).

Phosphatidyl choline non-raft fractions were enriched with AA in both ileum and colon, and raft fractions contained only low quantities of AA (Fig. 5E and 6E). Surprisingly, we couldn’t detect EPA and DHA in raft fractions of both ileum and colon. EPA was rich in non-raft fractions of Dn3 ileum and CON and Dn3 colon. DHA was three-fold higher in both non-raft fractions of ileum and colon compared to CON (Fig. 5E and 6E).

**Dietary n-3 PUFA and LPS challenge alter TLR4 and CD14 localization in the intestinal epithelium lipid rafts.** To pursue the mechanistic aspect of the difference in the LPS between the CON and Dn3 enriched pigs, lipid rafts were isolated from the apical membrane of ileum (Fig. 7) and colon (Fig. 8) mucosal scrapings. Purity of the membrane preparation from contamination by immune cells was ascertained by measuring the alkaline phosphatase levels in both ileum and colon apical membranes (Supplemental figure 1 and 2). Lipid raft and lipid insoluble (non-raft) fractions were identified by the presence of intestinal lipid raft marker protein galectin 4 and cholesterol. In our system and based on galectin 4 and cholesterol content, the raft fractions were identified as fractions 2-7, compared to the non-raft fractions 1 and 8-12. Irrespective of location, treatment with n-3 PUFA decreased TLR4 protein levels in the rafts of pig ileum (Fig. 7) and colon (Fig. 8) fractions. Interestingly, the colon tissues had higher TLR4 expression than the ileum. However, exposure to LPS for four hours reduced TLR4 protein expression in the CON lipid raft fractions, but not the Dn3 fractions. CD14 protein was found predominantly in the lipid raft fractions in both the ileum
(Fig. 7) and colon (Fig. 8). Similarly, LPS challenge also attenuated CD14 expression in these fractions.

**Modifications of lipid rafts reduce intestinal LPS permeability.** Lipid rafts have been shown to be essential for TLR4 recruitment and LPS signaling as well as permeability in immune cells (21, 22). Therefore, we wanted to further test the role of lipid rafts in ileum and colon LPS permeability. To do this, freshly isolated ileum and colon segments were treated on their mucosal side with the lipid raft modifier methyl-β-cyclodextrin (MβCD; 25 mM). Methyl-β-cyclodextrin is a carbohydrate molecule with a pocket for binding cholesterol and its mode of action depletes cholesterol from the lipid raft microdomain to dissociate rafts (9). We hypothesized that by increasing raft dissociation and reducing TLR4 recruitment into the lipid raft microdomain, intestinal transcellular LPS permeability would be attenuated. Thus, we have shown that incubation with MβCD significantly reduced the FITC-LPS permeability in both ileum and colon (Fig. 9). Interestingly, the effect was more pronounced in the colon compared to the ileum. This is indirect evidence that lipid raft play a significant role in intestinal LPS permeability.

**Discussion**

The health benefits of long chain n-3 PUFA such as those found in fish oil and algae, DHA and EPA, have been widely touted (23, 24). However, the biological impact of DHA and EPA enrichment into intestinal cell membranes and on LPS permeability and signaling has not been well established. We hypothesized that enrichment of intestinal epithelial
membrane phospholipids with DHA and EPA would alter membrane composition and function. Furthermore, this n-3 PUFA enrichment would reduce intestinal lipid raft mediated LPS permeability and signaling.

We have previously shown that dietary EPA and DHA are effective in influencing the inflammatory status and those pathways influenced by TLR4 signaling (25). Moreover, this is further supported by the high consumption of EPA and DHA, 7.6 g/d, which inhibited TLR4 and NOD2 signaling pathways and improved intestinal integrity under inflammatory conditions in pigs (26). Although not the main objective of the current study, the Dn3 pigs had a reduced LPS induced febrile and cytokine response to LPS compared to the CON pigs. Altogether, these data supports work that has shown n-3 PUFA to modulate the immune response and to be immuno-suppressive and anti-inflammatory (27, 28).

Interestingly, the signaling process for LPS is initiated in specialized membrane micro domains called lipid rafts (7). Lipid rafts are membrane regions rich in cholesterol, glycolipids, sphingolipids and saturated fatty acids, which result in a ‘rigid’ membrane structure compared to the adjacent ‘fluid’ regions (8). In immune cells, LPS triggers the recruitment of TLR4 into the lipid raft where it interacts with CD14 and other associated proteins such as MD-2 resulting in an inflammatory signaling cascade (29, 30). Thus, preventing LPS recognition by dissociating the lipid raft to attenuate TLR4 recruitment may have two major consequences. Firstly, reduced inflammatory signaling and secondly, attenuated LPS permeability.

Enrichment of the membrane phospholipids with n-3 PUFA have been shown to alter membrane composition and function (26, 31, 32). This is in agreement with our current work in which dietary n-3 PUFA in the Dn3 pigs increased intestinal membrane DHA and EPA
contents compared to the CON pigs. Importantly, n-3 PUFA enrichment results in the dissociation of lipid rafts in T-cells (10, 11) and decreases the recruitment of TLR4 to the raft region in macrophages (28). n-3 PUFA enrichment of phosphatidyl ethanolamine and phosphatidyl serine has been shown to dissociate the lipid rafts in T-cells (10). We also observed a similar result in our study. Therefore, lipid rafts may be a major target of DHA and EPA that lead to the down regulation of TLR4 signaling (33). Although TLR4 recruitment into the lipid rafts has been poorly characterized, we have demonstrated that this does also occur in the pig intestinal epithelium and that DHA and EPA enrichment attenuates TLR4 protein recruitment into raft microdomains. Changes in raft lipid composition by DHA and EPA affect both the size and order of rafts (21) and cellular function (9, 34, 35).

The permeability of gastrointestinal tract LPS has been shown to be modified by high dietary fat and caloric intake in humans and animals (36-38). Mechanistically, both bacteria and LPS intestinal permeability can occur via either a paracellular pathway through tight junction openings or transcellular pathways. Paracellular permeability is often associated with intestinal stress and hypoxia that modifies tight junction complexes and intestinal integrity (39). However, transcellular mediated LPS or bacterial permeability may also be independent to that of paracellular or tight junction facilitated permeability. Bacteria and LPS permeability across the intestinal epithelium has been shown to be mediated by TLR4 present in the IEC (14, 40). Additionally, lipids rafts have been found to be important in transcellular permeability of LPS and bacteria including *E. coli, Salmonella, Listeria* and *Mycoplasma* (41, 42). Thus, bacterial invasion and LPS permeability may exploit endocytic pathways facilitated by clustered lipid rafts. This notion is supported by the fact that when colon or ileum segments are treated with MβCD, we observed a significant reduction in FITC-LPS
permeability. Pretreatment of IEC cells with MβCD has also shown to reduce LPS-mediated NFκB activation in a dose dependent manner which was reversed with cholesterol addition because MβCD acts by decreasing the cholesterol content of the cell membrane (43). Collectively, these data suggest that LPS permeability and TLR4 mediated signaling in intestinal epithelial cells requires the presence of lipid rafts.

An intriguing result from our current study was the observation that a four hour LPS challenge decreased TLR4 localization into the lipid raft fractions. This decreased membrane protein localization must be a mechanism for LPS tolerance. Studies have shown that prior exposure to LPS leads to a transient state of refractoriness to further LPS re-stimulation and decreases cell surface expression of TLR4 (25, 44). However, LPS tolerance studies using human THP-1 cells have shown TLR4 surface expression is unchanged post LPS challenge, but LPS mediated TLR4 mobilization to lipid rafts was attenuated during tolerance (45). Furthermore, this tolerance appears to be protein kinase C (PKC)-ζ and the phosphatase SHIP dependent and is restored upon the restoration of PKC activity (45). Therefore, TLR4 appears to be not constitutively found within the rafts, but recruited following initial stimulation to initiate a receptor response.

In conclusion, our data demonstrates that EPA and DHA supplementation enriches specific intestinal epithelial membrane phospholipids. This results in dissociation of lipid rafts leading to dislocation of LPS signaling proteins like TLR4 and CD14 from the rafts. Thus, giving rise to decreased recognition, permeability and signaling of LPS. This may mechanistically explain why endogenous and exogenous LPS induced inflammatory and febrile responses are attenuated due to dietary DHA and EPA. Our results also indicate that
during an immune challenge, EPA and DHA supplemented animals are well equipped to fight the challenge.

Acknowledgments

VM, JH and NKG designed and conducted research presented. NKG was the principle investigator and NKG, JHH and JDS obtained funding for this work. VM was the graduate student supervised by NKG whom conducted most of the animal and laboratory work. TEW and JHH conducted aspects of the laboratory and data analysis. All authors contributed to the writing of the manuscript, read and approved the final manuscript. The authors would also like to thanks JBS United Inc., for supplying the pigs and feed for this project.

References


Table 1. Feed composition of the experimental diet (% as fed basis)

<table>
<thead>
<tr>
<th>Feed Component</th>
<th>Control</th>
<th>Dn3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>61.11</td>
<td>60.59</td>
</tr>
<tr>
<td>Soybean meal 48%</td>
<td>31.67</td>
<td>31.67</td>
</tr>
<tr>
<td>Meat &amp; bone Meal</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Salt</td>
<td>0.56</td>
<td>0.56</td>
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<tr>
<td>Gromega™ 1</td>
<td>-</td>
<td>0.53</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.41</td>
<td>0.41</td>
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<tr>
<td>L-Lysine</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>Threonine</td>
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<td>Premix 2</td>
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<td>0.36</td>
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<tr>
<td>Selenium</td>
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<td>0.05</td>
</tr>
<tr>
<td>Choline chloride</td>
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<td>0.03</td>
</tr>
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</table>

**Calculated composition**

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<thead>
<tr>
<th></th>
<th>Control</th>
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<tr>
<td>Energy, kcal/kg</td>
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<tr>
<td>Crude Protein, %</td>
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<td>SID Lysine, %</td>
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<td>Available Phosphorus, %</td>
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<td>Ether Extract, %</td>
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<tr>
<td>Crude Fiber, %</td>
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<td>2.36</td>
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1 Gromega™ was supplied by JBS United, Inc., containing approximately 14% EPA and 14% DHA of total fatty acids.
2 Supplied per kilogram of diet: vitamin A, 8364 IU; vitamin D₃, 1533 IU; vitamin E, 45 IU; vitamin K, 2.2 IU; choline, 6.5 mg; riboflavin, 4.2 mg; niacin, 21 mg; pantothenic acid, 17 mg; vitamin B-12, 28 mg; biotin, 1.6 mcg; folic acid, 0.0005 mg; Zn, 112 ppm as zinc sulfate and zinc oxide; Mn, 54 ppm as manganous oxide; Fe, 145 ppm as ferrous carbonate and ferrous sulfate; Cu, 20 ppm as copper chloride; I, 0.76 ppm as ethylenediamine dihydriodide; Se, 0.25 ppm as sodium selenite.
Table 2. Fatty acid composition of ileum and colon of mucosal scrapings in pigs fed either the control diet (CON) or a diet enriched with long chain n-3 PUFA (Dn3).

<table>
<thead>
<tr>
<th>Fatty Acid (%)</th>
<th>CON 1</th>
<th>Dn3 1</th>
<th>P-value 3</th>
<th>CON 1</th>
<th>Dn3 1</th>
<th>P-value 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.23 ± 0.09</td>
<td>0.57 ± 0.09</td>
<td>0.03</td>
<td>1.00± 0.14</td>
<td>0.76± 0.14</td>
<td>0.27</td>
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<tr>
<td>16:0</td>
<td>22.51 ± 0.86</td>
<td>25.29 ± 0.86</td>
<td>0.05</td>
<td>22.41± 0.28</td>
<td>22.32± 0.28</td>
<td>0.83</td>
</tr>
<tr>
<td>16:1</td>
<td>1.66 ± 0.31</td>
<td>2.09 ± 0.31</td>
<td>0.37</td>
<td>2.73 ± 0.32</td>
<td>2.64 ± 0.32</td>
<td>0.84</td>
</tr>
<tr>
<td>18:0</td>
<td>14.85 ± 0.63</td>
<td>16.00 ± 0.63</td>
<td>0.43</td>
<td>23.71 ± 0.74</td>
<td>18.9 ± 0.74</td>
<td>0.0018</td>
</tr>
<tr>
<td>trans-18:1n-9</td>
<td>3.13 ± 0.9</td>
<td>3.12 ± 0.9</td>
<td>0.83</td>
<td>4.43 ± 0.61</td>
<td>4.96 ± 0.61</td>
<td>0.24</td>
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<tr>
<td>cis-18:1n-9</td>
<td>18.99 ± 0.49</td>
<td>19.27 ± 0.49</td>
<td>0.98</td>
<td>22.80 ± 0.25</td>
<td>23.91 ± 0.25</td>
<td>0.18</td>
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<tr>
<td>18:3n-3</td>
<td>0.67 ± 0.07</td>
<td>0.63 ± 0.07</td>
<td>0.70</td>
<td>ND 2</td>
<td>ND 2</td>
<td></td>
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<tr>
<td>20:0</td>
<td>0.00 ± 0.07</td>
<td>0.15 ± 0.07</td>
<td>0.14</td>
<td>0.19 ± 0.08</td>
<td>0.00± 0.08</td>
<td>0.14</td>
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<tr>
<td>20:1</td>
<td>0.08 ± 0.11</td>
<td>0.26 ± 0.11</td>
<td>0.25</td>
<td>0.29 ± 0.12</td>
<td>0.20 ± 0.12</td>
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<td>20:2</td>
<td>0.67 ± 0.03</td>
<td>0.62 ± 0.03</td>
<td>0.13</td>
<td>0.47 ± 0.11</td>
<td>0.47 ± 0.11</td>
<td>0.98</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.57 ± 0.03</td>
<td>0.73 ± 0.03</td>
<td>0.006</td>
<td>0.41 ± 0.08</td>
<td>0.78 ± 0.08</td>
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<tr>
<td>20:4n-6</td>
<td>7.40 ± 0.38</td>
<td>5.83 ± 0.38</td>
<td>0.02</td>
<td>6.17 ± 0.42</td>
<td>4.64 ± 0.42</td>
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<td>20:5n-3 (EPA)</td>
<td>0.00 ± 0.07</td>
<td>1.18 ± 0.07</td>
<td>&lt; 0.0001</td>
<td>0.00 ± 0.04</td>
<td>0.87 ± 0.04</td>
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<td>22:0</td>
<td>0.47 ± 0.03</td>
<td>0.53 ± 0.03</td>
<td>0.15</td>
<td>0.27 ± 0.12</td>
<td>0.21 ± 0.12</td>
<td>0.77</td>
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<tr>
<td>24:0</td>
<td>0.85 ± 0.06</td>
<td>0.98 ± 0.06</td>
<td>0.14</td>
<td>0.81 ± 0.05</td>
<td>1.01 ± 0.05</td>
<td>0.03</td>
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<tr>
<td>24:1</td>
<td>0.37 ± 0.07</td>
<td>0.47 ± 0.07</td>
<td>0.35</td>
<td>0.36 ± 0.12</td>
<td>0.24 ± 0.12</td>
<td>0.51</td>
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<tr>
<td>22:6n-3 (DHA)</td>
<td>0.88 ± 0.24</td>
<td>1.72 ± 0.24</td>
<td>0.006</td>
<td>0.07 ± 0.11</td>
<td>1.31 ± 0.11</td>
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<tr>
<td>Saturated</td>
<td>39.00 ± 1.24</td>
<td>43.37 ± 1.24</td>
<td>0.04</td>
<td>49.66 ± 0.73</td>
<td>44.26 ± 0.73</td>
<td>0.0008</td>
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<tr>
<td>Σ(n-3) PUFA</td>
<td>1.56 ± 0.35</td>
<td>3.98 ± 0.35</td>
<td>0.001</td>
<td>0.07 ± 0.13</td>
<td>2.18 ± 0.13</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Σ(n-6) PUFA</td>
<td>29.35 ± 1.34</td>
<td>24.47 ± 1.34</td>
<td>0.03</td>
<td>15.77 ± 0.98</td>
<td>17.21 ± 0.98</td>
<td>0.33</td>
</tr>
<tr>
<td>(n-6)/(n-3)</td>
<td>20.51 ± 1.57</td>
<td>6.29 ± 1.57</td>
<td>0.0006</td>
<td>46.37 ± 1.7</td>
<td>8.11 ± 1.7</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>

1Fatty acid composition was measured by GC-MS on five pigs per treatment and expressed as a percentage of total fatty acids analyzed (mean fatty acid ± SEM).
2ND, not detected.
3Within tissue, significant difference between CONT verses Dn3.
Fig. 1. Dietary n-3 PUFA decreases endogenous serum LPS concentrations in pigs. Pigs were fed either a control (CON) or DHA and EPA rich (Dn3) diets for eight weeks. n= 5 pigs/trt. Different letters represent significant difference at P < 0.05.
Fig. 2. Dietary n-3 PUFA attenuates the febrile response in pigs induced by lipopolysaccharide (LPS) immune challenge. Pigs fed either a control (CON) or n-3 PUFA (Dn3) diet for eight weeks were immune challenged with LPS 10 µg/kg (LPS) or saline (SAL) control non-challenged. Rectal temperatures were measured every hour to assess the febrile response. Different letters a,b,c represent significant differences (P<0.05) within each hour. n = 5 pigs/trt/challenge/time.
Fig. 3. Effect of dietary n-3 PUFA supplementation and LPS challenge on transepithelial electrical resistance (TER) of the ileum measured in the Ussing chamber in pigs fed either control (CON) or n-3 PUFA (Dn3) diets and immune challenged with either saline (SAL) or Lipopolysaccharide 10 µg/kg (LPS) four hours post-challenge. n= 5 pigs/trt. Different letters represent significant difference at P < 0.05.
**Fig. 4.** Dietary n-3 PUFA supplementation and inflammatory challenge decrease LPS permeability in ileum and colon. Ex vivo ileum (a) and colon (b) LPS permeability in the Ussing chamber in pigs fed either control (CON) or n-3 FA’s rich (Dn3) diets and immune challenged with either saline (SAL) or lipopolysaccharide 10 µg/kg (LPS) four hours post challenge. n= 5 pigs/trt. Different letters represent significant difference at P < 0.05 and * represents tendency P < 0.10.
Fig. 5. Dietary n-3 PUFA supplementation enriches PUFA content of specific phospholipid classes of ileum. Lipid raft fractions were isolated from the apical membrane of ileum, phospholipids were isolated from the fractions using thin layer chromatography. Fatty acid composition of each phospholipid was identified and quantified. Phosphatidyl serine and phosphatidyl ethanolamine were enriched in n-3 PUFA.
Fig. 6. Dietary n-3 PUFA supplementation enriches PUFA content of specific phospholipid classes of colon. Lipid raft fractions were isolated from the apical membrane, phospholipids were isolated from the fractions using thin layer chromatography. Fatty acid composition of each phospholipid was identified and quantified. Phosphatidyl serine and phosphatidyl ethanolamine were enriched in n-3 PUFA.
Fig. 7. Dietary n-3 PUFA supplementation and immune challenge decrease the localization of LPS signaling proteins TLR 4 and CD 14. Lipid raft fractions were isolated from the brush border membrane of ileum, cholesterol and Galectin 4 were used as lipid raft markers. Fractions 2-7 were considered raft or lipid soluble fractions and 8-12 were considered non-raft or lipid insoluble fractions.
Fig. 8. Dietary n-3 PUFA supplementation and inflammatory challenge decrease the localization of LPS signaling proteins TLR 4 and CD 14. Lipid raft fractions were isolated from the apical membrane of colon, cholesterol and Galectin 4 were used as lipid raft markers. Fractions 2-7 were considered raft or lipid soluble fractions and 8-12 were considered non-raft or lipid insoluble fractions.
Fig. 9. Lipid raft modifier methyl-β-cyclodextrin (MβCD) decreases LPS permeability in ileum and colon. Ex vivo ileum and colon LPS permeability was measured in the Ussing chamber in tissues treated with either control (CON) or MβCD for thirty minutes. n= 7 pigs/trt. Different letters represent significant difference at P < 0.05.
Supplemental Fig. 1. Alkaline phosphatase activity in the CON and Dn3 treatment groups in the brush border membrane (BBM) and basolateral membrane (BLM) of ileum indicating the purity of the membrane preparations. Different letters represent significant difference at P<0.05.
Supplemental Fig. 2. Alkaline phosphatase activity in the CON and Dn3 treatment groups in the apical membrane and basolateral membrane (BLM) of colon indicating the purity of the membrane preparations. Different letters represent significant difference at P<0.05.
CHAPTER 5: MATERNAL n-3 PUFA SUPPLEMENTATION IN PIGS
ATTENUATES AN INFLAMMATORY CHALLENGE LATER IN LIFE²

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Abstract

Long-chain n-3 polyunsaturated fatty acids (PUFA) such as DHA and EPA possess strong anti-inflammatory properties. Together with the fact that the maternal diet can have beneficial effects on the offspring later in life, we hypothesized that maternal dietary EPA and DHA exposure would have beneficial anti-inflammatory effects in the offspring later in life. To test this, 30 piglets from the following treatment groups were used in a 3×2 factorial design. Ten pigs whose dams fed diets devoid of DHA and EPA (CON); 10 pigs which were from dams and themselves fed diets containing DHA and EPA (Cn3); and 10 pigs with only maternal dietary exposure to DHA and EPA (Mn3). All diets were corn-soybean meal based and the n-3 PUFA diets were the same supplemented with 0.5 % algal DHA and EPA in place of corn. At ten weeks of age, five pigs from each treatment were challenged with either

²This research was supported by the Iowa Pork Producers Association and the USDA/Iowa State University Nutrition and Wellness Research Center.
saline (SAL) or 10 µg/kg BW lipopolysaccharide (LPS). At 4h post challenge, blood was collected and pigs were euthanized. Overall, the Cn3-LPS and Mn3-LPS groups had a lower febrile and serum tumor necrosis factor-α (TNF-α) response. Further buffy coat mRNA expression of cytokines TNF-α, Interleukin (IL)-1β and IL-10 were attenuated compared to CON-LPS (P<0.05) in these two groups. Additionally, post inflammatory challenge mRNA for the LPS signaling proteins TLR4, CD14, and Myd88 were down-regulated in both n-3 PUFA treatments compared to the CON-LPS group (P<0.05). Dietary treatment did not affect the LPS detoxification mRNA abundance for acyloxyacyl hydrolase and the acute phase protein C-reactive protein (P>0.05). These results indicate that maternal n-3 PUFA supplementation can have beneficial effects in offspring towards attenuating LPS induced inflammation later in life.

**Key words:** Inflammation, Lipopolysaccharide, Maternal diet, n-3 PUFA,

**Introduction**

Western diets are low in n-3 polyunsaturated fatty acids (PUFA), and provide excessive amounts of n-6 fatty acids, such as linoleic acid and arachidonic acid, which leads to a very high n-6:n-3 ratio of up to 15:1 – 17:1(1). This is important as n-3 and n-6 fatty acids can regulate the expression and function of proteins and genes that control cell growth, metabolism and communication differentially (2). High n6:n3 ratio promotes the pathogenesis of many diseases, including cardiovascular diseases, cancer, diabetes and inflammatory diseases (3). A lower n-6:n-3 ratio is associated with decreased risk and this was highlighted in a cardiovascular disease prevention study, where a ratio of 4:1 was
associated with a major decrease in total mortality (4). The long chain PUFA eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3) have been shown to possess strong anti-inflammatory properties (5). However, these n-3 PUFA need to be supplemented through diet as they have limited synthesis in mammals due to low \( \Delta5 \) and \( \Delta6 \) desaturase enzyme expression (6). DHA and EPA exert their anti-inflammatory action through three key processes. Firstly by competition and suppression of arachidonic acid (AA) derived pro-inflammatory eicosanoids as they compete for cyclooxygenase and lipoxygenase enzymes (7). Secondly, via Toll-like receptor 4 (TLR4) and nuclear factor (NF)-\( \kappa\)B suppression that leads to decreased pro-inflammatory cytokine secretion (8). Thirdly, by incorporation into plasma membrane phospholipids and changing membrane structure and function that antagonizes inflammatory signaling (9, 10).

Inflammation is part of initial immune response to protect an animal from harmful agents such as lipopolysaccharide (LPS) as well as live pathogenic microbes (11). Irrespective of the stimuli the classical immune activation for inflammation starts with the sensing of the inflammatory agent by either the cell surface or intracellular TLR’s and NOD like receptors (12). The subsequent activation of the master transcription factor NF-\( \kappa\)B, results in the secretion of pro-inflammatory cytokines and inflammation (13, 14). If this is persistent, it has the potential to cause tissue damage and predisposing subjects to metabolic diseases such as diabetes, obesity and atherosclerosis (15, 16).

Infant and piglet survival is markedly dependent on the ability to transition from the prenatal to postnatal phase in which intestinal maturation is still occurring. During the transition from the prenatal to postnatal environment and from suckling to weaning, infants
undergo dramatic shifts in nutrient supply and profiles. These transition periods are associated with early inflammatory responses that may contribute to physiological and functional disorders (17, 18). Furthermore, studies have shown that maternal diet during gestation and lactation induces stable alterations to the physiology and phenotype of the offspring through epigenetic programming as well as enrichment of the fetal tissue (19). Fatty acids and their derivatives can influence both the early immune system development and maturation by regulating metabolic processes and the gene and protein expression of important enzymes and cytokines (20). It has been shown that maternal dietary fatty acid changes can modify neonatal neutrophil function in the offspring (21). This gives an opportunity where the young ones can be physiologically and immunologically prepared through maternal supplementation of n-3 PUFA for their early part of life when they are vulnerable to many diseases which are detrimental for the health and production performance later (22).

Therefore, based on previous research in which DHA and EPA attenuated LPS induced inflammation (23, 24), we hypothesized that maternal (gestation and lactation) supplementation of DHA and EPA would attenuate an inflammatory challenge by LPS and antagonize the production of pro-inflammatory cytokines and febrile response in offspring later in life.
Materials and Methods

Materials. All the chemicals used for the experiment were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. The dietary source of n-3 PUFA, Gromega™ (JBS United Inc., Sheridan, IN) contained 14% DHA and 14% EPA.

Animals and Experimental Design. All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee and adhered to the ethical and humane use of animals for research. Five sows were fed a basal diet and 10 sows a diet enriched with n-3 PUFA upon confirmation of pregnancy: 1) standard corn-soybean meal diets were fed to one group (CON) and 2) the basal diet supplemented with 0.5% Gromega™ (JBS United Inc., Sheridan, IN), which is an enriched source of algal EPA and DHA. Upon farrowing, litters were standardized to 10 piglets per litter within 24 h of birth, with cross fostering only occurring within treatment. At weaning (18-21 days of age), two piglets from each sow were separated and individually penned during the ten week nursery period. In the nursery, CON pigs stayed on a DHA and EPA devoid diets (n=10), while piglets from five n-3 PUFA fed sows (n=10) were also switched to the CON diet in the nursery (Mn3). The 10 remaining piglets were fed nursery diets supplemented with 0.5 % Gromega™ (Cn3). In all diets, Gromega™ was mixed in the basal diet in substitution to corn and formulated to meet or exceeded the nutrient requirements of the respective growth period (25). An example of the later nursery diet is given in Table 1.

Pigs were fed ad libitum and had free access to water all the time. After 8 weeks in the nursery and performance data collection, all pigs were fasted overnight and challenged with either an intramuscular injection of saline (SAL) or lipopolysaccharide (LPS, from
*Escherichia coli* Serotype 055:B5, 10 µg/kg BW, Sigma-Aldrich, St. Louis, MO). Following the peak febrile response at four hours as measured by rectal temperature, blood was collected for isolating buffy coat and blood assays.

**Buffy coat isolation.** Twenty mL of blood was mixed with 30 ml of RBC lysis buffer (1.45 M NH₄Cl, 0.1M KHCO₃, 5 mM EDTA, pH 8.0) and vigorously shaken for 15 min. The tubes were centrifuged at 300 g for 5 min at 4°C. The supernatant was discarded leaving the white blood cells and 20 mL of ice cold RBC lysis buffer was added again. This was vortexed for few minutes and centrifuged at 300 x g for 5 min at 4°C. Supernatant was discarded and 20 mL of room temperature phosphate buffered saline (PBS; containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·2H₂O, 2 mM KH₂PO₄, pH 7.4) was added. This tube was vortexed and centrifuged at 300 x g for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 mL of PBS and stored at -80°C until further analysis.

**Serum inflammatory cytokine concentration.** Tumor necrosis factor-α levels were quantified from the serum collected after four hours of immune challenge with LPS. A quantitative sandwich enzyme immunoassay ELISA was performed using a commercially available kit (R&D Systems Inc., Minneapolis, MN).

**RNA isolation and quantitative PCR.** Total RNA was isolated from tissue samples using Trizol (Invitrogen, Inc., Carlsbad, CA) reagent according to the manufacturer’s protocol and the RNA pellets were resuspended in nuclease free water. Total RNA was quantified by measuring the absorbance at 260nm using a spectrophotometer (ND-100, NanoDrop
Technologies, Rockland, DE) and the purity was assessed by determining the ratio of the absorbance at 260 and 280 nm (NanoDrop). All samples had 260/280 nm ratios above 1.8. The integrity of the RNA preparations was verified by visualization of the 18S and 28S ribosomal bands stained with SYBR® Safe DNA gel stain (Life technologies, Carlsbad, CA) after electrophoresis on 2% agarose gels. Total RNA (1μg) was transcribed in a reaction combining genomic DNA elimination using a commercially available cDNA synthesis kit (Quantitect® reverse transcription kit, Qiagen, Valencia, CA). cDNA was quantified using NanoDrop and used for real-time quantitative PCR reaction. Amplification was carried out in a total volume of 25 μL containing 2X quantitect SYBR Green PCR master mix (Quantitect® SYBR® Green PCR kit, Qiagen, Valencia, CA), forward and reverse primers (0.3 μM) and 400 ng of cDNA. The primers used are listed in Table 1. The cycling conditions were, after an initial 15 min denaturation step at 95°C, the reactions were cycled 50 times under the following parameters: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Optical detection was carried out at 72°C. At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. A non-template control was run with every assay and all determinations were performed in triplicate. The mRNA abundance values for each sample were normalized to RPL32 according to the 2^{-ΔΔCT} method (26).

Fatty acid analysis. Fatty acid profiles of the whole blood were determined and analyzed by GC-MS (27, 28). 0.2 g of buffy coat tissue was mixed with 2.5 ml of 4:1 hexane and 125 μg/L heptadecanoic acid was added to each sample as an internal standard. FAME were analyzed by GC on a Hewlett-Packard model 6890 fitted with an Omegawax 320 (30-m × 0.32-mm i.d. 0.25 um) capillary column. Hydrogen was the carrier gas. The temperature
program ranged from 80 to 250°C with a temperature rise of 5°C/min. The injector and detector temperatures were 250°C and 1 µL of sample was injected and run split. Fatty acids methyl esters were identified by their relative retention times on the column with respect to appropriate standards and heptadecanoic acid.

Statistical Analyses. All data are expressed as means ± SEM. The main effects of dietary treatment and LPS challenge were determined by the Proc Mixed procedure in SAS (Cary, NC), and treatment differences were established using the least significant difference. Differences were deemed significant at P < 0.05 and tendencies at P < 0.10.

Results

Fatty acid composition. Fatty acid profiles were measured in the buffy coats in all three dietary treatment groups of pigs to ascertain the difference in n-3 PUFA enrichment or depletion (Table 3). As expected, the Cn3 group had a high EPA and DHA content compared to the CON (P<0.0001). Interestingly, the Mn3 buffy coats also had moderate amounts of EPA and DHA ten weeks after last dietary exposure from maternal milk (P<0.05). However, the DHA and EPA content of Mn3 treatment was only half of the Cn3 treatment content (P<0.05). As such, all three treatment groups differed in total n-3 PUFA content, with the CON having the lowest, Mn3 and Cn3 treatments having the highest (1.53 vs. 3.70 vs. 5.98, respectively, P<0.0001). The CON group also had the highest n6:n3 ratio, followed by the Mn3 and CON groups (P=0.0003). The four hour LPS challenge (SAL vs. LPS) did not alter these fatty acid profiles (data not shown).
Effect of dietary fat on the febrile response induced by LPS. To assess the anti-inflammatory effect of maternal and continuous dietary n-3 PUFA on nursery pigs, an acute systemic LPS challenge was given. Irrespective of diet, LPS challenged pigs had higher febrile response as assessed by rectal temperature changes compared to the SAL groups (P < 0.001, Fig. 1). After 1 h, all three LPS challenged groups had significantly elevated rectal temperatures compared to the SAL groups. Moreover, the Cn3-LPS pigs had a lower febrile response within the four hour challenge period than the CON-LPS pigs (P = 0.011). While the Mn3-LPS group tended to have a comparatively lower febrile response compared to the CON-LPS pigs (P < 0.10, Fig. 1).

Blood cytokine response to inflammatory challenge. Tumor necrosis factor-α concentration, a marker of systemic inflammation, was measured in serum collected 4 h post challenge (Fig. 2). As expected all the LPS challenged pigs had an augmented TNF-α concentration compared to the SAL pigs (P<0.0001). Dietary n-3 PUFA supplementation attenuated serum TNF-α concentration (P<0.0073) and there was a diet and challenge interaction (P<0.0172). Furthermore, CON-LPS pigs had a significantly higher TNF-α concentration than both the Mn3-LPS and Cn3-LPS pigs (P<0.01).

Buffy coat LPS signaling pathway mRNA expression. Lipopolysaccharide challenge caused an increase in the mRNA abundance of major LPS signaling molecules such as TLR4, CD14 and Myd88 in the peripheral blood cells irrespective of the dietary treatment (P=0.0003,
There was no dietary effect on mRNA abundance. However, there was a diet by LPS interaction for these three genes (P<0.0012). Interestingly, TLR4, CD14 and Myd88 mRNA abundance followed the same pattern as the febrile response and serum TNF-α concentration. The Cn3-LPS and Mn3-LPS mRNA levels were lower than CON-LPS (P<0.05).

As expected, the LPS challenge also caused an increase in the mRNA abundance of major pro-inflammatory cytokines TNF-α and IL-1β, and the anti-inflammatory cytokine IL-10, irrespective of the dietary treatment (P<0.05, Table 3). There was no dietary effect but there was a diet by LPS interaction. All cytokine mRNA expression also followed the same trend as the LPS signaling genes with CON-LPS group having higher expression compared to a significantly lower expression in Mn3-LPS and Cn3 LPS groups (P<0.05) indicating the pro-inflammatory effect of LPS challenge and anti-inflammatory effect of supplementing n-3 PUFA either maternally or continuously. Inducible nitric oxide synthase (iNOS) expression was not affected by the LPS challenge (P=0.15) or dietary treatment (P=0.47).

Acyloxyacyl hydrolase (AOAH) is a major LPS detoxification enzyme present in the liver and immune cells and C-reactive protein (CRP) is one of the major acute phase proteins secreted during inflammatory challenge by liver and immune cells. The LPS challenge resulted in an approximately 5 fold increase in the AOAH mRNA expression and more than a 1000 fold increase in CRP mRNA expression, irrespective of the dietary treatment (P<0.05). There was no diet by LPS interaction (P>0.05), for both genes.
Discussion

Long chain n-3 PUFA such as DHA and EPA are known to attenuate LPS induced inflammation (24, 29-32). Recently, fish oil has been shown to directly inhibit TLR4 and NOD2 signaling and inflammation in pigs (23). Moreover, we and others have also shown that maternal supplementation of n-3 PUFA has beneficial effects in the offspring (20, 33). In pigs, maternal n-3 PUFA supplementation increases glucose absorption in weanling piglets and helps maintain the nutritional status and health during this early weaning transition period (28). Therefore, this study herein was conducted to examine whether maternal supplementation of DHA and EPA during gestation and lactation would have long lasting protective effects against acute inflammatory challenges and inflammation in the offspring later in life.

Nutritional manipulation of tissue fatty acid profiles in utero is not a new phenomenon. Providing supplemental fat to sows in late gestation and lactation not only improves body condition scores and productivity, but also enhances piglet performance (34-36). It is also well established that the fatty acid content of the suckling piglet is determined by the fatty acid content of the sow diet (37-39). Rooke et al., (38, 40, 41) and Fritsche et al., (39), fed sows salmon and tuna oils late in gestation and showed both sow and neonatal tissue enrichment of DHA and EPA. In our study, the evidence of continuous supplementation of n-3 PUFA was obvious in the Cn3 treatment in which enrichment with EPA and DHA was clearly seen. Surprisingly, the Mn3 treatment also had significant DHA and EPA content, even after supplementation of diets devoid of DHA and EPA for seven weeks post weaning. Interestingly, it has been shown that maternal supplementation does not
enrich the fatty acid composition of all the tissues evenly. Liver n-3 PUFA enrichment is highest followed by muscle and then the subcutaneous adipose tissue (42, 43). Usually, when supplementation of n-3 PUFA is stopped, the fatty acid composition of the tissues change rapidly reflecting the current dietary fatty acid intake (34). Lymphocytes have long life span of about 30 days (44). So, the possibility of immune cells enriched through maternal supplementation present in the circulation for two months is remote. However, Hoile et al., (45) showed that maternal n-3 PUFA supplementation causes stable DNA methylation pattern changes in Fads2 gene which encodes Δ6 desaturase, a rate limiting enzyme in n-3 PUFA synthesis. This epigenetic mechanism may also partially explain the higher DHA and EPA content of the buffy coat in our Mn3 pigs.

Piglet survival is markedly dependent on the ability to transition in the pre and postnatal phases (46). This is even more critical as early weaning (15-21 days of age) stress can result in sustained impairment in intestinal barrier function and heightened inflammation post weaning (47). Interestingly, fatty acids and their derivatives could be used to influence the early immune system development and maturation by regulating metabolic processes and the gene and protein expression of important enzymes and cytokines (20). It has been shown that maternal dietary fatty acid changes can modify neonatal neutrophil function (21). Epigenetic studies have also shown that some nutrients consumed during pregnancy and/or lactation induces stable alterations to the physiology and phenotype of the offspring (19). Along these lines, evidences currently exists for maternal n-3 PUFA supplementation to cause stable alterations in the epigenetic machinery that the beneficial effect is carried over for a long duration (45). The higher n-3 PUFA content of buffy coat from maternally
supplemented pigs in our study confirms that epigenetic mechanism may be involved. We currently have collaborators examining DNA methylation profiles in these pigs to test this theory (Boddicker and Ross, unpublished).

Protective effect of maternal n-3 PUFA supplementation towards inflammation was studied by administering an LPS challenge and collecting blood samples at the peak immune response. Webel et al. (48) have shown that the peak inflammatory response induced by exogenous LPS occurs at 2-4 h post challenge in pigs. This is associated with elevated body temperature and blood TNF-α, cortisol, and IL-6 concentrations. Blood urea nitrogen concentrations are elevated compared to the control between 6-12 h post challenge. Knowing the peak response occurs at 2-4 h, by design we examined the impact Cn3 and Mn3 treatments have on LPS induced febrile response and inflammation markers. As expected, LPS induced an elevated febrile and serum cytokine response over the 4 h period. As hypothesized, Mn3 and Cn3 supplementation attenuated both responses. This is in agreement with other DHA and EPA research showing n-3 PUFA decreasing the LPS induced inflammatory response (23, 24, 29). Additionally, EPA and DHA can replace arachidonic acid in the cell membrane and act as competitive agonist for cyclooxygenase and lipoxygenase enzymes which otherwise synthesize pro-inflammatory mediators from the arachidonic acid (49). These pro-inflammatory mediators stimulate the secretion of cytokines such as TNF-α and IL-1β. This competition leads to the production of less inflammatory or anti-inflammatory eicosanoids and resolvins (50). Furthermore, DHA and EPA can also decrease the action of pro-inflammatory transcription factor NF-κB and activate the nuclear
receptor PPARγ which reduces the secretion of pro-inflammatory cytokines and increasing the anti-inflammatory cytokine secretion (10).

Lipopolysaccharide, when present in the circulation is bound to LPS binding protein (LBP) which presents the LPS monomer to CD14. Toll like receptor 4 recognizes the LPS presented by CD14 in association with MD-2 and then the signal is transmitted intracellularly through Myd88 dependent and independent pathways both ultimately stimulating the secretion of cytokines (14, 51). Recent evidences indicate that saturated and n-3 PUFA can reciprocally modulate the expression and signaling of LPS signaling receptor TLR4 (52). Additionally, n-3 PUFA enrichment in macrophage and T cell membranes dissociate the lipid raft, preventing the TLR4 localization into the raft which will attenuate the LPS recognition and signaling, and cell function (32, 53).

All evidences point to the fact that under n-3 PUFA supplementation decreased expression and signaling of TLR4 occurs. We also observed a decreased mRNA expression of TLR4 and other LPS signaling pathway proteins CD14 and Myd88 under n-3 PUFA supplementation in both the continuous and maternal supplemented pigs. Since maternal supplemented pig buffy coat cells are also enriched with n-3 PUFA it is a plausible mechanism through which they might attenuate the inflammation by decreasing the expression of these LPS signaling genes either through the decreased expression of pro-inflammatory mediators or through activation of nuclear receptor PPARγ (10).

Lipopolysaccharide detoxification enzyme AOAH is secreted by monocyte-macrophages, dendritic cells and neutrophils. Acyloxyacyl hydrolase removes the secondary acyl chains of the lipid-A component of LPS rendering it unable to be bind to TLR4 (54).
Faster LPS detoxification is essential after an inflammatory challenge to limit the detrimental effects of LPS. Higher mRNA expression of AOAH in LPS treated pig immune cells indicate the immune cells trying to limit the negative effects of LPS via increased detoxification. However, diet had no influence on AOAH mRNA expression. Further, acute phase protein C-reactive protein (CRP) mRNA levels in the blood cells were very highly elevated indicating the acute phase response happening after the LPS challenge. Acute phase proteins are inflammatory mediators mainly secreted by liver and also by peripheral immune cells which usually bind to the immune stimulant which in turn helps facilitate its neutralization through the complement system (55). C-reactive protein (CRP) is a major acute phase protein and shown to be secreted by immune cells in response to an LPS challenge (56, 57). n-3 PUFA supplementation did not have any impact on the secretion of CRP again indicating the need for a maximum response from the acute phase proteins.

Overall, our results indicate that maternal n-3 PUFA supplementation enriches the immune cells and attenuates the adverse effects of an acute inflammatory challenge even ten weeks after birth (58). Maternal supplementation with n-3 PUFA may provide protective effect like a regular n-3 PUFA supplementation later in the life, importantly during the early stages of life which might be important for maintaining the health of the animal during the crucial transition period.

Literature Cited


37. Rooke JA, Bland IM, Edwards SA. Relationships between fatty acid status of sow plasma and that of umbilical cord, plasma and tissues of newborn piglets when sows were fed on diets containing tuna oil or soyabean oil in late pregnancy. Br J Nutr. 1999 Sep;82:213-21.


Table 1. Example of the nursery diet feed composition (% as fed basis)

<table>
<thead>
<tr>
<th>Feed Component</th>
<th>Control</th>
<th>Dn3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>61.11</td>
<td>60.59</td>
</tr>
<tr>
<td>Soybean meal 48%</td>
<td>31.67</td>
<td>31.67</td>
</tr>
<tr>
<td>Meat &amp; bone Meal</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Salt</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>Gromega™ 1</td>
<td>-</td>
<td>0.53</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>Threonine</td>
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<td>0.11</td>
</tr>
<tr>
<td>Premix²</td>
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<td>0.36</td>
</tr>
<tr>
<td>Selenium</td>
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<td>0.05</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.03</td>
<td>0.03</td>
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**Calculated composition**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dn3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kcal/kg</td>
<td>3,238</td>
<td>3,238</td>
</tr>
<tr>
<td>Crude Protein, %</td>
<td>22.36</td>
<td>22.36</td>
</tr>
<tr>
<td>SID Lysine, %</td>
<td>1.35</td>
<td>1.35</td>
</tr>
<tr>
<td>Available Phosphorus, %</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Ether Extract, %</td>
<td>3.80</td>
<td>3.80</td>
</tr>
<tr>
<td>Crude Fiber, %</td>
<td>2.36</td>
<td>2.36</td>
</tr>
</tbody>
</table>

1 Gromega™ was supplied by JBS United, Inc., containing approximately 14% EPA and 14% DHA of total fatty acids.

2 Supplied per kilogram of diet: vitamin A, 8364 IU; vitamin D3, 1533 IU; vitamin E, 45 IU; vitamin K, 2.2 IU; choline, 6.5 mg; riboflavin, 4.2 mg; niacin, 21 mg; pantothenic acid, 17 mg; vitamin B-12, 28 mg; biotin, 1.6 mcg; folic acid, 0.0005 mg; Zn, 112 ppm as zinc sulfate and zinc oxide; Mn, 54 ppm as manganese oxide; Fe, 145 ppm as ferrous carbonate and ferrous sulfate; Cu, 20 ppm as copper chloride; I, 0.76 ppm as ethylenediamine dihydroiodide; Se, 0.25 ppm as sodium selenite.
Table 2. Primer sequences for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5' - 3')</th>
<th>Antisense (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>GAA TAT TTT TCT AAC CTG CCC AAC CTG GAG</td>
<td>CCA GCC AGA CCT TGA ATA CAA GTT TTC ATT ACA TC</td>
</tr>
<tr>
<td>CD14</td>
<td>TGG ACC TCA GTC ACA ACT CG</td>
<td>CCT TTA GGC ACT TGC TCC AG</td>
</tr>
<tr>
<td>Myd88</td>
<td>AAG TTT GCC CTC AGC TCT CTC TCT CCA</td>
<td>ACA GAC AGT GAT GAA CCG CAG GAT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ACT CGG AAC CTC ATG GAC AG</td>
<td>AGG GGT GAG TCA GTG TGA CC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AAA GGG GAC TTG AAG AGA G</td>
<td>CTG CTT GAG AGG TGC TGA TAT</td>
</tr>
<tr>
<td>IL-10</td>
<td>ATG GGC GAC TTG TTG CTG AC</td>
<td>CAC AGG GCA GAA ATT GAT GAC A</td>
</tr>
<tr>
<td>iNOS</td>
<td>ATG TCC GAG GCA ACC ACA ACC ACA TTC</td>
<td>GCA TGC TGC TGA GAG CTT TGT TGA</td>
</tr>
<tr>
<td>AOAH</td>
<td>TCA GGG GGA CAG AAA TAT GG</td>
<td>CCA GAA TCA CGC AGA ATC AC</td>
</tr>
<tr>
<td>CRP</td>
<td>TGC CCA GAC AGA CAT GAT CGG AAA</td>
<td>TGA GCC TTG CAG TCA GAC TCA CAT</td>
</tr>
</tbody>
</table>

Primer Abbreviations: TLR4, Toll like receptor4; CD14, Cluster of differentiation 14; Myd88, Myeloid differentiation factor 88; TNF-α, Tumor Necrosis Factor-α; IL-1β, Interleukin-1β; IL-10-Interleukin-10; iNOS, inducible nitric oxide synthase; AOAH, Acyloxyacyl hydrolase; CRP-C-Reactive Protein.
**Table 3.** Buffy coat fatty acid composition of pigs fed either the control diet (CON) or a diet enriched with long chain n-3 PUFA only through maternally (Mn3) or continuously (Cn3).

<table>
<thead>
<tr>
<th>Fatty Acid (%)</th>
<th>CON</th>
<th>Mn3</th>
<th>Cn3</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>20.10 ± 0.34</td>
<td>20.61 ± 0.34</td>
<td>21.41 ± 0.34</td>
<td>0.33</td>
</tr>
<tr>
<td>18:0</td>
<td>17.36 ± 1.40</td>
<td>14.22 ± 1.39</td>
<td>16.89 ± 1.39</td>
<td>0.30</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.87 ± 0.09</td>
<td>0.84 ± 0.09</td>
<td>1.00 ± 0.09</td>
<td>0.42</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.29 ± 0.17</td>
<td>0.28 ± 0.17</td>
<td>0.21 ± 0.17</td>
<td>0.94</td>
</tr>
<tr>
<td>20:2</td>
<td>0.69 ± 0.27</td>
<td>1.24 ± 0.27</td>
<td>0.33 ± 0.27</td>
<td>0.14</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>1.26 ± 0.25</td>
<td>1.51 ± 0.25</td>
<td>0.85 ± 0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>11.23 ± 0.30</td>
<td>11.35 ± 0.30</td>
<td>10.67 ± 0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>0.00 ± 0.03</td>
<td>0.45 ± 0.03</td>
<td>1.10 ± 0.03</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>22:6n-3 (DHA)</td>
<td>0.00 ± 0.06</td>
<td>1.10 ± 0.06</td>
<td>2.09 ± 0.06</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Saturated</td>
<td>34.07 ± 0.62</td>
<td>34.46 ± 0.62</td>
<td>35.85 ± 0.62</td>
<td>0.18</td>
</tr>
<tr>
<td>ω-3 PUFA</td>
<td>1.53 ± 0.26</td>
<td>3.70 ± 0.26</td>
<td>5.98 ± 0.26</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>ω-6 PUFA</td>
<td>42.96 ± 0.46</td>
<td>42.32 ± 0.46</td>
<td>42.65 ± 0.46</td>
<td>0.63</td>
</tr>
<tr>
<td>(ω-6)/(ω-3)</td>
<td>23.80 ± 1.36</td>
<td>11.43 ± 1.36</td>
<td>7.13 ± 1.36</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

1Fatty acid composition was measured by GC-MS on three pigs per treatment and expressed as a percentage of total fatty acids (mean ± SEM).
Table 4. Effects of maternal n-3 PUFA supplementation on mRNA expression of major LPS signaling molecules, cytokine genes, LPS detoxification gene AOAH and acute phase protein gene CRP under an acute inflammatory challenge in peripheral immune cells.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Control</th>
<th>Maternal</th>
<th>Continuous</th>
<th>SEM</th>
<th>Diet</th>
<th>Challenge</th>
<th>Diet*Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>LPS</td>
<td>Saline</td>
<td>LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>1.00a</td>
<td>18.54b</td>
<td>2.16a</td>
<td>8.45c</td>
<td>1.41a</td>
<td>8.51c</td>
<td>2.74</td>
</tr>
<tr>
<td>CD14</td>
<td>1.00a</td>
<td>5.41b</td>
<td>2.30a</td>
<td>3.49b</td>
<td>1.65a</td>
<td>2.34c</td>
<td>0.98</td>
</tr>
<tr>
<td>Myd88</td>
<td>1.00a</td>
<td>18.59b</td>
<td>2.29a</td>
<td>8.80c</td>
<td>2.84a</td>
<td>10.19cb</td>
<td>2.93</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.00a</td>
<td>5.07b</td>
<td>0.25a</td>
<td>1.79c</td>
<td>0.88a</td>
<td>1.57c</td>
<td>0.94</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.00a</td>
<td>2.27b</td>
<td>0.40a</td>
<td>1.38c</td>
<td>0.27a</td>
<td>1.45c</td>
<td>0.28</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.00</td>
<td>1.14</td>
<td>1.31</td>
<td>0.34</td>
<td>1.64</td>
<td>0.36</td>
<td>0.53</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.00a</td>
<td>4.34b</td>
<td>0.59a</td>
<td>2.61bc</td>
<td>0.99a</td>
<td>2.21c</td>
<td>0.69</td>
</tr>
<tr>
<td>AOAH</td>
<td>1.00</td>
<td>6.73</td>
<td>1.05</td>
<td>4.80</td>
<td>1.07</td>
<td>6.21</td>
<td>1.28</td>
</tr>
<tr>
<td>CRP</td>
<td>1.00</td>
<td>1215</td>
<td>1.33</td>
<td>1205</td>
<td>1.17</td>
<td>1140</td>
<td>90.43</td>
</tr>
</tbody>
</table>

Values are mean and pooled SE, n=5 pigs/trt. Means in the same row without a common letter differ, P < 0.05. All data were acquired using real time PCR. RPL32 was used as the housekeeping gene and the Control-Saline treatment was used as the reference sample per gene. The expression values were normalized to RPL32 housekeeper.
Figure 1. Febrile response of pigs fed either control (CON) or n-3 PUFA rich diets until the lactation through maternal supplementation (Mn3) or throughout the life (Cn3) and challenged with either saline (SAL) or LPS 10 µg/kg (LPS) after eight weeks. n = 5 pigs/trt. Different letters a,b,c represent significant differences within each hour (P<0.05). Diet P = 0.011, Challenge P < 0.001, Time P < 0.001.
Fig. 2. Effects of maternal n-3 PUFA on serum tumor necrosis factor (TNF)-α concentration after a 4 hour LPS challenge in pigs. Pigs fed either control (CON) devoid of DHA and EPA, n-3 PUFA diets throughout life (Cn3) or only during maternal period of gestation and lactation (Mn3). Pigs where challenged with either saline (SAL) or LPS 10 µg/kg (LPS) at 11 weeks of age. n = 5 pigs/trt. Different letters a,b,c represent significant differences at (P<0.05). Diet P = 0.0073, Challenge P < 0.0001, Diet*Challenge P < 0.0172.
CHAPTER 6: INTESTINAL INTEGRITY, ENDOTOXIN TRANSPORT AND DETOXIFICATION IN PIGS DIVERGENTLY SELECTED FOR RESIDUAL FEED INTAKE

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Abstract

Microbes and microbial components potentially impact the performance of pigs through immune stimulation and altered metabolism. These immune modulating factors can include endotoxin from gram negative bacterial outer membrane component, commonly referred to as lipopolysaccharide (LPS). In this study, our objective was to examine the relationship between intestinal barrier integrity, endotoxin and inflammation with feed efficiency (FE), using pig lines divergently selected for residual feed intake (RFI) as a

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model. Twelve gilts (62 ± 3 kg BW) from the low RFI (LRFI, more efficient) and 12 from the high RFI (HRFI, less efficient) were used. Individual performance data was recorded for five weeks. At the end of the experimental period, ADFI of LRFI pigs was lower (P < 0.001), average daily gain (ADG) not different between the two lines (P = 0.72) but the Gain: Feed ratio of LRFI pigs was higher than for HRFI pigs (P = 0.019). Serum endotoxin concentration (P<0.01) and the acute phase protein haptoglobin (P<0.05) were higher in HRFI pigs. Transepithelial resistance of the ileum, transport of FITC-Dextran and FITC-LPS in ileum and colon, as well as tight junction protein mRNA expression in ileum, did not differ between the lines, indicating the two lines did not differ in transport characteristics at the intestinal level. Ileum inflammatory markers, myeloperoxidase and IL-8, were found to be higher in HRFI pigs (<0.05). Alkaline phosphatase (ALP) activity was significantly increased in the LRFI pigs in ileum and liver tissues and negatively correlated with blood endotoxin (P<0.05). Lysozyme activity in the liver was not different between the lines, however, the LRFI pigs had a twofold higher lysozyme activity in ileum (P<0.05). Despite the difference in their activity, ALP or lysozyme mRNA expression was not different between the lines in either tissue. Lower endotoxin and inflammatory markers and the enhanced activities of antimicrobial enzymes in the LRFI line may not fully explain the difference in the feed efficiency between the lines; but they have the potential to prevent the growth potential in HRFI pigs. Further studies are needed to identify the other mechanisms that may contribute to the higher endotoxin levels in the HRFI pigs and the higher feed efficiency in the LRFI pigs.

Key words: Endotoxin • Feed efficiency • Intestinal integrity • Pig
Introduction

Feed efficiency has become a major goal in pig research and breeding programs for economic, environmental and food security reasons. Residual feed intake (RFI) has been adopted as a reliable method for measuring, selecting for and studying FE. Pigs with low RFI (LRFI) consume less feed for a given amount of growth and backfat as pigs with higher RFI (HRFI) (Cai et al., 2008; Gilbert et al., 2007). Yorkshire pigs selected for LRFI for 5 generations, differed by up to 124 g/day in ADFI with no significant reduction in weight gain compared to randomly selected HRFI pigs (Boddicker et al., 2011). However, the physiology that underlies this improved FE has been poorly defined.

Inflammation can have a major impact on growth performance and feed efficiency (Schinckel et al., 1995) and could contribute to lower maintenance nutrient requirements in pigs selected for reduced RFI (Barea et al., 2010; Boddicker et al., 2011). Lipopolysaccharide (LPS), a Gram negative bacterial outer membrane component referred also as endotoxin, is a chronic innate immune stimulator in pigs (Gabler et al., 2008; Webel et al., 1997; Weber and Kerr, 2008). Importantly, Gram negative bacteria are present in the gastrointestinal tract in large amounts and can serve as a major source of systemic endotoxin (Cani and Delzenne, 2010; Ravin et al., 1960). However, intestinal barrier integrity plays critical host defense functions against luminal immunogens such as endotoxin. Once in circulation, endotoxin activates the immune system via toll like receptors (TLR), which results in repartitioning of nutrients for immune function, rather than towards anabolism (Kimball et al., 2003; Rakhshandeh and de Lange, 2012). Additionally, detoxification processes can prevent the negative effects of endotoxin (Elsbach, 2000). Therefore, our objective was to examine the
relationship between intestinal barrier integrity, endotoxin and inflammation with FE, using pig lines divergently selected for RFI as a model.

**Materials and Methods**

All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee and adhered to the ethical and humane use of animals for research. All chemicals used for the experiment were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Animals and Experimental Design**

Twelve pigs per line (62 ± 3 kg, BW) were selected and matched across lines for age and weight from the seventh generation of the Iowa State University RFI selection project (Cai et al., 2008). Pigs were individually penned and had free access to water and feed at all times. All pigs were fed a common commercial corn-soybean meal-distillers dry grain with soluble diet formulated to meet or exceed the nutrient requirements for this size pig (NRC, 1998). Feed intake and body weights were recorded on a weekly basis and used to calculate average daily gains and RFI for each pig, as previously described (Cai et al., 2008; Young et al., 2011).

After five weeks, all pigs were fasted overnight and whole blood (10 mL) was collected via venipuncture and serum separated by centrifugation at 2000 × g at 4°C. Thereafter, pigs were euthanized via captive bolt followed by exsanguination. Immediately following euthanasia, segments of ileum and mid colon were collected and flushed with ice
cold Krebs-Henseleit buffer (consisting of, in mmol/L: 25 NaHCO₃, 120 NaCl, 1 MgSO₄, 6.3 KCl, 2 CaCl₂, 0.32 NaH₂PO₄; pH 7.4) to remove any undigested food material. Fresh ileum and colon segments were used for *ex vivo* integrity measures and for mucosal scrapings. A 20 cm segment of ileum 150 cm from the ileal-cecal junction and a 10 cm segment of proximal colon 60 cm from the rectum were isolated. Ileum and colon segments were flushed with ice cold Krebs-Henseleit buffer. For mucosal scrapings, intestinal segments were then cut open longitudinally along the mesenteric border and the epithelial layer gently scrapped with a glass slide without disturbing the underlying lamina propria. Ileum and colon samples that were not used for *ex vivo* work were then snap frozen in liquid nitrogen and stored at -80°C until analysis.

**Intestinal Integrity**

Electrophysiological measurements were taken using modified Ussing chambers as previously described (Albin et al., 2007; Gabler et al., 2009; Moeser et al., 2012). Briefly, fresh segments of the ileum and colon were removed and placed on ice in Krebs-Henseleit buffer for transport to the laboratory while under constant aeration until clamped in the modified Ussing chambers. To assess tight junction integrity and the mucosal to serosal endotoxin transport, tissues stripped of outer serosal layers were immediately mounted in a modified Ussing Chamber, with each chamber connected to a pair of dual channel current and voltage electrodes submerged in 3% noble agar bridges and filled with 3M potassium chloride for electrical conductance (Physiologic Instruments Inc., San Diego, CA and World Precision Instruments Inc., New Haven, CT). Each segment (0.71 cm²) was bathed on its mucosal and serosal sides with Krebs buffer and constantly gassed with 95% O₂-5% CO₂
mixture. The temperature of all tissues and apparatus was constantly maintained at 37 °C using circulating warm water. A short circuit current was established and stabilized for about 10 minutes and transepithelial resistance (TER) was measured using the included software (Acquire and Analyze, Physiological instruments, San Diego, CA).

After recording the basal electrophysiological measurements, the mucosal to serosal macromolecule transport of fluorescein isothiocyanate labeled dextran (4.4 KDa; FITC-Dextran) was assessed to measure the integrity of both ileum and colon, as previously described (Wang et al., 2001). Briefly, the mucosal chambers were treated with 2.2 mg/mL FITC-Dextrans, and chamber samples from both sides were collected every 10-15 min. The relative fluorescence was then determined using a fluorescent plate reader (Bio-Tek, Winooski, VT), with excitation and emission wavelengths of 485 and 520 nm, respectively. An apparent permeability coefficient (Papp) was then calculated using the area of the membrane and rate of FITC-Dextran transport, where \( \frac{dQ}{dt} \) = transport rate (µg/min); \( C_0 \) = initial concentration in the donor chamber (µg/mL); \( A \) = area of the membrane (cm²):

\[
Papp = \frac{dQ}{(dt \times A \times C_0)}
\]

The mucosal to serosal transport of LPS was also assessed as previously described by Tomita et al., (2004). Briefly, the mucosal chambers were challenged with 20 µg/mL fluorescein isothiocyanate labeled LPS (FITC-LPS) and chamber samples from both sides were collected every 10-15 min. The fluorescence and apparent permeability coefficient was calculated as described for FITC-Dextran.
**Circulating Endotoxin**

Serum endotoxin concentrations were measured by an end point fluorescent assay using the recombinant factor C (rFC) system (Lonza, Basel, Switzerland). Briefly, the serum samples were diluted 1000X in pyrogen free water and 100µL of the samples and standards were added to a 96 well round bottom plate and incubated at 37°C for 10 min. After incubation, 100 µL of rFC enzyme, rFC assay buffer and rFC substrate were added at a ratio of 1:4:5 to the plate and an initial reading were taken followed by 1h incubation at 37°C. Thereafter, the relative fluorescence unit (RFU) for each well was determined (excitation 380 nm and emission 440 nm). The concentration of the endotoxin was interpolated from the standard curve constructed from the standards and corrected for sample dilution.

**Alkaline Phosphatase activity**

Alkaline phosphatase (ALP) activity was measured using the Quantichrom ALP assay kit (DALP-250, Gentaur, Bioassay systems, Hayward, CA). Protein was extracted using potassium phosphate buffer (PPB), pH 6.0 from liver and ileum and the protein concentration was determined using BCA assay (Pierce, Rockford, IL) and 50 µL of sample was added to a 150 µL working solution containing magnesium acetate, p-nitrophenyl phosphate and assay buffer in a 96 well plate. The optical density at 405 nm was measured at time 0 and after 4 minutes using a Synergy 4 microplate reader (Bio-Tek, Winooski, VT) and ALP activity was calculated according to the manufacturer’s instructions.
Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was measured in the whole ileal tissue as an indicator of inflammation and neutrophil infiltration using an o-dianasidine assay (de La Serre et al., 2010; Suzuki et al., 1983). Tissue samples were homogenized in PPB pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and then freeze-thawed on ice and vortexed three times. Samples were then centrifuged for 15 min at 10,000 x g. The resulting supernatant was transferred to a new tube and the remaining pellet was resuspended in 500 µL of PPB + 0.5% HTAB. The re-suspended pellet was freeze-thawed and homogenized twice and 500 µL of this solution was transferred to a new tube. Samples were then centrifuged again at 10,000 x g for 15 min and the supernatant was collected. The final supernatant was mixed with o-dianasidine dihydrochloride and 0.005% hydrogen peroxide. One unit of MPO activity was expressed as the amount of MPO needed to degrade 1 µmol of hydrogen peroxide/min/mL. Absorbance was read at 460 nm for 10 min reaction time and absorbance was calculated on mL sample/mg tissue basis.

Lysozyme activity

Whole ileum and liver samples were analyzed for lysozyme activity using the EnzChek fluorescent assay which compares sample lysozyme activity to lysozyme activity on Micrococcus Lysodeikticus cell walls (Invitrogen-Molecular Probes, Carlsbad, California). Samples were diluted and fluorescence was measured using excitation emission wavelengths of 485 and 530nm and the lysozyme activity were interpolated from the standard curve constructed from the standards and corrected for sample dilution.
**Interleukin-8 and Haptoglobin Assays**

Ileal protein (100 μg) extracts were analyzed for interleukin-8 (IL-8) concentration using a porcine-specific ELISA (DuoSet® Porcine IL8, catalog number DY535, R&D systems, Minneapolis, MN, USA) per the manufacturer’s instructions. Haptoglobin was analyzed using a commercially available ELISA (ALPCO diagnostics, Salem, NH). Briefly, samples were added to wells adsorbed with anti-porcine haptoglobin antibodies. After washing, horseradish peroxidase (HRP) conjugated anti haptoglobin antibodies were added to the plate. After another washing, the HRP was assayed by the addition of the chromogenic substrate 3,3’,5,5’-tetramethylbenzidine (TMB) and the absorbance was measured at 450 nm. The quantity of haptoglobin in the test sample was interpolated from the standard curve constructed from the standards and corrected for sample dilution.

**Quantitative real-time PCR**

Total RNA was isolated from tissue samples using Trizol (Invitrogen Inc., Carlsbad, CA) reagent according to the manufacturer’s protocol and the RNA pellets were resuspended in nuclease free water. To eliminate potential genomic DNA contamination, the RNA samples were treated with a DNase I kit (DNA-free, Ambion Inc., Austin, TX) per the manufacturer’s instructions. Total RNA was quantified by measuring the absorbance at 260nm using a spectrophotometer (ND-100, NanoDrop Technologies, Rockland, DE) and the purity was assessed by determining the ratio of the absorbance at 260 and 280 nm. All samples had 260/280 nm ratios above 1.8. The integrity of the RNA preparations was verified by visualization of the 18S and 28S ribosomal bands stained with ethidium bromide after electrophoresis on 1.2% agarose gels (E-gel; Invitrogen Inc., Carlsbad, CA). A good
preparation was indicated by the presence of 28S and 18S bands that were not smeared and by the 28S band stained with a greater intensity than the 18S band. Total RNA (1 μg) was reverse transcribed using a commercially available cDNA synthesis kit (iScript, BioRad Laboratories, Hercules, CA). The iScript kit used a blend of oligo (dT) and random hexamer primers for cDNA synthesis and the reverse transcriptase is RNAs H+ to ensure removal of the RNA template. The primers used for real-time RT-PCR are presented in Table 1.

Amplification was carried out in a total volume of 25 μL containing 1X iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA), forward and reverse primers (0.1 μg/μL) and 1 μL of the 20 μL cDNA reaction. After an initial 5 min denaturation step at 95°C, the reactions were cycled 40 times under the following parameters: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Optical detection was carried out at 72°C. At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. A non-template control was run with every assay and all determinations were performed in duplicate. Presence of a single PCR product of the correct size for each primer set was verified by visualizing the PCR products via electrophoresis on 1% agarose gels stained with ethidium bromide. The mRNA abundance values for each sample were normalized to β2 microglobulin according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

**Statistical Analyses**

All results were expressed as LS means ± SEM. The main effect of line (HRFI versus LRFI) was determined by the Proc Mixed procedure in SAS (Cary, NC) with age matched pairs (repetition) as a random effect. However, for Ussing chamber data tissue (colon versus ileum) was also included as a fixed effect. Statistical significance of differences was
determined by Tukey’s range test for pair wise comparisons. Differences were deemed significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$. Phenotypic correlations between serum endotoxin, ileum Endotoxin permeability, ALP and lysozyme with RFI, ADG, ADFI and G:F were computed based on residuals derived from the above models using the CORR procedure of SAS.

**Results**

Twelve gilts per line weighing approximately (62±3, kg BW) were used for the study. At the end of the experimental period, growth performance was measured. As expected, the LRFI pigs consumed less feed than their HRFI counterparts (ADFI: 1.90 vs. 2.23 kg/day, $P < 0.001$). However, the ADG did not differ between the lines (0.67 vs. 0.65, kg/day, $P = 0.72$). Since the LRFI pigs consumed less feed for the same ADG, their G:F ratio was higher than the HRFI pigs (0.35 vs. 0.29, $P = 0.019$). These results confirm that the effect of selection for RFI was maintained in the gilts used for this study.

Intestinal integrity was assessed by measuring the TER and macromolecule permeability in freshly isolated ileum and colon samples. Transepithelial resistance, an electrophysiological measure of intestinal integrity, was not different in either the ileum or colon of pigs divergently selected for RFI (Ileum: 108 vs. 103 $\Omega/cm^2$, Colon: 78 vs. 73 $\Omega/cm^2$, Table 1). However, irrespective of line, colon TER was significantly lower than in the ileum ($P < 0.05$). Further, these data were supported by additional ex vivo analysis of intestinal integrity using FITC-Dextran, a macromolecule permeability marker. No
differences between lines were observed in Papp for either the ileum or colon (P > 0.05, Table 1).

Ex vivo intestinal endotoxin transport characteristics were also assessed using FITC-LPS in modified Ussing chambers in the two RFI lines. Similar to the TER and FITC-Dextran values, we found no differences between the lines in either ileum and colon FITC-LPS transport (P > 0.05, Table 2). The tight junction proteins claudin 3 and 4 and occludin are three important proteins involved in intestinal barrier function and integrity. Gene expression analysis of these tight junction proteins also indicated no difference between the lines (P > 0.05, Table 3).

Serum endotoxin concentrations were found to be lower in the LRFI than in the HRFI gilts (6.19 vs. 17, EU/ml, P < 0.05, Figure 1). Furthermore, the serum acute phase protein, haptoglobin, was also lower in LRFI pigs than in HRFI gilts (P ≤ 0.05, Figure 2). The presence of a higher endotoxin load and the consequent increase in haptoglobin secretion has the potential to cause a generalized inflammation. The ileum was assessed for the presence of general inflammatory markers. Ileum myeloperoxidase activity was lower in the LRFI pigs than in the HRFI pigs (P = 0.047, Figure 3). The proinflammatory cytokine, IL-8 protein expression, also tended to be lower in the ileum of LRFI versus HRFI pigs (1.7 vs. 1.1, µg/g protein, P = 0.062, Figure 4).

Alkaline phosphatase activity (Figure 5) was measured and found to be significantly increased in the LRFI pigs in both liver (62 vs. 93 mU/mg protein, P<0.018) and ileum (597 vs. 947 mU/mg protein, P<0.01). Lysozyme activity in the liver was not different between the lines but ileum lysozyme activity in LRFI pigs was almost twice as high as in HRFI pigs (8.7 vs. 14.4, U/mg protein, P<0.01, Figure 6). Although we saw differences in activity of
these enzymes, surprisingly, no difference was found in the mRNA expression in either ileum or liver (Table 4). mRNA expression of LPS detoxification gene acyloxyacyl hydrolase (AOAH) present in liver and ileum tissues was quantified to assess differences between the two lines. The expression profiles of these genes was not different, indicating that at the mRNA level, LPS detoxification enzyme AOAH expression was not altered due to blood endotoxin, selection for RFI, or FE (Table 4).

Residual correlations of performance traits with endotoxin transport and serum concentrations, ALP and RFI were generally moderate (Table 5). Interestingly, this is the first data to our knowledge in swine that shows a significant positive correlation between intestinal endotoxin transport and circulating endotoxin (P = 0.019). Weak negative correlations were observed with FE measures and endotoxin permeability and circulating concentrations. Furthermore, ileum ALP activity was moderately negatively correlated with endotoxin concentration (P = 0.027). Lysozyme activity was poorly correlated with all parameters measures (P > 0.05).

**Discussion**

The current study was conducted to identify if differences in intestinal integrity, circulating endotoxin and its associated inflammatory markers may partially be responsible for difference in FE between pigs that were divergently selected for RFI. The physiological mechanisms underlying RFI or FE in swine are poorly defined. However, differences in nutrients and energy digestibility, metabolic efficiency of nutrient use, basal metabolic rate and energy expenditure are presumed to be contributing to differences in RFI, either alone or
in combination (Barea et al., 2010; Boddicker et al., 2011; Herd and Arthur, 2009). Endotoxin and its associated inflammation can reduce digestibility and alter intestinal nutrient transport and post-absorptive metabolism (Rakhshandeh and de Lange, 2012). The net effect of increased circulating endotoxin or endotoxemia is reduced growth, increased energy expenditure and antagonizing lean tissue accretion (Orellana et al., 2007). Thus, it results in partitioning energy and other nutrients away from growth and towards immune system requirements and thus may contribute to differences in FE.

The gastrointestinal tract contains large quantities of both gram positive and negative bacteria in which bacterial populations gradually increase from approximately $0-10^3$ per mL of luminal contents in the duodenum to $10^{11}$ per mL in the colon (Berg, 1999). The gram-negative bacterial family *Enterobacteriaceae*, includes genera such as *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Citrobacter* and *Enterobacter*, and serves as a major source of endotoxin (Billiar et al., 1988). Some of these strains are commensals, while others can be classified as opportunistic pathogens. The outer leaf of the gram negative bacterial outer membrane is mainly composed of a unique glycolipid generally known as endotoxin, or if purified, often referred to as LPS. Lipid A is the bioactive-immunogenic component that can stimulate localized or systemic inflammation via its recognition through toll like receptors (TLR) and other immune system sensing mechanisms (Beutler, 2000). Endotoxin may enter systemic circulation through routes including: 1) nonspecific paracellular transport through the tight junctions; 2) transcellular transport, potentially through lipid raft membrane domains via TLR4 mediated endocytosis (Tomlinson and Blikslager, 2004; Triantafilou et al., 2002); or 3) chylomicron facilitated transport into circulation during fat digestion (Kelly et al., 2012). All three transport routes may be altered by diet, management conditions and
environmental stressors (Mani et al., 2012). Furthermore, although characterization of intestinal microbial populations was not within the scope of this study, we cannot rule out RFI line specific differences in *Enterobacteriaceae* populations that may partially explain the increase in circulating endotoxin.

Interestingly, the presence of higher circulating endotoxin also has the potential to affect intestinal health and function (Gardiner et al., 1995). Ileal MPO activity and IL-8 protein expression were lower in the LRFI pigs. Both of these markers are commonly used for assessing intestinal inflammation and neutrophil infiltration (Suzuki et al., 1983). A “leaky” or porous intestine allows the harmful agents into the circulation, which can cause localized inflammation of the intestine and systemic inflammation (Vaarala et al., 2008). Further, decreased expression of tight junction proteins claudin 3 and 4 during inflammation have been implicated in increased intestinal permeability (Pinton et al., 2010). In our study, TER was not different between the lines and this was corroborated further by no differences in the paracellular transport of FITC-Dextran or FITC-LPS transport. Furthermore, mRNA expression of the tight junction proteins claudins 3 and 4 and occludin were not different between the lines. Together, these data indicate that in healthy pigs, intestinal integrity is tightly controlled and may not contribute significantly to differences in FE.

The presence of higher blood endotoxin has the potential to contribute to the development of an inflammatory state and reduce growth potential. In our study, serum endotoxin and the acute phase protein, haptoglobin, were found to be significantly lower in the LRFI pigs. Repeated exposure to endotoxin augments haptoglobin concentration compared to a single endotoxin exposure (Dritz et al., 1996; Wright et al., 2000). As haptoglobin is a good marker of tissue damage and the acute phase response in swine (Hall et
al., 1992), this supports our serum endotoxin data, which suggest that the LRFI pigs were exposed to comparatively lower levels of endotoxin intermittently, although the two lines shared the same environmental conditions (i.e. housing and diets). Furthermore, LRFI pigs appear to be experiencing a comparatively lower acute phase response. However, the high immune response of HRFI pigs appear not to be a result of increased intestinal permeability or endotoxin transport but could be a result of differences in detoxification processes.

Animals and humans harbor low concentrations of circulating endotoxin even in normal and healthy conditions (Erridge et al., 2007). Intriguingly, we found that the LRFI pigs had lower endotoxin levels compared to the HRFI pigs, with no difference in the intestinal integrity and transport characteristics between the lines. The potential biological process that might account for the lower endotoxin levels in the LRFI pigs may include the efficiency of endotoxin clearance, neutralization or detoxification. These processes occur in various tissues, including immune cells, liver, kidney and intestine. Furthermore, various binding proteins and enzymes such as ALP, lysozyme, and AOAH are involved (Munford et al., 2009). Alkaline phosphatase is a hydrolase enzyme present in liver, intestine and kidney tubules and it dephosphorylates bacterial LPS and reduces its toxicity (Bates et al., 2007; Poelstra et al., 1997). Further, in the intestine ALP reduces trans-mucosal passage of bacteria and also protects against LPS-induced inflammation (Lallès, 2010). Higher ALP activity in both liver and ileum of LRFI pigs indicates that they deactivated or neutralized endotoxin more efficiently. This may be affirmed by the moderately negative correlation between ALP and serum endotoxin concentration. Further evidences suggest that ALP can also mitigate body weight loss after an immune challenge (Bol-Schoenmakers et al., 2010).
To further explore the difference in endotoxin metabolism, we measured AOAH mRNA expression. Acyloxyacyl hydrolase is an important lipase enzyme that selectively removes the secondary fatty acyl chains attached to the primary chains in the lipid A moiety and detoxifies endotoxin (McDermott and Fenwick, 1992). This leads to an LPS molecule which could bind the signaling proteins MD2/TLR4 but does not have the potential to initiate the signal or can only be a partial agonist (Lu et al., 2005). The fact that we did not see evidence of differential AOAH mRNA expression in the intestine or liver was surprising. However, AOAH mRNA and activity appear to be correlated in a tissue or cell specific manner (Feulner et al., 2004). Therefore, liver and intestinal mRNA expression may not be correlated in swine or immune and Kupffer cell specific expression needs to be determined, where activity and expression of AOAH are greater.

Lysozyme is another important antimicrobial peptide secreted by various cells of the body, including cells in the intestine and liver. Lysozyme regulates microbial populations by lysing the bacterial cell wall component peptidoglycan and it also binds to and detoxifies LPS (Takada et al., 1994). In our study, although lysozyme activity in the liver was not different between the two lines, ileum lysozyme activity was higher in LRFI pigs, which may explain the lower serum endotoxin and haptoglobin levels in the LRFI pigs and higher FE. Supporting this hypothesis, a recent study found that feeding lysozyme to young pigs improved their health and FE (May et al., 2012). Surprisingly, although ALP and lysozyme activity was different between the two lines in ileum and liver, mRNA expression of both enzymes were not different in the liver and ileum, indicating a post-translational mechanism which may act differently in the two lines of pigs.
In conclusion, our results indicate that LRFI pigs seem to have a more robust intestinal and liver endotoxin detoxification and higher active anti-microbial enzymes including ALP, ileum lysozyme and the inflammatory mediator enzyme myeloperoxidase, and that HRFI pigs seem to be undergoing a greater level of basal inflammation. Although lower serum endotoxin and the associated decreased inflammatory markers and the enhanced activities of antimicrobial enzymes in the LRFI pigs may not explain the line difference in FE wholly, it has the potential to be a significant contributing factor. Further studies are needed to identify other mechanisms that contribute to the lower endotoxin levels in the LRFI pigs and how this is associated with their higher FE.

**Literature Cited**


Figure 1. Circulating serum endotoxin levels in pigs divergently selected for high (HRFI) or low (LRFI) residual feed intake. N= 12 pigs/line.
Figure 2. Serum haptoglobin concentration in pigs divergently selected for high (HRFI) or low (LRFI) residual feed intake. N= 12 pigs/line.
Figure 3. Ileum myeloperoxidase activity in pigs divergently selected for high (HRFI) or low (LRFI) residual feed intake. N = 12 pigs/line.
Figure 4. Ileum interleukin 8 protein concentration in pigs divergently selected for high (HRFI) or low (LRFI) residual feed intake. N= 12 pigs/line.
Figure 5. Liver and Ileum alkaline phosphatase activity in pigs divergently selected for high (HRFI) or low (LRFI) residual feed intake. N= 6 pigs/line.
Figure 6. Liver and ileum lysozyme activity in pigs divergently selected for high (HRFI) or low (LRFI) residual feed intake. N= 6 pigs/line.
Table 1. Primer sequences for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sense (5’- 3’)</th>
<th>Antisense (5’- 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOAH (159 bp)</td>
<td>TCAGGGGGACAGAAATATGG</td>
<td>CCAGAATCAGCAGAATCAC</td>
</tr>
<tr>
<td>Lysozyme (NM_214392.2; 80 bp)</td>
<td>CGGTGCGAGTTCGAGAATTC</td>
<td>AAACACACCAAGTGCCAGGC</td>
</tr>
<tr>
<td>Intestinal alkaline phosphatase (XM_003133729; 139 bp)</td>
<td>GTCAGAACGGAGTTCCAGGAAG</td>
<td>AGGCCCATGAGGTGTTAC</td>
</tr>
<tr>
<td>Claudin 3 (NM_001160075; 95 bp)</td>
<td>CATCGGCAGCAGCATTATC</td>
<td>ACACHTTGCACTGACTCTG</td>
</tr>
<tr>
<td>Claudin 4 (NM_001161637.1; 110 bp)</td>
<td>AGGTGATGGGATCAGCTTCGAGGC</td>
<td>CGACGTAGCAGATGTTGCTG</td>
</tr>
<tr>
<td>Occludin (NM_00163647; 115 bp)</td>
<td>ATCATGAGGGTGTGGGATTG</td>
<td>ACTGTTGCAAGGGCATAG</td>
</tr>
<tr>
<td>β-2-Microglobulin</td>
<td>TGGTCTTTCTCCTTTGTCGG</td>
<td>TGTGATGCCGGTGTAGTGCT</td>
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</tbody>
</table>
**Table 2.** Intestinal integrity is not altered in gilts divergently selected for low (LRFI) and high (HRFI) residual feed intake

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ileum</th>
<th>Colon</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LRFI(^1)</td>
<td>HRFI(^1)</td>
<td>LRFI(^1)</td>
<td>HRFI(^1)</td>
</tr>
<tr>
<td>TER(^2) (Ω/cm(^2))</td>
<td>108</td>
<td>103</td>
<td>78</td>
<td>73</td>
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<tr>
<td>FITC-Dextran(^3) (Papp)</td>
<td>0.48</td>
<td>0.52</td>
<td>0.13</td>
<td>0.19</td>
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<tr>
<td>FITC-LPS(^3) (Papp)</td>
<td>2.76</td>
<td>3.63</td>
<td>3.95</td>
<td>3.39</td>
</tr>
</tbody>
</table>

\(^1\) n=6 pigs/line.

\(^2\) Transepithelial resistance (TER).

\(^3\) Papp=Apparent permeability coefficient (µg/mL/min/cm).
Table 3. Ileum tight junction protein gene expression pigs selected for high (HRFI) or low residual feed intake (LRFI).

<table>
<thead>
<tr>
<th>Gene</th>
<th>HRFI&lt;sup&gt;1,2&lt;/sup&gt;</th>
<th>LRFI&lt;sup&gt;1,2&lt;/sup&gt;</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin 3</td>
<td>14.76</td>
<td>14.41</td>
<td>0.530</td>
<td>0.65</td>
</tr>
<tr>
<td>Claudin 4</td>
<td>8.09</td>
<td>8.00</td>
<td>0.874</td>
<td>0.94</td>
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<tr>
<td>Occludin</td>
<td>13.75</td>
<td>12.01</td>
<td>0.936</td>
<td>0.22</td>
</tr>
</tbody>
</table>

<sup>1</sup> N=6 pigs/line.
<sup>2</sup> Mean gene expression (ΔΔCT) from β-2-microglobulin housekeeper.
Table 4. Lipopolysaccharide and Gram positive bacteria detoxification mRNAs in pigs divergently selected for high (HRFI) and low (LRFI) residual feed intake

<table>
<thead>
<tr>
<th>Gene</th>
<th>HRFI$^{1,2}$</th>
<th>LRFI$^{1,2}$</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver Acyloxyacyl hydrolase</td>
<td>5.38</td>
<td>6.34</td>
<td>0.87</td>
<td>0.30</td>
</tr>
<tr>
<td>Ileum Acyloxyacyl hydrolase</td>
<td>7.45</td>
<td>6.98</td>
<td>0.35</td>
<td>0.37</td>
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<tr>
<td>Ileum Lysozyme</td>
<td>8.22</td>
<td>7.41</td>
<td>0.97</td>
<td>0.57</td>
</tr>
<tr>
<td>Ileum Alkaline Phosphatase</td>
<td>12.38</td>
<td>11.97</td>
<td>0.77</td>
<td>0.71</td>
</tr>
</tbody>
</table>

$^1$ N=6 pigs/line.
$^2$ Mean gene expression ($\Delta \Delta CT$) from β-2-microglobulin housekeeper.
Table 5. Residual correlations of performance parameters, endotoxin transport and serum concentration, and alkaline phosphatase in gilts divergently selected for residual feed intake$^1$

<table>
<thead>
<tr>
<th></th>
<th>ADFI$^2$</th>
<th>ADG$^2$</th>
<th>Gain:Feed$^2$</th>
<th>RFI Index$^3$</th>
<th>Serum endotoxin$^2$</th>
<th>Ileum Endotoxin permeability$^2$</th>
<th>Ileum Alkaline Phosphatase$^2$</th>
<th>Ileum Lysozyme$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADFI$^1$</td>
<td>1.000</td>
<td>0.35</td>
<td>-0.58</td>
<td>0.65</td>
<td>0.57</td>
<td>0.50</td>
<td>-0.45</td>
<td>-0.21</td>
</tr>
<tr>
<td>ADG$^1$</td>
<td></td>
<td>1.00</td>
<td>0.55</td>
<td>0.02</td>
<td>0.14</td>
<td>-0.29</td>
<td>0.28</td>
<td>0.40</td>
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<tr>
<td>Gain:Feed$^1$</td>
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<td></td>
<td>1.00</td>
<td>-0.55</td>
<td>-0.40</td>
<td>-0.47</td>
<td>0.47</td>
<td>0.34</td>
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<tr>
<td>RFI Index$^2$</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
<td>0.34</td>
<td>0.57</td>
<td>-0.43</td>
<td>-0.26</td>
</tr>
<tr>
<td>Serum endotoxin$^1$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.13</td>
<td>0.052</td>
<td>0.17</td>
<td>0.41</td>
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<tr>
<td>Ileum Endotoxin permeability$^1$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
<td>-0.63</td>
<td>-0.43</td>
</tr>
<tr>
<td>Ileum Alkaline Phosphatase$^1$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
<td>0.25</td>
</tr>
<tr>
<td>Ileum Lysozyme$^2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>

$^1$Upper row = residual correlations. Bottom row = P - values.

$^2$ADFI = kg/d, ADG = g/d, Serum endotoxin = EU/mL, Ileum Endotoxin permeability = Papp, Ileum Alkaline Phosphatase = mU/mg; Ileum Lysozyme = U/mg.

$^3$Residual feed intake (RFI) = adfi-$\beta_1$(ontest wt deviation)+$\beta_2$(offtest wt deviation)+$\beta_3$(metabolic mid-wt)+$\beta_4$(adg)+$\beta_5$(offtest backfat)
CHAPTER 7. GENERAL CONCLUSIONS

A multitude of factors affect the health and production performance of farm animals and human health and wellbeing. Lipopolysaccharide and its associated inflammation is one such factor that has received much attention. This thesis research provided further understanding into the mechanism by which LPS enters the body and its ability to induce inflammation as well as the impact dietary fat has on modulation of intestinal LPS permeability and signaling. A particular emphasis was placed on the role DHA and EPA n-3 PUFA have on these processes.

Recent evidence suggests that high caloric and high dietary fat increases serum LPS concentration and induce acute low grade inflammation, a predisposing factor for common metabolic diseases such as obesity, insulin resistance and atherosclerosis (Erridge et al., 2007; Ghanim et al., 2010). In Chapter 3 of this thesis, we first sought to explore whether fatty acid composition of common household dietary oils differentially modulates intestinal LPS permeability using \textit{in vivo} and \textit{ex vivo} pig models. Dietary oils were orally administered to pigs and postprandial serum LPS concentration determined. Additionally, freshly isolated ileum tissues were pretreated \textit{ex vivo} in modified Ussing chambers with different dietary oils and FITC-LPS permeability was assessed. These experiments indicated that oils rich in n-3 PUFA, such as fish and cod liver oil decreased postprandial LPS concentration. Oils rich in saturated fatty acids, such as coconut oil, increased the ileum LPS permeability. However, oils rich in monounsaturated fatty acids, i.e. corn oil and olive oil, did not alter LPS permeability or circulating LPS concentration. Interestingly, corn oil has been previously
Figure 1. Schematic representation of lipopolysaccharide (LPS) intestinal permeability and systemic signaling under normal, n-3 PUFA supplemented and LPS challenge conditions. Under normal conditions, lipid rafts are enriched with cholesterol, n-6 and saturated fatty acids and they are stable or ordered to facilitate membrane signaling. Lipopolysaccharide stimulation causes toll-like receptor (TLR)-4 localization into lipid rafts which facilitate receptor mediated LPS permeability or signaling. This results in increased pro-inflammatory cytokine production and immune activation. However, n-3 PUFA such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) enrich membrane phospholipids and cause lipid rafts to become less ordered and dissociated. This leads to decreased localization of TLR4 into the rafts which eventually results in attenuated LPS signaling and permeability. Alternatively, after LPS challenge, decreased localization of TLR4 into raft occurs which causes an attenuated inflammatory response and desensitization to subsequent LPS. The dotted lines and boxes indicate mechanisms which are not completely understood yet.
shown to increase postprandial endotoxemia (Cani et al., 2007; Laugerette et al., 2012). This discrepancy may be due to the different species of animals used in these papers and divergent experimental conditions. Finally, in this Chapter we explored the role of saturated fatty acids and lipid raft mediated LPS permeability in ex vivo intestinal segments. We showed that saturated fatty acids stabilize the lipid rafts which enhance LPS permeability and dissociation of lipid raft lead to decreased saturated fatty acid mediated LPS permeability implicating lipid raft’s essential role in LPS permeability.

Lipopolysaccharide enters the circulation from the intestine through three major routes. 1) Receptor mediated endocytosis through toll like receptor (TLR) 4 mediated mechanisms; 2) paracellular permeability through tight junctions if the intestine is damaged; or 3) postprandial permeability through micelles (Kelly et al., 2012; Tomita et al., 2004). Paracellular and micellar mode of entry may not account completely for the decreased LPS permeability we observed with dietary n-3 PUFA in Chapter 3. To explore the mechanisms by which of n-3 PUFA attenuate intestinal LPS permeability, we raised pigs on either a corn-soybean or EPA and DHA enriched diets (Chapter 4). Thereafter, we stimulated an inflammatory response in these pigs with LPS and measured their inflammatory parameters. These data suggest n-3 PUFA attenuated the LPS induced febrile and inflammatory response. This work agrees with research by Huang et al in which DHA and EPA reduced LPS signaling and inflammation (Huang et al., 2012).

Interestingly, n-3 PUFA supplemented pigs also had lower endogenous circulating LPS concentration and reduced \textit{ex vivo} intestinal LPS permeability (Chapter 4). Intestinal epithelial cells are part of innate immune system and express TLRs similar to immune cells
such as macrophages (Abreu, 2010). In macrophages, DHA attenuated LPS mediated immune signaling by preventing the localization of the LPS receptor TLR4 into the cell membrane lipid raft microdomains (Fan et al., 2004; Wong et al., 2009). Therefore, we explored whether the same phenomena might be exhibited in the intestinal epithelium and that n-3 PUFA dissociation of the lipid raft order may explain attenuated transcellular LPS permeability. Lipid raft from the intestinal epithelium were isolated and the LPS signaling and permeability proteins (TLR4 and CD14) localization into lipid rafts were measured. Herein, we report that dietary n-3 PUFA enrichment decreased localization of TLR4 and CD14 in the lipid rafts. Phospholipid fatty acid composition of the lipid raft fractions revealed the enrichment of phosphatidyl ethanolamine and phosphatidyl serine with DHA and EPA which has the potential to dissociate the lipid rafts and prevent the localization of proteins (Fan et al., 2003). Therefore, n-3 PUFA enrichment associated destabilization and dissociation of the lipid raft results in decreased LPS signaling and permeability which might account for the decreased LPS permeability and circulating LPS concentrations in our pig models.

An acute systemic inflammatory challenge resulted in the removal of intestinal TLR4 and CD14 from the lipid rafts of control pigs making the animal vulnerable to any further immune challenge whereas n-3 PUFA supplemented pigs had TLR4 and CD14 still present in the rafts giving the advantage of defending the next immune challenge. Our results indicate that enrichment of intestinal epithelial cell membrane phospholipids with n-3 PUFA destabilizes and dissociates the lipid raft resulting in decreased LPS signaling and permeability.
Long term supplementation of high dietary saturated fat shifts the microbial population of the intestine resulting in the death of gram negative bacteria in large numbers which subsequently become sources of serum LPS as well as decreasing the beneficial gram positive bacteria which would contribute to gram negative bacterial overgrowth (Cani et al., 2007; Kim et al., 2012; Laugerette et al., 2012). Interestingly, long term n-3 PUFA supplementation has not been shown to change the microbial population of the intestine (Geier et al., 2009; Li et al., 2011). So, in our study n-3 PUFA supplementation may not have shifted the microbial population towards more Gram negative organisms which might have led to more LPS production. The differences we observed with regard to the serum LPS might have come mostly from differences in intestinal TLR4 localization in the lipid rafts and transcellular LPS permeability.

Maternal supplementation of n-3 PUFA during gestation and lactation has been shown to have beneficial effects in the offspring immediately after birth as well as later in life such as increasing IQ and controlling obesity and neutrophil infiltration (Helland et al., 2003; Taylor and Poston, 2007). In Chapter 5 of our research we examined the effect of maternal n-3 PUFA supplementation during both gestation and lactation modulating LPS induced inflammation later in life. Maternal n-3 PUFA supplementation had an anti-inflammatory effect which is almost equivalent to the continuously supplemented pigs. Maternal n-3 PUFA supplementation induced the n-3 PUFA enrichment of buffy coat, decreased the febrile response, decreased the serum cytokine levels for TNF-α and mRNA abundance of cytokines TNF-α, IL-1β and IL-10 in buffy coat cells. Further supplementation also decreased expression of LPS signaling proteins including TLR4, CD14 and Myd88 in
buffy coat cells under LPS challenge. Our research indicates that maternal supplementation with n-3 PUFA provides protective advantages against inflammation later in life.

To understand the relationship between intestinal barrier integrity, endotoxin and inflammation with feed efficiency, in Chapter 6 we used pigs divergently selected for residual feed intake (RFI, with low RFI being more efficient compared to high RFI). These two lines of pigs provide an excellent model to study the physiological mechanisms behind feed efficiency in pigs. HRFI pigs exhibited a greater level of basal inflammation compared to their LRFI counterparts. Furthermore, the LRFI pigs had lower circulating endotoxin concentration and more robust LPS detoxification and anti-microbial enzyme machinery. Chronic immune stimulation like persistent presence of higher endotoxin in the serum has the potential to divert nutrients away from anabolic processes to immune functions resulting in reduced growth (Buchanan and Johnson, 2007; Gabler and Spurlock, 2008). Lower endotoxin and inflammatory markers and the enhanced activities of antimicrobial enzymes in the LRFI line may not fully explain the difference in the feed efficiency between the lines; but they have the potential to prevent the growth potential in HRFI pigs.

Overall, this research lays the foundation for several hypothesis driven objectives which will have immense beneficial effects for humans and livestock. The first line of research could be to measure the exact contribution of LPS and its associated inflammation on feed efficiency, appetite and lean tissue accretion in livestock. This work could be done with animals of same genetic background and raised in germ free environment administered with a known dose(s) of LPS.
A second line of research could identify the changes in intestinal bacterial populations and their contribution to endotoxemia. Little is known about the effects of dietary fats on intestinal microbiota. Metagenomic and bacteria viability assays could be used to determine the dynamic shift in bacterial populations during fatty acid administration. Further, whether processed oils have the same biological effects as unprocessed oils should also be investigated as cooking at high temperatures is known to degrade unsaturated fatty acids which might have a different biological function and also has the potential for negative health consequences.

Thirdly, one could identify the exact percentage of n-3 PUFA need to be supplemented to give the beneficial anti-inflammatory effects and also does not compromise the carcass quality. Further, studying immune cell infiltration into the intestinal epithelium and the extent of intestinal inflammation contributes towards intestinal integrity and function warrants more attention. Further, investigation of mechanisms of anti-inflammatory effect of n-3 PUFA in the intestine will have beneficial effects and therapeutic applications in two most important human intestinal diseases like infectious bowel disease and ulcerative colitis.

Finally, Mechanism of n-3 PUFA epigenetic modifications during maternal supplementation and the anti-inflammatory effect in the offspring of n-3 PUFA could also be a potential avenue for research. Changes in methylation pattern through binding assays, assessing chromatin remodeling through histone modifications using chromatin immune precipitation assay as well as deep sequencing for identifying single nucleotide polymorphisms under n-3 PUFA supplementation can be carried out.
In conclusion, this dissertation presented evidence that the presence of LPS is correlated to feed efficiency. Furthermore, dietary fat in particular saturated and n-3 PUFA differentially modulates intestinal LPS permeability and signaling. These fatty acids appear to play a critical role in lipid raft mediated signaling and assembly of signaling proteins. By studying the role of lipid rafts and dietary fatty acids in LPS and bacterial pathogenesis, gives us opportunities to better understand, prevent and treat disease and inflammation. This research will then enable us to improve livestock performance and human health and wellbeing.

Reference


The modulation of intestinal endotoxin transport by different dietary fats

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Experimental Biology, Anaheim, CA, April, 2010,

Intestinal derived endotoxin and the subsequent endotoxemia are considered to be major predisposing factors for diseases such as atherosclerosis, sepsis, obesity and diabetes. Therefore, the aim of this study was to assess the effects of dietary fat on intestinal endotoxin transport. We hypothesized that saturated fat (SF) would augment, while n-3 fatty acids (FA) would attenuate endotoxin transport across the small intestine of pigs. Eleven pigs (three-four weeks of age) were euthanized and jejunum segments were mounted in modified Ussing chambers. Segments were treated with either no oil (CON), 12.5% (v/v) vegetable oil (VO), DHA (DO), cod liver oil (CLO), coconut oil (CO) or olive oil (OO) in 20 mM bile acid. Ex vivo endotoxin transport was measured by the addition of fluorescein isothiocyanate labeled lipopolysaccharide (FITC-LPS, 20 µg/mL) and serial sampling of the mucosal and serosal chambers. Fluorescence was measured in a plate reader and an apparent permeability coefficient (Papp) was calculated using the surface area and transport rate. Compared to the CON, DO and CLO reduced the endotoxin Papp by 50% (P<0.01). However, versus the CON, CO increased the Papp by 60% (P=0.008). OO and VO Papp were not different from the CON. Overall, these data indicate that fat high in n-3 FA can attenuate intestinal endotoxin transport, while SF augments endotoxin transport. This work was supported by the USDA and NWRC.
Dietary n-3 fatty acids attenuates colon endotoxin transport in growing pigs

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Midwest Animal Science Meeting, Des Moines, IA, March, 2010

Intestinal absorption of the bacterial endotoxin plays an important role in the development of intestinal dysfunction, inflammation, peripheral tissue catabolism and reduced pig performance by activating the immune system. However, the ability of dietary n-3 fatty acids (FA), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), to regulate intestinal endotoxin transport in growing pigs is unknown. Therefore, this study aimed to evaluate the ability of dietary n-3 FA to attenuate large intestine endotoxin transport and circulating endotoxin levels in growing pigs under basal or immune challenged conditions. Ten pigs (22±2.4 kg BW) were fed one of two diets, either a standard corn-soybean control diet (CON) or the CON plus 0.5% Gromega™ (JBS United Inc., Sheridan, IN), which was high in DHA and EPA (GRO). After 8 weeks of feeding, the CON and GRO groups were sub-divided and challenged (n=5 pigs/trt) with an intramuscular injection of either saline (SAL) or lipopolysaccharide (CH; LPS 10 µg/kg BW). The febrile response was measured by rectal temperature hourly, blood was collected at 0 and 4 h for serum endotoxin levels and the pigs were euthanized 4 hrs post-challenge. Segments of the mid-colon were isolated, flushed and mounted in modified Ussing chambers to measure ex vivo FITC-LPS endotoxin transport and an apparent permeability coefficient (Papp) was calculated using the area of the membrane and transport rate. Average rectal temperatures tended to be reduced in
the GRO vs CON pigs, $P<0.055$. Furthermore, CON pigs had higher endotoxin transport compared to the GRO pigs (7.4 vs 2.0, respectively, $P=0.02$). Irrespective of diet, CH lowered the endotoxin transport compared to the SAL pigs ($P=0.02$). There is significant difference between CON-SAL and GRO-SAL colon Papp (11.4 vs 3.3, respectively, $P<0.01$), however, there were no differences between the CON-CH and GRO-CH pigs. Four hour post-challenge serum endotoxin level tended to five-fold higher in the CON vs GRO pigs ($P=0.07$). Overall, these data indicate that n-3 FA may mitigate colon transport and serum levels of endotoxin
Bitter compounds decrease gastric emptying and influence intestinal nutrient transport

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Taste alters food perception and may contribute to body weight regulation. Bitter taste receptor expression has been demonstrated in the tongue, stomach, intestine, and lung. Bitter compounds increase intestinal hormone secretions of GLP-1, PYY, GIP and CCK, which are associated with gastric emptying, intestinal motility and satiety. Therefore, the aim of this study was to assess the effect of bitter compounds on gastric emptying (GE) and nutrient transport (NT) in pigs. Sixteen pigs (35±3 kg body weight) were fasted overnight and fed a 600 g meal that was either a control meal or an identical meal containing 1 mM phenylthiocarbamide (PTC). Exactly 45 min after completing the meal, all pigs were euthanized and the weight of gastric contents measured. Further, jejunum segments were excised and mounted into modified Ussing chambers for NT. The jejunum was pre-treated for 30 minutes with or without 5 mM PTC before measuring glucose, lysine and glutamine transport. All pigs ate their respective meal. However, the PTC meal decreased gastric emptying by 30% compared to the control (P < 0.01). Glucose, lysine and glutamine NT was increased by 250% compared to the control pigs (P<0.05). However, ex vivo jejunum pre-treatment with PTC had no effect on NT (P > 0.1). Overall, these data indicate that feeding PTC decreases GE and increases NT in the intestine, but localized exposure of the intestine to PTC doesn’t have any effect.
The effects of immune stressors on porcine intestinal epithelial cell integrity and inflammation

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Intestinal epithelial cells continually encounter luminal pathogens, immunogens and toxins. However, data regarding the effects of these substances on intestinal integrity and function in pigs are limited. Our study objective was to examine the effect of immunogens on barrier integrity and inflammation in IPEC-J2 cells. Cells were plated on 0.4 µm pore size collagen coated transwells, where they form a single confluent monolayer, polarize and form tight junctions (TJ). The transepithelial electrical resistance (TER) was measured to evaluate TJ formation and integrity along with FITC-Dextran (FD, 4 kDa) macromolecule permeability. When the cells attained peak TER, approximately 9 days post confluence, cells were treated with the immune agonists lipopolysaccharide (LPS, 10 µg/ml, E.coli 055:05), PolyI:C (PIC, 20 µg/ml), zymosan (ZYM, 100 µg/ml) and deoxynivalenol (DON, 20 µm) on the luminal side, or with tumor necrosis factor \( \alpha \) (TNF\( \alpha \)) and interleukin 1\( \beta \) (IL1\( \beta \)) on the basolateral side for 48 hours. The TER and FD permeability was assessed for membrane integrity. Interleukin 8 (IL-8) secreted into the media was measured as a marker of inflammation. After 48 h of DON or TNF\( \alpha \) treatment, TER was significantly reduced compared to the non-challenged control (P<0.05; 53 and 63\%, respectively). The TER was not different from the control when cells were exposed to ZYM, PIC or IL1\( \beta \). Further, FD permeability did not differ
between the treatments. Compared to the control, media IL-8 concentrations were increased by TNFα and LPS (P<0.05; 0.03, 2.68 and 0.96 ng/ml, respectively). Treatment with PIC and ZYM did not increase IL-8 secretion (P>0.10; 0.61 and 0.31 ng/ml respectively). These data indicate that IPEC-J2 cells are particularly responsive to inflammation and barrier integrity modifications induced by DON, TNFα and LPS. However, barrier integrity appears to be maintained under most challenge conditions.
Lipopolysaccharide and n-3 fatty acids alter intestinal Toll like receptor 4 (TLR4) recruitment and function

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Previously we reported that dietary n-3 fatty acids (FA) decrease intestinal lipopolysaccharide (LPS) transport (LT) and serum endotoxin in pigs. Endotoxin or LPS is recognized by TLR4 to initiate an innate immune response. Localization of TLR4 to lipid raft (LR) membrane micro domains is critical for cellular LT and signaling in numerous cells. Our objective was to examine the effects of n-3 FA and LPS on intestinal TLR4 LR recruitment and LT. Twenty pigs (22±2.4 kg) were fed two diets: 1) control (CON); 2) CON plus 0.5% Gromega™ (GRO, JBS United Inc.), high in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) n-3 FA. After eight weeks, the CON and GRO pigs were challenged (n=5 pigs/trt) with either an I.M. injection of E. coli LPS (CH; 10 µg/kg BW) or saline (SAL). Four hours after CH or SAL, pigs were euthanized and ileum and colon segments mounted into Ussing chambers to measure ex vivo FITC-LPS apparent permeability coefficient (Papp) as a marker of LT. Ileum and colon mucosa were assessed for n-3 FA enrichment, LR isolated and membrane localization of TLR4 determined. Compared to the CON, pigs fed GRO had increased ileum and colon EPA, DHA and total n-3 FA content (P<0.05; 200, 250, 300%, respectively). Overall, ileum LT did not differ due to FA or CH treatments. However, GRO-SAL treated pigs tended to have decreased LT by 37%
compared to the CON-SAL pigs (P=0.06). Pigs injected with CH had attenuated colon LT (P=0.02). Pigs fed GRO also had reduced colon LT compared to the CON (P=0.03; 2.0 vs. 7.4 Papp, respectively). Compared to CON-SAL treated pigs, ileum and colon TLR4 recruitment into LR micro domains was decreased in the GRO-SAL pigs. However, CH reduced ileum LR TLR4 protein in CON, but not GRO fed pigs. Localization of TLR4 into LR didn’t differ in the colon of CON-CH and GRO-CH treatment groups. These data indicate that n-3 FA decrease TLR4 recruitment into intestinal LR. This may explain how DHA and EPA attenuate receptor mediated LT and LPS induced febrile response. Furthermore, reduced LR localization of TLR4 post CH, may describe an LPS tolerance mechanism.