

**EFFECT OF DIETARY SOURCE AND CONCENTRATIONS OF COPPER,
MANGANESE, AND ZINC ON GROWTH PERFORMANCE AND IMMUNE
RESPONSE OF NURSERY PIGS**

By

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Abstract: Two experiments were conducted to evaluate dietary source and concentrations of copper, manganese, and zinc on growth performance and the immune response of nursery pigs. Experiment 1 and 2 evaluated two sources of copper, manganese, and zinc: sulfate and chloride. Both sources were then evaluated in two dietary concentrations. To study the acute immune response, all pigs in both experiments were subjected to acute immune challenge by single intraperitoneal injection of lipopolysaccharide (LPS). Additionally, pigs from experiment 2 were subjected to multiple intramuscular injections of LPS to evaluate the chronic immune response. The data from experiment 1 and 2 relative to growth performance and acute immune challenge were combined for statistical analysis. Overall, BW, ADG, ADFI, and G:F ratio were not different among dietary treatment groups. Following the acute immune challenge, pigs fed both dietary concentrations of chlorides produced lower concentrations of TNF- α and IL-1 β . A concentration effect was also observed, with higher dietary concentrations of both sources producing less TNF- α and IL-1 β . Total-SOD activity was not affected by dietary treatments following the acute immune challenge. During the multiple LPS injections, growth performance was negatively affected; however, no differences were observed among dietary treatment groups. Numerically, TNF- α concentration was lower and total-SOD activity was greater in both dietary concentrations of chlorides. IL-1 β was not affected by dietary treatment group during the chronic immune challenge. In conclusion, chloride sources are able to alleviate the acute immune response by decreasing the proinflammatory cytokine production regardless of the dietary concentration, suggesting a higher bioavailability over sulfate sources. Besides that, high dietary concentration of copper, manganese, and zinc showed more promising results, indicating that the immune response may have a greater requirement than that needed for growth. Following a chronic immune challenge, chloride sources showed a numerical advantage over sulfates, suggesting less stimulation of the inflammatory response.

TABLE OF CONTENTS

Chapter	Page
I. INTRODUCTION	1
II. REVIEW OF LITERATURE	4
Weaning process and its complications	4
Review of the immune system	7
Superoxide dismutase – manganese and copper-zinc SOD.....	10
Proinflammatory cytokines – TNF- α and IL-1 β	13
Copper	17
Copper and performance	17
Copper and immune system	23
Manganese	25
Manganese and performance	25
Manganese and immune system	27
Zinc	29
Zinc and performance	29
Zinc and immune system	35
Lipopolysaccharide challenge in pigs	38
Summary	42
III. EFFECT OF DIETARY SOURCE AND CONCENTRATIONS OF COPPER, MANGANESE, AND ZINC ON GROWTH PERFORMANCE AND IMMUNE RESPONSE OF NURSERY PIGS FOLLOWING ACUTE AND CHRONIC LIPOPOLYSACCHARIDE CHALLENGE	44
Abstract	44

Chapter	Page
Introduction	47
Materials and Methods	48
Results	56
Growth performance	56
Acute LPS challenge – Body weight and rectal temperature changes ...	
.....	57
Acute LPS challenge – Blood analytes	58
Growth performance following chronic LPS challenge	60
Chronic LPS challenge – Blood analytes	60
Discussion	61
Conclusion	69
 IV. SUMMARY	 93
 REFERENCES	 94
 APPENDICES	 118
Appendix 1 – Experiment 1	118
Appendix 2 – Experiment 2	127
Appendix 3 – Pilot Study	145

LIST OF TABLES

Table	Page
 CHAPTER III	
III.1. Nutrient composition of the diets	70
III.2. Chemical composition of the basal diets.....	71
III.3. Pens allotment for chronic LPS challenge	72
III.4. Effect of source and dietary concentrations of copper, manganese, and zinc on growth performance of nursery pigs	73
III.5. Effect of source and dietary concentrations of copper, manganese, and zinc on body weight and rectal temperature of nursery pigs following an acute LPS challenge	74
III.6. Effect of source and dietary concentrations of copper, manganese, and zinc on blood analytes and total-SOD activity of nursery pigs following an acute LPS challenge	75
III.7. Effect of multiple intramuscular injections of LPS or saline solution on growth performance of nursery pigs	76
III.8. Effect of source and dietary concentrations of copper, manganese, and zinc on growth performance of nursery pigs following a chronic LPS challenge	77

Table	Page
III.9. Effect of source and dietary concentrations of copper, manganese, and zinc on blood analytes and total-SOD activity of nursery pigs following a chronic LPS challenge	78

LIST OF FIGURES

Figure	Page
CHAPTER II	
II.1. Signaling recognition of lipopolysaccharide (LPS) and activation of proinflammatory cytokines production	41
CHAPTER III	
III.1. Effect of dietary source and concentrations of copper, manganese, and zinc on body weight of nursery pigs following acute lipopolysaccharide challenge	80
III.2. Effect of dietary source and concentrations of copper, manganese, and zinc on rectal temperature of nursery pigs following acute lipopolysaccharide challenge.	81
III.3. Effect of dietary source and concentrations of copper, manganese, and zinc on serum tumor necrosis factor- α (TNF- α) of nursery pigs 3 hours following acute lipopolysaccharide challenge.....	82
III.4. Effect of dietary source and concentrations of copper, manganese, and zinc on serum tumor necrosis factor- α (TNF- α) fold of nursery pigs 3 hours following acute lipopolysaccharide challenge.	83
III.5. Effect of dietary source and concentrations of copper, manganese, and zinc on serum interleukin-1 β (IL-1 β) of nursery pigs 3 hours following acute lipopolysaccharide challenge.....	84

Figure	Page
III.6. Effect of dietary source and concentrations of copper, manganese, and zinc on serum interleukin-1 β (IL-1 β) fold of nursery pigs 3 hours following acute lipopolysaccharide challenge.....	85
III.7. Effect of dietary source and concentrations of copper, manganese, and zinc on serum total superoxide dismutase (total-SOD) activity of nursery pigs 3 hours following acute lipopolysaccharide challenge.	86
III.8. Effect of dietary source and concentrations of copper, manganese, and zinc on serum tumor necrosis factor- α (TNF- α) of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34	87
III.9. Effect of dietary source and concentrations of copper, manganese, and zinc on serum tumor necrosis factor- α (TNF- α) fold of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34	88
III.10. Effect of dietary source and concentrations of copper, manganese, and zinc on serum interleukin-1 β (IL-1 β) of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34.....	89
III.11. Effect of dietary source and concentrations of copper, manganese, and zinc on serum interleukin-1 β (IL-1 β) fold of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34	90
III.12. Effect of dietary source and concentrations of copper, manganese, and zinc on serum total superoxide dismutase (total-SOD) activity of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34	91
III.13. Effect of dietary source and concentrations of copper, manganese, and zinc on changes in serum total superoxide dismutase (total-SOD) activity of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34	92

CHAPTER I

INTRODUCTION

The weaning process for pigs is associated with several stress factors which contribute to intestinal and immune system dysfunctions resulting in reduced feed intake, growth, and intestinal diseases (Boudry et al., 2004; Campbell et al., 2013; Lalles et al., 2004). This transient growth check can last days or weeks, depending on how susceptible the weaned pig becomes to all these stress factors (Lalles et al., 2007; Pluske et al., 1997). At this moment, the weaned pig immune system is not completely mature yet (Kick et al., 2012), even though there is a high demand for adequate immune response and mechanisms to combat the oxidative stress (Zhu et al., 2012). Several nutritional strategies to improve health status and growth performance have been studied and used to minimize the short and long-term effects of weaning (de Lange et al., 2010; Heo et al., 2013).

Micronutrients, such minerals, are required by cells in small amounts, and regulate many physiological processes (Afacan et al., 2012). The mineral functions range from structural functions to a broad variety of regulatory processes, including the efficiency of the use of protein and energy (NRC, 2012).

Copper, manganese, and zinc are cofactors of several metalloenzymes involved in protein, carbohydrate, lipid metabolism, and also oxidative enzymes (Suttle, 2010; Underwood & Suttle, 1999). Although these minerals are added in small amounts in the diet, they are important to maintain normal metabolism and growth (Klasing, 2013; Suttle, 2010). However, meeting the physiological mineral requirements of pigs is certainly influenced by the bioavailability of the mineral sources (Cohen, 2014; Klasing, 2013; NRC, 2012).

Moreover, there is a strong relationship between nutrition, metabolism and the immune response (Chandra, 1997; Stafford et al., 2013). Trace mineral nutrition affects a large number of biological processes vital to the immune response, including gene expression, protein synthesis, signal transduction, and cellular proliferation and survival (Afacan et al., 2012; Wintergerst et al., 2007). As research in the area of nutritional immunology has increased, it is becoming apparent that nutrient needs for immune response do not coincide with those required for growth or protein accretion (Johnson, 1998; Kidd, 2004; Sun et al., 2009). A better understanding of this scenario will allow nutritionists to formulate diets to improve immunity and overall health in swine production. However, there is not much information regarding the effect of different sources of copper, manganese, and zinc on the immune response of weaned pigs subjected to an immune challenge.

Therefore, the objective of this study was to evaluate dietary source and concentrations of copper, manganese, and zinc on growth performance and the immune response of nursery pigs following an immune challenge. Two

experiments were performed and the combined data is presented. The first experiment evaluated, beyond growth performance, the acute immune response following intraperitoneal lipopolysaccharide (LPS) injection, while the second experiment also evaluated the chronic immune response following multiple intramuscular lipopolysaccharide (LPS) injections.

CHAPTER II

REVIEW OF LITERATURE

Weaning process and its complications

The weaning process is a complex period during which piglets face social and physical stressors, such as maternal and littermate separation, and changes in the environment (Campbell et al., 2013; Lalles et al., 2007). Intensive production systems normally wean at an early age which exacerbates the stress (Lalles et al., 2007).

Weaned pigs have to switch from sow's milk, which is highly digestible, to a less digestible and more complex solid feed (Lalles et al., 2007; Pluske et al., 1997). Furthermore, the lack of some components of milk (e.g., secretory immunoglobulins and enzymes) makes them vulnerable to opportunistic pathogens as well as delay the maturation of the intestinal cells (Pluske et al., 1997). As a result, weaning affects intestinal development and immediately decreases feed intake leading to undernutrition and to a transient growth check (Campbell et al., 2013; Lalles et al., 2004). Brooks et al. (2001) studied the feed intake of pigs after weaning. Although 50% of weaned pigs consumed their

first meal within 24 h post-weaning, 10% did not eat until 48 h afterwards.

Besides reduced feed intake, water intake is also compromised which impairs growth performance as well (Pluske et al., 1997).

According to Kelly et al. (1992), the ingestion, as well as the physical presence of food in the gastrointestinal tract, are necessary for structural and functional maintenance of the intestinal mucosa. In the long term, a reduction in feed intake is expected to decrease the rate of cell production and decrease cell renewal in the small intestine (Pluske et al., 1997). Boudry et al. (2004) studied the changes in intestinal physiology and morphology after weaning. Two weeks after weaning, they observed an acute transient as well as long-lasting changes, mainly in the small intestine. These changes probably are related to post-weaning fasting, followed by a period of intestinal maturation corresponding to voluntary feed intake restart (Lalles et al., 2007). These marked changes that occur in gut structure and function after weaning, such as villous atrophy and crypt hyperplasia, cause a temporary decrease in digestive and absorptive capacity of the small intestine (Pluske et al., 1997).

Additionally, Nabuurs et al. (1993b) suggested that villous height and crypt depth may influence the pathogenesis of diarrhea after weaning. They postulated that the relationship between intestinal morphology and diarrhea may be influenced by the function of villous enterocytes and crypt cells, since shorter villi and deeper crypts have fewer absorptive and more secretory cells resulting in a decreased absorption and increased secretion capacity. A reduction in digestion and absorption would sustain the development of an osmotic diarrhea, while

unabsorbed dietary material would act as a substrate for enterotoxigenic *Escherichia coli* in the gut (Boudry et al., 2004).

Post-weaning diarrhea is one of the most common causes of morbidity and mortality for weaning piglets, and hence the reduced performance in pigs (Lalles et al., 2004; Pluske et al., 1997). This condition is associated with proliferation of enterotoxigenic *Escherichia coli* (*E. coli*) in the small intestine of affected pigs, and has been well documented throughout the world as a cause of significant economic loss in swine production systems (Pluske et al., 1997). Although specific serotypes of *E. coli* have a central role in the etiology of post-weaning diarrhea (PWD), diarrhea may also be caused by rotavirus, and the role of this pathogen in the etiology of PWD should also be considered (Lecce et al., 1983). Nabuurs et al. (1993a) reported that rotavirus and enterotoxigenic *E. coli* (ETEC) were generally detected when pigs had diarrhea; however, they were also encountered in normal feces from healthy pigs.

In addition to pathologic conditions, early weaning also induces substantial changes in the intestinal bacterial population (Campbell et al., 2013). Following weaning, lactobacilli were detected at significantly lower levels in the gastrointestinal tract when compared to unweaned piglets where lactobacilli were abundant colonizers (Konstantinov et al., 2004). Furthermore, Konstantinov et al. (2006) observed an emergence of clostridia and *E. coli* in intestinal samples of piglets after the early post-weaning period. This beneficial microbiota, such as lactobacilli, is especially important during weaning (Konstantinov et al., 2006), when the animal still has an immature immune system and depends on certain

compounds in the sow's milk to prevent the growth of opportunistic bacteria (Lalles et al., 2007).

Undoubtedly, development of immune competence is an absolute requirement for optimum growth and performance (Lalles et al., 2007). Furthermore, weaned pigs have very high demand for antioxidant defense (Hill et al., 2014) since the weaning process increases the oxidative stress and free radical metabolism (Zhu et al., 2012). Beyond the oxidative stress, elevation of cortisol concentrations (Kick et al., 2012) along with other stress hormones, may reduce response to growth hormone (Luo & Murphy, 1989), increase energy expenditure and loss of body nitrogen (Bessey et al., 1984), retarding performance of weaned pigs.

Several nutritional strategies to alleviate the weaning transition and minimize enteric diseases have been tested in the past decade (Campbell et al., 2013; de Lange et al., 2010; Heo et al., 2013; Lalles et al., 2007). Nutrients play an important role in the functionality of the immune system, as they are the decisive factors in expression of immunity (Chandra, 1997; Stafford et al., 2013). Also, several micronutrients play a crucial role in maintenance of optimum immune response (Chandra, 1997; Wintergerst et al., 2007).

Review of the immune system

The immune response can be divided in two interactive major systems: the innate or non-specific immune system and the acquired or specific immune system (Wintergerst et al., 2007). The innate immune system is present since

birth and its non-specific defense mechanisms are not influenced by previous exposure to pathogens (Chandra, 1997). It is considered the first line of defense for the body and retards the establishment of infections (Bonham et al., 2002). Besides cellular components (e.g. phagocytic cells), the innate immune system has structural barriers such as skin and mucous membranes (Chandra, 1997), as well as physiological barriers, such as pH and differing oxygen levels (Bonham et al., 2002). In addition, a non-cellular component with recognition molecules (C-reactive protein, serum amyloid protein, mannose-binding protein) and complement is involved in phagocytosis, pinocytosis and the inflammatory response (Wintergerst et al., 2007).

Polymorphonuclear cells (PMNs), macrophages and natural killer (NK) cells are the first innate immune cells acting in pathogen recognition and elimination once pathogens have entered the body. Through phagocytosis process they eliminate pathogens by producing reactive oxygen species (ROS) which neutralizes their activity (Arango Duque & Descoteaux, 2014). Macrophages also comply with essential protective functions involved in host defense and inflammation. Once activated, macrophages produce different cytotoxic molecules, such as cytokines. Interleukin 1 (IL-1), interleukin 6 (IL-6), tumor necrosis factor- α (TNF- α) are examples of cytokines that assist in the activation of T cells and act as proinflammatory molecules. Macrophages also present antigen physically on their surface and bind directly to T cells (Arango Duque & Descoteaux, 2014).

Cytokines are small soluble proteins that are crucial for cell signaling among immune and non-immune cells (Johnson, 1997). Cytokines are mainly produced by macrophages and lymphocytes, although they can also be produced by polymorphonuclear (PMNs), endothelial and epithelial cells, adipocytes, and connective tissue (Arango Duque & Descoteaux, 2014). They are crucial for the regulation of the immune and inflammatory responses (Wintergerst et al., 2007).

The adaptive immune response is divided into cell-mediated and antibody-mediated immunity constituted by highly specialized cells: the T lymphocytes (T cells originating from bone marrow and maturing in the thymus) and B lymphocytes (B cells originating and maturing in the bone marrow) (Bonham et al., 2002; Chandra, 1997; Wintergerst et al., 2007). Following maturation, these cells enter the pool of native cells in the peripheral lymph nodes to be able to respond to pathogens (Wintergerst et al., 2007). The cells of the acquired immune system are responsible for synthesizing antibodies, providing memory, and killing invading micro-organisms after being stimulated by exposure to infectious agents (Bonham et al., 2002). B lymphocytes play a role in the humoral immune response producing antibodies specifically directed against an antigen, whereas T lymphocytes are involved in cell-mediated immune responses by activation of other immune cells, such as T helper lymphocytes (CD4+), and by the production of toxic granules in cytotoxic T lymphocytes (CD8+) (Bonaventura et al., 2015; Bonham et al., 2002; Wintergerst et al., 2007). T lymphocytes are only able to recognize antigens presented on cell surfaces. After recognition, the

antigen is complexed with MHC class I (endogenous antigens) or MHC class II (exogenous antigens). Antigen presenting cells are B cells, macrophages, and dendritic cells (Wintergerst et al., 2007).

Dendritic cells (DC) connect the innate and adaptive immune systems. They circulate as immature cells and after contact with the antigen, DC start expressing major histocompatibility complex (MHC) molecules and co-receptors on their cell surface for the activation and stimulation of T cells (Bonaventura et al., 2015). Both systems, the innate and the acquired, act together to provide an integrated and efficient defense for the host (Bonham et al., 2002).

Superoxide Dismutase – manganese and copper-zinc SOD

Reactive oxygen species (ROS) are produced by many physiological processes, which include aerobic metabolism, oxidative phosphorylation, and in stimulated macrophages and neutrophils during the immune response (Fridovich, 1995). Oxidative damage, however, may occur when antioxidant properties are decreased and/or when oxidative stress is increased, which increases free radical production. Free radical-induced oxidative damage has been implicated in the pathogenesis of a number of injury and diseases states (Ibrahim et al., 2000).

Therefore, the management of the reactive oxygen species is vital for proper cellular function and integrity (Marikovsky et al., 2003). If uncontrolled, reactive oxygen species, including the superoxide radical, can result in

inflammation, lipid peroxidation, and oxidation (Li & Zhou, 2011), and cell injury, including DNA damage and cell death (Holley et al., 2011).

The immune system is particularly susceptible to oxidative stress, since immune cells depend on cell signaling via membrane receptors. Interference with the signaling system is harmful and results in an impaired immune response (Wintergerst et al., 2007). To protect themselves from the constant oxidative challenge, cells have developed defense mechanisms that ensure a proper balance between pro and antioxidant molecules (Forman & Torres, 2001; Marikovsky et al., 2003).

Superoxide dismutase (SOD) protects cells from reactive oxygen species by catalyzing the superoxide radicals into molecular oxygen and hydrogen peroxide (Fridovich, 1995; Perry et al., 2010). The elimination of superoxide radicals by SOD can, therefore, be considered an anti-inflammatory activity (Li & Zhou, 2011). Superoxide dismutase can be classified regarding its localization within the cell as well as regarding its mineral cofactor (Fridovich, 1995): copper - zinc (Cu, Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD) (Ibrahim et al., 2000). Copper and zinc containing SOD (Cu/Zn SOD or SOD1) is primarily found in the cytoplasm (Slot et al., 1986), although small amounts of Cu/Zn SOD have been identified in the intermembrane space of mitochondria (Okado-Matsumoto & Fridovich, 2001). Extracellular SOD (SOD3) shares significant amino acid structure with Cu/Zn SOD (40–60%), contains both copper and zinc in its active site, but is found in the extracellular region of the cell (Slot et al., 1986). Mn SOD

is a manganese containing enzyme localized exclusively in the mitochondrial matrix (Okado-Matsumoto & Fridovich, 2001; Perry et al., 2010).

Manganese superoxide dismutase (Mn-SOD) is considered the principal detoxifying enzyme due to its localization inside the mitochondria (Ibrahim et al., 2000). This SOD scavenges superoxide radicals produced within the organelle and protects the mitochondria from the harmful effects of ROS (Perry et al., 2010). According to Holley et al. (2011) any alteration in its function or expression may impact mitochondrial processes and the overall health of cells due to oxidative damage. Thus, according to Ibrahim et al. (2000) it is possible that increases in Mn SOD activity may yield increased protection against oxidative stress.

The activity of the cytosolic SOD enzyme has been found to be influenced by the concentration of dietary copper. Inadequate concentrations of this trace mineral in the diet have been associated with a reduction of enzyme activity (Bonham et al., 2002). Additionally, Yin et al. (2014) considered that plasma SOD activity is also mediated by other factors, such as plasma zinc levels as well as by the expression of SOD related genes. Marikovsky et al. (2003) demonstrated that alterations in Cu/Zn SOD activity indeed affected the inflammatory response through alterations in the potential of the cell to deal with increased superoxide radical production.

The same may occur with a diet deficient in manganese. Rosa et al., (1980) reported that, as with copper-zinc SOD, the activity of manganese-SOD may be regulated by the availability of its component trace mineral. The

membrane damage observed in manganese deficient mice and chickens was due to the accumulation of free radicals (O_2^-) inside the mitochondria. Altered Mn-SOD expression or activity as well as factors that affect manganese availability can also compromise cellular and mitochondrial lipid integrity (Holley et al., 2011). Also, manganese deficiency induced an increase in the Cu-Zn enzyme activity (Rosa et al., 1980). The authors concluded that the intracellular accumulation of free radicals (O_2^-), as a consequence of the decreased activity of Mn-SOD, induced a compensatory increase in the cytosolic form of this enzyme.

Proinflammatory cytokines - TNF – α and IL-1 β

Cytokines are low molecular weight proteins that play a role in cell signaling as well as mediate an effective immune response through the connection of innate and adaptive immune systems (Huynh et al., 2007; Johnson, 1997). Their production takes place in the immune cells, such as macrophages, lymphocytes, and polymorphonuclear leukocytes (PMN), although cytokines can also be produced by endothelial and epithelial cells, adipocytes, and connective tissue (Arango Duque & Descoteaux, 2014).

These molecules play a vital role in regulating the immune response, such as local and systemic inflammation, cellular proliferation, metabolism, chemotaxis, and tissue repair (Arango Duque & Descoteaux, 2014; Spurlock, 1997). For this reason, they can be classified as proinflammatory and anti-inflammatory cytokines (Arango Duque & Descoteaux, 2014). Different cytokines

share similar functions and they may act on many different cell types (Huynh et al., 2007). Furthermore, cytokines may induce other immune modulators such as glucocorticoids, prostaglandins, and catecholamines, which may affect cell metabolism and growth (Spurlock, 1997). Cytokines trigger neuro mediated events either by directly accessing the central nervous system (CNS) or by triggering the synthesis of cytokines by cells in the CNS (Johnson, 1997). In both situations, cytokines in the CNS alter the neuroendocrine system, reducing growth hormone secretion and increasing plasma corticosteroids (Spurlock, 1997).

An increased production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), and interleukin 12 (IL-12) is observed following an immune system stimulation (Johnson, 1997). Among their local effects, increased vascular permeability and recruitment of inflammatory cells are induced by these molecules (Johnson, 1997). Furthermore, systemic effects such as fever and the production of acute inflammatory response proteins are also related to proinflammatory cytokines (Arango Duque & Descoteaux, 2014).

Tumor necrosis factor- α presents regulating properties related to the inflammatory response since this cytokine is able to stimulate the production of other inflammatory molecules, including IL-1, IL-6, platelet-derived growth factor, tumor growth factor, and arachidonic acid metabolites, such as prostaglandin E2 and prostacyclin. Therefore, TNF- α initiates a cascade of responses that contribute to the recruitment and activation of inflammatory cells and immune

reactions (Marikovsky et al., 2003). Tumor necrosis factor- α is also considered a potent pyrogenic molecule and is one of the main cytokines responsible for septic shock (Roth et al., 1994). In the hypothalamus, TNF- α stimulates the corticotrophic releasing hormone, suppresses appetite, and induces fever. In the liver, it stimulates the acute inflammatory response by elevating the synthesis of C-reactive protein and other mediators (Arango Duque & Descoteaux, 2014).

Regarding IL-1, three forms are known: IL-1 α , IL-1 β and IL-1Ra. Although both IL-1 α and IL-1 β are strongly proinflammatory, perform many of the same functions and bind to the IL-1 receptor (IL-1R), there is only 25% amino acid homology between them (Arango Duque & Descoteaux, 2014). Similarly to TNF- α , IL-1 β is also an endogenous pyrogen that is produced and released at the early stages of the immune response. Although monocytes and macrophages are the main sources of IL-1 β , it is also released by NK cells, B cells, dendritic cells, fibroblasts, and epithelial cells. Similar to TNF- α , IL-1 β stimulates the production of acute phase proteins from the liver and acts on the central nervous system to induce fever and prostaglandin secretion. In mast cells, IL-1 β induces the release of histamine, which initiates vasodilation and localized inflammation. IL-1Ra when linked to the IL-1 receptor does not induce the proinflammatory signaling induced by IL-1 α and IL-1 β (Arango Duque & Descoteaux, 2014).

Both IL-1 β and TNF- α may induce resistance of hepatic growth hormone (GH) receptors leading to a significant reduction in plasma insulin-like growth factor 1 (IGF-I), which impairs nutrient utilization for growth (Johnson, 1997). It is also known that TNF- α has a major role in regulating lipid metabolism. Tumor

necrosis factor- α increases hepatic fatty acid synthesis, but in cultured fat cells it decreased the activity of lipoprotein lipase, inhibited de novo fatty acid synthesis, and stimulated lipolysis (Spurlock, 1997). Hardardottir et al. (1994) also reported that TNF- α and IL-1 induce hypertriglyceridemia by decreasing lipoprotein lipase activity and by increasing the rate of hepatic fatty acid synthesis and their subsequent incorporation into very low-density lipoprotein. Moreover, according to Johnson (1998), TNF- α induces adipocytes to secrete leptin, a factor that acts centrally reducing food intake and increasing energy expenditure. Therefore, in immunological challenged pigs, immune system activity and energy balance may be coupled by leptin (Johnson, 1998).

The inflammatory response is beneficial for the host when the proinflammatory cytokines are produced in adequate amounts (Johnson, 1997). Otherwise, elevated amounts may trigger an acute generalized inflammatory response, such as septic shock and multi-organ failure due to excessive production of IL-1 β and TNF- α (Arango Duque & Descoteaux, 2014).

Additionally, overproduction of these cytokines may adversely affect growth and feed efficiency (Williams et al., 2009). In immune system challenged pigs, the increased release of proinflammatory cytokines caused reduced feed intake, growth performance and impairment in feed efficiency, mainly due to anorexia and fever (Johnson, 1997; Webel et al., 1997). Because proinflammatory cytokines have pronounced effects on amino acid, protein, and fat metabolism in rodents, they have been linked to the depression in lean growth in immunologically challenged pigs (Johnson, 1997). Muscle protein degradation

is a characteristic of an inflammatory response and it is also mediated by IL-1 and TNF- α (Spurlock, 1997). Therefore, the modulation of these proinflammatory cytokines may alleviate the negative effects induced by an immunological challenge (Elsasser et al., 2008; Johnson, 1998; Liu et al., 2003).

Copper

Copper and performance

Copper plays a significant role in synthesis and activation of several oxidative enzymes required for normal metabolism in pigs, including ceruloplasmin, cytochrome c oxidase, and superoxide dismutase (Suttle, 2010). As a cofactor, copper is indispensable for enzymatic and non-enzymatic copper-dependent proteins involved in mitochondria respiration, neurotransmitter synthesis, connective tissue formation, and pigmentation (McDowell, 2003). Along with iron, copper is important for hemoglobin synthesis. Although copper is not contained in hemoglobin, a trace of this mineral is necessary to catalyze the iron utilization by the body for hemoglobin formation (NRC, 2012).

According to the NRC (2012), the nutritional copper requirement for weaned pigs is 5 to 6 mg of copper per kg of diet during the nursery phase. A deficiency of copper leads to poor iron mobilization, impairing hematopoiesis. Also, depigmentation, poor keratinization, decreased synthesis of collagen, elastin, and myelin may be observed. Additionally, deflected legs, spontaneous fractures, cardiac and vascular disorders are consequences of copper deficiency (McDowell, 2003; NRC, 2012; Suttle, 2010; Underwood & Suttle, 1999). The

maximum tolerable concentration for pigs is 250 ppm. Above that, copper may be toxic, especially if fed during extended periods of time (NRC, 2012).

In swine nutrition, two major copper sources have been used: copper sulfate (CuSO_4) and tribasic copper chloride (TBCC) (Huang et al., 2015). Both present similar growth-promoting effects in weanling pigs (Cromwell et al., 1998). However, these two sources differ greatly in their water solubility (Miles et al., 1998). Copper sulfate is hygroscopic and very soluble in water, while TBCC is not hygroscopic and poorly soluble in water, but soluble under acidic conditions (Miles et al., 1998). Besides, TBCC is a more concentrated form of copper than copper sulfate (58% versus 25% Cu). Because it has low hygroscopicity and solubility in water, it should be a less reactive and less destructive form of copper when combined with vitamins in the diet (Ammerman et al., 1995).

Similar to zinc oxide, copper sulfate is commonly added at pharmacological concentrations to nursery diets due to its growth promoter effect. Studies have shown that copper supplementation at 100 to 250 mg/kg of diet increases growth rate, feed efficiency, and stimulates feed intake in pigs (Cromwell et al., 1998; Fry et al., 2012). Cromwell et al. (2001) summarized the results from 23 experiments showing that supplementation of copper sulfate (200 to 250 ppm of copper from CuSO_4) improved pig performance from 8 to 20 kg of body weight. Average daily gain (ADG) was increased by 40 g/pig and a 4.5% improvement in feed efficiency was also observed due to CuSO_4 addition in nursery diets.

Previously, Coffey et al. (1994) supplemented nursery diets with different inclusions of dietary copper from CuSO₄ or Cu-lysine (50, 100 or 200 ppm Cu plus basal diet with 18 ppm of copper from CuSO₄). An 11.5% improvement in growth rate, 8.7% increasing feed intake, and 2.4% improvement in feed efficiency was observed in diets supplemented with 100 and 200 ppm Cu compared to pigs fed no additional copper. Regardless of copper source, 100 ppm of copper was as efficacious as 200 ppm of copper in stimulating growth of weaned pigs (Coffey et al., 1994).

Tribasic copper chloride (TBCC) is nearly as efficacious as copper sulfate at the same inclusion (200 ppm), resulting in improvements of 8% in growth rate, 5% in feed intake, and 4% in feed efficiency as compared with control pigs not supplemented with additional copper. Furthermore, 100 ppm of copper from TBCC is similar in efficacy as 200 ppm, allowing a lower inclusion and resulting in a lower excretion (Cromwell et al., 1998). In 2012, Fry et al. compared TBCC (225 ppm) and CuSO₄ (225 ppm) in nursery diets. The results suggested that during phase 1 (d 0 to 6), average daily gain and feed efficiency were greater in nursery pigs supplemented with TBCC (225 mg/kg diet). Average daily feed intake was not affected by copper amount or source. During phase 2 (d 7 to 21), pigs supplemented with TBCC tended to gain less and consume less feed than pigs supplemented with CuSO₄. Pig growth performance was not affected by source or by dietary concentration of copper during phase 3 (d 22 to 35).

According to Zhou et al. (1994), high concentrations of copper act directly on the growth regulatory system. When injected intravenously, copper had

similar effects on growth performance compared to dietary copper intake (250 ppm). They observed a stimulus on serum mitogenic activity, and also an increase in growth hormone mRNA concentrations. Therefore, a mode of action that does not involve antimicrobial activity may be considered (Zhou et al., 1994). Yang et al. (2011) also observed an effect on growth performance through the action of copper on growth hormone levels. They found that the diets supplemented with 125 ppm of copper methionine or 125 ppm of copper sulfate increased the secretion of growth hormone-releasing hormone (GHRH) mRNA, influencing its transcription and subsequent synthesis. Over the entire experimental period, average daily gain (ADG) was higher in copper supplemented groups than in the control group, as well as feed efficiency was improved. Between copper sources, there was no significant difference in ADG or feed efficiency between 125 ppm copper sulfate and 125 ppm copper methionine supplemented groups.

High dietary concentrations of copper also stimulate feed intake in pigs by upregulating neuropeptide Y (NPY) mRNA expression and enhancing NPY concentration in the hypothalamus (Li et al., 2008). Their results showed that average daily gain (ADG) and average daily feed intake (ADFI) were higher and feed to gain (F:G) ratio was lower in pigs fed diets supplemented with 125 or 250 ppm copper than pigs fed the control diet with 10 ppm copper. However, there was no significant difference in growth performance between the 125 ppm and the 250 ppm copper groups. The higher NPY concentrations and NPY mRNA expression levels in the hypothalamus was observed in both groups fed high

concentrations of copper compared to the control group. This link between dietary copper, NPY mRNA expression and appetite was also observed by Zhu et al., (2011). In this study, they observed that high dietary copper (250 ppm) increased NPY mRNA expression levels in the pig hypothalamus through the down-regulation of leptin receptor mRNA expression, which might contribute to the stimulation of appetite.

Moreover, the addition of high concentrations of copper to diets of weaned pigs improves the digestibility of dietary fat by stimulating lipase and phospholipase A activities in the small intestine. According to Luo and Dove (1996) the addition of 250 ppm of copper (as CuSO_4) improved apparent fat digestibility and apparent nitrogen retention in diets containing 5% added animal fat. The same dietary concentration of copper increased its levels in plasma, liver, and kidney and decreased iron in plasma and liver. No effect on pancreatic lipase or phospholipase activities and no effect on trypsin, chymotrypsin, or amylase activities in the small intestine or the pancreas were observed in this study. They concluded that the improved fat digestibility and the enhanced enzymes activities could lead to a better absorption of fatty acids and fat-soluble vitamins and affect other pathways of nutrient metabolism, and therefore stimulate growth of weaned pigs (Luo & Dove, 1996).

Recently, in a multi trial analysis, Ma et al. (2015) confirmed that improvement in growth performance by high concentrations of copper is enhanced by pharmacological concentrations of zinc oxide in nursery diets. A greater response to added copper was observed in phases I and II. Namkung et

al., (2006) concluded that the addition of 3000 ppm of zinc and 250 ppm of copper to the diet improved growth performance of piglets during the first 2 weeks post-weaning. However, thereafter and when pigs were fed reduced concentrations of both minerals no effect of previous zinc and copper supplementation levels were observed.

Hill et al. (2000) observed that average daily gain, feed intake, and feed efficiency were improved by the addition of pharmacological concentrations of zinc (3000 ppm) and (or) copper (250 ppm) in nursery diets. They also observed that the responses to zinc and copper were independent and not additive, meaning that the combination of both minerals did not result in an additive growth response. However, they did observe that copper and zinc were additive in improving feed efficiency in the second week post-weaning.

The opposite was observed by Perez et al. (2011). The data from their 4 experiments consistently showed that dietary copper and zinc have additive effects in growth promotion, improving performance of weanling pigs. These additive effects can be explained as the sum of their different effects to promote growth. The mode of action of zinc seems to be restricted to the intestine, whereas copper may also have a systemic effect (Perez et al., 2011; Yang et al., 2011).

When supplemented together, high doses of zinc oxide (2500 ppm) and copper sulfate (175 ppm) reduce the size of some commensal groups of bacteria in the gastrointestinal tract, such as lactobacilli and streptococci (Højberg et al., 2005). However, the reduced level of these commensal bacteria may benefit the

pig by allocating more nutrients for growth performance (Gaskins, et al. 2002). As suggested by the author, reduced fermentation of digestible nutrients in the proximal part of the gastrointestinal tract may provide more energy available for the pig contributing to the growth promoting effect of high dietary zinc oxide doses. The influence of zinc oxide in the gastrointestinal microbiota is similar to some growth promoting antibiotics, which suppress gram positive commensals rather than potentially pathogenic gram negative microorganisms (Hojberg et al., 2005). Copper sulfate reduced the number of coliforms in the large intestine, which may be part of other mechanisms, such as the suppression of specific pathogens and the induction of resistance by the animal to pathogen adhesion, invasion as well as toxins (Carlson et al., 2004).

Once again, although supplementation of high concentrations of zinc oxide and copper sulfate has shown positive impact on weaned pig performance and health status, this practice results in significant excretion of these minerals becoming an environmental problem (Carlson et al., 1999; Hill et al., 2000). In this way, this use has been decreased or limited in nursery diets (Jondreville et al., 2003).

Copper and immune system

Copper has been shown to play a role in the development and maintenance of the immune system (Percival, 1998). Its deficiency impacts functions of multiple immune cell types of innate and acquired immunity, such as neutrophil, monocyte, T-cell (Wintergerst et al., 2007), and especially

macrophage functions (Percival, 1998). According to Stafford et al. (2013) macrophages may utilize copper through several mechanisms during host defense, such as acute and delayed generation of ROS. Also, many of the copper transport genes have also been implicated in macrophage mediated host defense (Stafford et al., 2013).

During copper deficiency, the capacity of neutrophils to reach the site of infection, adhere and transmigrate across to the endothelium to phagocytize and eliminate the pathogen is decreased (Karimbakas et al., 1998). Additionally, a decrease in the number of circulating neutrophils, a condition termed neutropenia, as well as in the number of red blood cells is related to copper deficiency (Bonham et al., 2002).

Copper is also present in the copper/ zinc-containing enzyme SOD (Okado-Matsumoto & Fridovich, 2001), which is an essential defense against reactive oxygen substances (ROS) decreasing damage of lipids, proteins, and DNA, participating actively during inflammatory response (Perry et al., 2010).

Piglets that were supplemented with high concentrations of copper (250 ppm) and zinc (3000 ppm) had decreased plasma cortisol levels and cytokine circulation after lipopolysaccharide challenge, suggesting that both minerals may alleviate the stress response induced with bacterial endotoxin (Namkung et al., 2006).

Gonzales-Eguia et al. (2009) compared two sources of copper regarding the immune response. They supplemented weaned pig diets with nano copper or copper sulfate at 50 ppm of copper. Their results showed a significant increase in

level of γ -globulin in the nanoCu group when compared to the control. IgG levels in both nanoCu and CuSO₄ groups was higher than that in the control group. However, they did not observe significant differences between the two copper sources. Compared to the control group, both nano Cu and CuSO₄ supplementation significantly increased the SOD activity in the blood serum of piglets, and the nano copper group showed the highest activity.

Manganese

Manganese and performance

Manganese is an essential trace mineral that participates as a cofactor in several enzymatic reactions involved in carbohydrate, lipid, and protein metabolism (NRC, 2012). Manganese plays a role in intermediary energy metabolism through the activity of metalloenzyme pyruvate carboxylase, which is required for normal lipid and carbohydrate metabolism (Suttle, 2010). In addition, manganese is a necessary cofactor for biosynthesis of mucopolysaccharides in the organic matrix of bones through the activation of glycosyltransferase which attaches modified sugars to proteins (NRC, 2012). Moreover, manganese is an essential constituent in the metalloenzyme superoxide dismutase (Rosa et al., 1980), which adds protection against oxidative stress associated with inflammatory responses (Suttle, 2010).

The nutritional manganese requirement for weaned pigs with body weights ranging between 5 and 25 kg is 3 to 4 mg of manganese per kg of diet. (NRC, 2012). Swine diets deficient in manganese impair growth, reduce feed efficiency,

and impact negatively the reproductive performance (McDowell, 2003). Although the toxic concentration of manganese is not well documented, reduced feed intake and growth rates have been observed when pigs were fed 4,000 ppm of manganese (Leibholz et al., 1962). Grummer et al. (1950) reported that 500 ppm of manganese reduced growth rate and resulted in limb stiffness in growing pigs.

There is limited research on the performance of nursery pigs related to manganese supplementation in the diet. Few studies documented that performance of baby pigs and sows were not improved by dietary supplementation of manganese (Plumlee et al., 1956). Leibholz et al. (1962) reported that 0.4 ppm of manganese was sufficient for young pig performance. However, long-term feeding of a diet containing only 0.5 ppm of manganese resulted in abnormal skeletal growth, increased fat deposition, irregular or absent estrous cycles, resorbed fetuses, weak pigs at the birth, and reduced milk production in female pigs fed a semi-purified diet from 3 weeks of age throughout growing, gestation and lactation periods (Plumlee et al., 1956).

Grummer et al. (1950) fed pigs a basal diet containing 12 ppm of manganese supplemented with 40, 80 and 160 ppm of manganese. Pigs consuming diets supplemented with 40 ppm had increased average daily gain and improved overall feed efficiency when compared to pigs fed the basal diet. No additional improvement in pig performance was observed with higher concentrations of manganese. The opposite was observed by Plumlee et al. (1956). No difference in growth rate and feed efficiency was found in pigs fed

semi-purified diets from weaning to market weight when concentrations of manganese ranged from 0.5 to 40 ppm.

In a growing-finishing trial, Sawyer et al. (2007) supplemented manganese at 350 ppm from manganese sulfate or from manganese amino acid complex. The performance results showed that manganese supplementation during the early grower phase did not impact ADG, ADFI, or F:G. However, during the later grower phase, pigs fed basal diet including manganese had lower intake than pigs fed basal diets without supplementation of manganese. Additionally, there was a tendency for pigs fed diets supplemented with manganese to be more efficient during the later grower phase than those pigs fed the basal diet without manganese. Even though pig performance was not improved during the early-finishing phase, pigs fed basal diets containing manganese grew faster and had higher intake than pigs fed basal diets lacking supplemental manganese during the late-finishing phase. Across the entire growing-finishing period, however, ADG, ADFI, and F:G were not affected by supplemental manganese source.

Manganese and immune system

Regarding the immune system, manganese has not been broadly studied in swine. However, research has been performed in other species. Smialowicz et al. (1985) suggested that manganese may play a significant role as an immunomodulator. After a single intramuscular injection in mice, manganese chloride enhanced macrophage phagocytic activity as well as tumoristatic and tumoricidal activity.

Son et al. (2007) reported that rats supplemented with high doses of manganese (500 ppm) and magnesium (1000 ppm) for 12 weeks significantly decreased the percentage of macrophage death compared to the control. The preventive effect on cell death was more pronounced in the high dose of manganese treated group. Thus, they reported that the high dose manganese seems to be more useful to maintain macrophages viability. Moreover, a significant decrease on intracellular reactive oxygen species (ROS) and nitric oxide (NO) level was observed with the supplementation of the high dose of mineral as well. Cytotoxicity of macrophages was also increased in treated rats. Son et al. (2007) concluded that the immune system had positive effects after supplementation with manganese. However, the exact explanation for the modulation of immune response was not defined.

Sunder et al. (2006) supplemented manganese sulfate at different concentrations in diets of broiler chickens to evaluate the mineral retention by tissues and immune competence. Regarding immune response, they concluded that supplementation of manganese at 100 ppm was essential for enhanced immune response affecting both humoral and cell mediated immune responses. The immune response was also improved in a similar study conducted by Bozkurt et al. (2015). Broilers supplemented with dietary manganese produced higher levels of antibodies after vaccination than chickens fed a control diet with no manganese supplementation.

Zinc

Zinc and performance

The nutritional zinc requirement for weaned pigs with body weights ranging between 5 and 11 kg is 100 mg of zinc per kg of diet. At the end of the nursery phase, a reduction to 80 mg of zinc per kg of diet occurs when the body weight is around 25 kg (NRC, 2012).

Zinc is a ubiquitous element in cells being present in the cytoplasm and in most organelles (Bonaventura et al., 2015). Zinc plays an important role in regulating gene expression, nutrient metabolism, immune function, and health (Rink & Kirchner, 2000). Zinc is a cofactor of more than 300 metalloenzymes (Suttle, 2010), particularly those involved in protein, carbohydrate and lipid metabolism such as carbonic anhydrase, alkaline phosphatase and zinc-binding proteins, including metallothionein (McDowell, 2003). DNA and RNA synthetases and transferases have zinc as a cofactor as well (NRC, 2012). Two key structural proteins, collagen and keratin, both require zinc for their synthesis. Keratin is the major structural protein of the hoof and skin, while collagen is the major structural protein of the extracellular matrix and connective tissues, including cartilage and bone (Underwood & Suttle, 1999).

Moreover, zinc is necessary for adequate differentiation of epithelial cells and to promote wound healing (Chandra, 1997; Jensen-Waern et al., 1998). According to Huang et al., (1999), zinc has a primary effect on tissues with a high turnover rate, such as the gastrointestinal tract and immune system, which have a significant requirement for DNA and protein synthesis. Zinc contributes to

normal intestinal barrier function, and also to the regeneration of damaged gut epithelium. Additionally, dietary zinc has been shown to reduce intestinal permeability after weaning (Huang et al., 1999; Zhang & Guo, 2009).

Zinc also plays a role in the production, storage, and secretion of individual hormones as well as in the effectiveness of receptor sites (McDowell, 2003). Among the most notable effects of zinc deficiency on hormone production and secretion are those related to testosterone, insulin, and adrenal corticosteroids (McDowell, 2003; NRC, 2012).

Zinc deficiency results in impairment of immune function (Rink & Kirchner, 2000), parakeratosis, diarrhea in young pigs, growth retardation and depressed feed intake (McDowell, 2003; NRC, 2012). The growth efficiency may be affected by the impairment of amino acid utilization or protein synthesis by animals during zinc deficiency (McDowell, 2003).

Zinc toxicity depends upon the zinc source, dietary concentration, the duration of feeding, and the concentrations of other minerals in the diet. The maximum tolerable dietary concentration for swine has been set at 1000 ppm with exception of zinc oxide, which may be included at higher concentrations for several weeks (NRC, 2012).

During absorption, zinc interacts competitively with several elements, such as calcium, copper, and iron. Excessive intake of zinc may thus result in copper and iron deficiency (Jensen-Waern et al., 1998). Hill et al. (2001) observed that the increase in plasma zinc and the concurrent decline in plasma copper began

when dietary zinc concentrations were higher than 1000 ppm and was exacerbated as the dietary zinc concentration increased.

Zinc bioavailability is influenced by source and dietary concentration. Hahn & Baker (1993) and Carlson et al. (1999) observed that plasma zinc concentration increased as the dietary concentration of zinc increased, particularly when diets contained above 1000 ppm of zinc. Additionally, the form of zinc in the diet affected its bioavailability and subsequent plasma concentration (Hahn & Baker, 1993). Zinc oxide had lower bioavailability, which resulted in lower plasma zinc concentrations compared to zinc sources chemically bound with sulfate or lysine (Hahn & Baker, 1993). Besides that, zinc from grains and plant protein also has low bioavailability, however, it may be enhanced by microbial phytase addition to the diet (NRC, 2012). According to McDowell (2003), cereal grain contains phytic acid which impairs zinc digestion. Among other sources of zinc, zinc sulfate is highly hygroscopic, resulting in several reactions with metal ions and in the breakdown of vitamins, fatty acid, and other nutrients in the diet, while tetrabasic zinc chloride (TBZC) is insoluble in water, therefore, more chemically stable in the diets (Zhang & Guo, 2007).

Zinc oxide is the most widely studied source of zinc in nursery diets; however, other zinc sources can substitute for zinc oxide at the same or even lower concentration (Mavromichalis et al., 2001). Zinc oxide is commonly used as a pharmacological agent while zinc sulfate is used as an inorganic nutritional source (Hill et al., 2014). The availability of organic sources of zinc for dietary supplementation has become popular in the last 20 years, although data with the

comparison of organic and inorganic zinc sources in nursery diets are limited (Hill et al., 2014).

Mavromichalis et al. (2001) compared growth performance of weaned pigs after supplementation of zinc oxide (ZnO) and tetrabasic zinc chloride (TBZC) in pharmacologic concentrations (1500 or 3000 mg Zn/ kg). Both sources of zinc increased weight gain and feed efficiency, however feed efficiency was improved to a greater result by TBZC. In a similar study, Zhang & Guo (2007) fed pharmacological concentrations of zinc from ZnO or TBZC during the first 4 weeks after weaning. They concluded that TBZC could be a desirable zinc source compared with ZnO for nursery diets to enhance growth performance at lower dosage. During the entire experimental period, supplementation of Zn at 2250 mg/kg of diet from ZnO increased ADG and ADFI by 27% and 19%, respectively, while there were about 33% increase in ADG and 20% increase in ADFI by supplementing 1500 mg Zn/kg of diet as TBZC.

Pharmacological concentrations of zinc (2000 to 3000 ppm) from zinc oxide (ZnO) have been widely used in nursery diets due to its effects on enhancing growth performance and alleviating the incidence of diarrhea (Hill et al., 2001; Lalles et al., 2004; Shelton et al., 2011). At high concentrations, zinc oxide increases weight gain and improves feed efficiency in weaned pigs regardless of diarrhea incidence (Carlson et al., 1999; Smith et al., 1997). The oxide form of the mineral seems to be critical in achieving these response benefits and is less toxic than other inorganic zinc sources (Hahn & Baker, 1993).

Poulsen (1995) and Smith et al. (1997) reported that growth rate was increased by 10-26% when nursery pigs were fed diets with 2500 to 4000 ppm of zinc oxide. Other studies also demonstrated that pharmacological doses of zinc oxide improve growth performance in weanling pigs during the first 2 weeks post-weaning (Case & Carlson, 2002; Mavromichalis et al., 2001; Perez et al., 2011).

Hahn & Baker (1993) reported a 17% increase in daily weight gain and 14% increase in daily feed intake in weaned pigs supplemented with 3000 ppm of zinc oxide compared to pigs fed lower concentrations of zinc oxide, zinc sulfate or zinc-lysine. Hill et al. (2001) also reported improvement in growth rates in response to feeding pharmacological concentrations of ZnO. Moreover, Hill et al. (2001) reported that pigs weaned early (less than 15 d of age) had greater benefits of feeding high amounts of zinc oxide than pigs weaned at 20 d of age. Previously, Carlson et al. (1999) reported that both early and later weaned pigs responded in a beneficial manner to supplemental zinc oxide from weaning to 14 d post-weaning with minimal differences in responses afterwards.

An increase in feed intake was also observed by Yin et al. (2009) after supplementation of 2000 ppm of zinc oxide in nursery diets. The increase in plasma concentrations of ghrelin, a molecule that stimulates feed intake, may explain the increased feed intake and increased growth rate of weaned pigs. Hedemann et al. (2006) reported that zinc increases the activity of several pancreatic enzymes. A high concentration of zinc (2500 ppm) in nursery diet resulted in a greater activity of 5 of 7 measured enzymes in pancreatic tissue homogenate, such as amylase activity, carboxypeptidase A, chymotrypsin,

trypsin, and lipase, which may increase the digestibility of other nutrients in the diet. Li et al. (2016) reported that zinc supplementation in nursery diets improved nutrient digestibility regardless of the zinc source when compared to the control group without any zinc addition in the diet. They also observed that crude protein, crude fat and phosphorus digestibility were higher in the groups supplemented with zinc than those in the control group.

The intestinal morphology is also affected by feeding pharmacological concentrations of zinc oxide. Li et al. (2001) reported that high concentrations of zinc oxide (3000 ppm) fed to weaned pigs increased villus height and decreased crypt depth at 11 d post-weaning compared with pigs without supplementation of zinc. In this study, zinc oxide supplementation altered the mucosal morphology, especially in the jejunum. Previously, in 1999, Carlson et al. suggested a potential role of zinc on improving intestinal morphology as well.

Under a natural diarrhea outbreak due to *E. coli*, Perez et al. (2011) observed that high dietary zinc oxide supplementation (3000 ppm) was effective in improving pig health. In a previous study, Katouli et al. (1999) observed that feeding 2500 ppm of zinc oxide was able to reduce post-weaning diarrhea by improving the stability of the intestinal flora and maintaining high diversity of coliforms, a factor which promotes an active competition for colonizing receptor sites of pathogenic strains. Several mechanisms for the mode of action of zinc to reduce diarrhea have been suggested including a reduced secretory capacity in the small intestine epithelium (Carlson et al., 2004), protection of intestinal cell membrane integrity by preventing their rupture, inhibiting bacterial adhesion and

invasion, and reducing cytokine induced inflammation (Roselli et al., 2003). Zinc oxide also decreased the response to *E. coli* infection by reducing plasma cortisol (Namkung et al., 2006).

Furthermore, Huang et al. (1999) reported that supplementation of 3000 ppm of zinc oxide may prevent septicemia by reducing translocation of *E. coli* and *Enterococcus spp.* from small intestine to the corresponding lymph nodes in weaned pigs. Bacteria were cultured from ileum lymph nodes in 3 of 9 pigs fed supplemental zinc oxide while 8 of 9 pigs of the control group (149 ppm of zinc oxide) had bacterial growth. The numbers of bacteria in the ileum lymph nodes tended to be lower in zinc oxide supplemented pigs (15 ± 7 CFU per g of tissue) than in control pigs (387 ± 194 CFU per g of tissue).

Although supplementation of pharmacological concentrations of zinc oxide has shown positive impact on weaned pig performance and health status, it results in elevated excretion of this mineral in the manure becoming an environmental problem (Carlson et al., 1999; Hill et al., 2000). Because of this reason, this practice has been criticized worldwide, and many countries have limited or banned the use of high concentrations of zinc oxide in nursery diets (Jondreville et al., 2003).

Zinc and immune system

Zinc is an essential micronutrient (McDowell, 2003), and its homeostasis plays an important role in the regulation of the immune system (Wellinghausen et

al., 1997). The majority of immunological events are somehow influenced by the availability of zinc (Haase & Rink, 2014).

Zinc is essential for cell-mediated innate immunity, affecting the function of neutrophils, natural killer cells and macrophages (Rink & Haase, 2007). Immune cells contain a wide number of different zinc containing enzymes and zinc finger proteins (Haase & Rink, 2014). Some requirements for zinc in immune function are related to its role as an enzymatic cofactor (Rink & Kirchner, 2000) as well as to its additional role in regulating immune cell signaling (Haase & Rink, 2014). Zinc executes its functions in two different ways: as a neurotransmitter, being the first messenger in cell to cell communication, or as an intracellular signaling molecule (as the secondary messenger) (Bonaventura et al., 2015).

Zinc participates in the chemo attractant process between the immune cells, therefore, its deficiency leads to reduced polymorphonuclear cells chemotaxis (Rink & Kirchner, 2000). Zinc induces monocytes to produce cytokines such as interleukin IL-1 β , IL-6 and TNF- α (Haase & Rink, 2014; Rink & Kirchner, 2000; Wellinghausen et al., 1997). Furthermore, the monocytes function and amount is also affected by zinc availability (Stafford et al., 2013). On the other hand, it has been reported that zinc supplementation may reduce the gene expression of proinflammatory cytokines such as TNF- α , IL-1 β , and IL-8, controlling their overproduction during immune response (Bao et al., 2003). Zinc also interacts with macrophages enhancing their microbicide activity (Stafford et al., 2013). Van Heugten et al. (2003) reported increased phagocytic capability of peritoneal exudate cells when weaning piglets were fed 160 ppm of zinc sulfate.

The effect of zinc on phagocytosis is probably mediated by zinc proteins involved in this process.

The secretion of thymulin is affected by zinc availability, which stimulates T-cell production (Wellinghausen et al., 1997). Therefore, dietary zinc deficiency results in a profound suppressive effect on thymic function, T-lymphocyte development and proliferation, as well as resistance to infections (Kubena & McMurray, 1996). Supplementation of different sources of zinc significantly improved immune traits (goat red blood cells antibody titer, IgG and γ -globulin) and the cell-mediated and humoral components of the immune system in weaned pigs (Li et al., 2016).

Sun et al. (2009) evaluated the effect of zinc supplementation on growth performance of pigs after an immunological challenge. Zinc supplementation did not relieve daily feed intake depression during d 7 to 14 after lipopolysaccharide challenge, but there was a trend for average daily gain to be enhanced with supplemental zinc. This indicated that zinc supplementation may alter the negative effects of an immunological stress (Sun et al., 2009). In this study, they also observed a trend for lymphocyte proliferation to be enhanced with supplemental zinc. Likewise, Van Heugten et al. (2003) reported that pigs supplemented with zinc (control + 80 ppm added Zn from Zn methionine) had greater lymphocyte proliferation than pigs fed the control diet (basal concentration of 80 ppm added Zn from ZnSO₄).

Lipopolysaccharide Challenge in Pigs

Lipopolysaccharide (LPS), an intrinsic component of the outer membrane of gram-negative bacteria, has frequently been used as a model to study immune and neuroendocrine interactions in pigs (Tuchscherer et al., 2004; Wright et al., 2000). LPS induces sickness behaviors, including vomiting, diarrhea, lethargy, increased body temperature, and reduced feed intake in pigs (Johnson & Von Borell, 1994; Webel et al., 1997; Wright et al., 2000), mimicking a natural bacterial infection (Mandali et al., 2000). Reductions in feed intake are mostly dose-dependent in intensity and duration (Frank et al., 2005; Johnson & Von Borell, 1994), and the major changes are typically alleviated 24-48 h after the challenge (Wright et al., 2000).

The bacterial lipopolysaccharide is recognized by a receptor complex composed of CD14, Toll-like receptor (TLR4) and MD-2. Many different cell types express CD14, TLR4 and MD2, including monocytes, macrophages, lymphoid cells and cells that are not part of the immune system, such as epithelial, endothelial and vascular smooth-muscle cells (Bryant et al., 2010). When LPS is circulating, the lipopolysaccharide-binding protein (LBP) accelerates the binding of the endotoxin to CD14 (Fitzgerald et al., 2004). CD14 facilitates the transfer of LPS to the TLR4/MD-2 receptor complex and modulates LPS recognition (Lu et al., 2008). Then, the TLR4 is activated via pathogen associated molecular patterns (PAMP) (Lippolis, 2008). The LPS molecule is composed by lipid A, O side chain, and core oligosaccharide (Freudenberg et al., 2008). The lipid A is the PAMP that activates TLR4. It initiates a signaling cascade that leads to activation of nuclear factor kappa B (NF κ B), which leads to the activation of

genes encoding various cytokines and chemokines that are central to an immune response (Lippolis, 2008). Among them, there are proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) as well as cyclooxygenase (COX)-2 and the subsequent induction of the eicosanoid pathway, including prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs), which are collectively referred to as eicosanoids. Arachidonic acid (AA) is the main source of eicosanoids. The PGE₂ induces the endotoxic febrile response. Additionally, an important upregulation and synthesis of a variety of proteins, the acute phase proteins (APP), occurs in the liver. In pigs, C-reactive protein (CRP), serum amyloidA (SAA), haptoglobin (Hp) and pig major acute phase protein (pig-MAP) have been identified as major positive APP (Wyns et al., 2015).

Lipopolysaccharide also activates the hypothalamus-pituitary-adrenal (HPA) axis via pro-inflammatory cytokine stimulation, resulting in increased secretions of glucocorticoids (Johnson et al., 1997), which may reduce response to growth hormone (Luo & Murphy, 1989) and increase energy expenditure, muscle protein degradation and loss of body nitrogen (Williams et al., 2009).

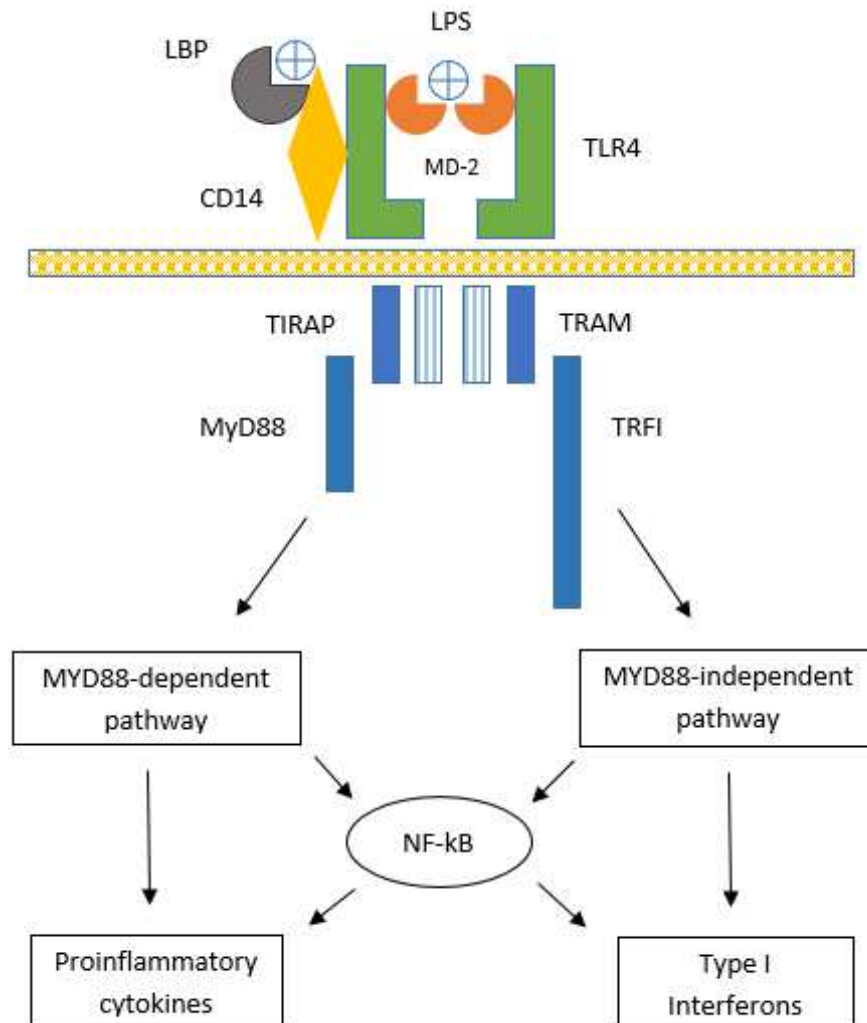
The response of proinflammatory cytokines and various acute phase proteins has been studied by several researchers who have used broad ranges of LPS dosages (from 25 to 200 $\mu\text{g/kg}$) and serotypes (Wyns et al., 2015) as well as different administration routes in pigs (intravenous, intramuscular, intraperitoneal and subcutaneous) (Frank et al., 2005; Kanitz et al., 2002; Liu et al., 2003; Namkung et al., 2006; Tuchscherer et al., 2004; Wright et al., 2000). The LPS serotype O111:B4 is the most frequently used in swine research

(Williams et al., 2009). While a single LPS administration better reflects the sequence of events following an acute LPS challenge, a multiple administration with increasing amounts of LPS imitates more accurately a clinical endotoxemia, since endotoxin remains in circulation for a longer period and it is continuously produced and released during a gram-negative bacterial infection (Rakhshandeh & de Lange, 2012; Wyns et al., 2015). In chronic studies, the LPS dose should be increased at every injection time to avoid resistance (Liu et al., 2003; Rakhshandeh & de Lange, 2012). Low dose endotoxin challenge (5 – 10 µg/kg) have also been successfully used to study the time response of TNF- α , IL-6 and cortisol in pigs as an indicators of stimulation of sub-acute infection (Warren et al., 1997; Webel et al., 1997).

Liu et al. (2008) described that LPS challenge (100 µg/kg, intraperitoneal route) severely decreased performance of weaned pigs during 48 h post-challenge. In 2006, Namkung et al. challenged weaned pigs with 75 µg/kg of LPS intramuscularly on d 13 and 19 after weaning, observing that LPS significantly decreased average daily gain and average daily feed intake of pigs during 7 d following the challenge. These findings were consistent with some previous studies in pigs (Johnson, 1997; Liu et al., 2008).

Regarding immunological parameters, Namkung et al. (2006) observed that LPS injection also resulted in an increased lymphocyte proliferation, concentration of plasma cytokines (IL-1 β and TNF- α) and cortisol within 3 h after the injection indicating an activation of the immune system. Wright et al. (2000), reported maximum cortisol concentrations at 2 h after LPS challenge (100 µg/kg,

Figure II.1. Signaling recognition of lipopolysaccharide (LPS) and activation of proinflammatory cytokines production.



(Adapted from Lu et al., 2008)

intraperitoneal route) in weaned pigs, returning to control levels by 16 h after treatment. Also, the stimulation of the acute response was supported by a significant rise in plasma TNF- α concentration at 2 and 4 h after LPS injection.

The outcome of the activation of the immune system is that nutrients are directed to support its function rather than to growth (Spurlock, 1997), which

impairs nutrient utilization and performance (Johnson, 1997; Liu et al., 2003). Liu et al. (2003) observed depression of weight gain and feed intake in weaned pigs after an immunological challenge with LPS (200 µg/kg, intraperitoneal route) on d 14 and 21 after weaning, as well as increased lymphocyte proliferation, IL-1 β and cortisol concentration. Wright et al. (2000) reported a reduction in plasma IGF-I with a concomitant elevation of plasma cortisol during both the first and the second LPS challenges periods, which may indicate the repartition of nutrients away from normal growth towards the immune response (Liu et al., 2003).

Summary

Nursery pigs are subjected to several stress factors following weaning, which demand an adequate immune response. Nutritionally, minerals such as copper, manganese, and zinc play an important role in many metabolic processes necessary to maintain a normal growth rate and performance. Beyond that, these minerals are involved in the maintenance of immune system function.

However, little research has been performed regarding the interaction between nutritional concentrations of these trace minerals and the immune response in nursery pigs. The availability of information is related mainly to zinc and copper when added in high concentrations in the diets. Manganese, on the other hand, has been studied in other species. Furthermore, more studies concerning the bioavailability of minerals in nursery diets are necessary, since some publications have shown that it varies according to the mineral source.

Therefore, there is a relevant opportunity to enhance the knowledge related to feeding nutritional concentrations of trace minerals in nursery diets and the impact on performance and immune response as well.

CHAPTER III

Effect of dietary source and concentrations of copper, manganese, and zinc on growth performance and the immune response of nursery pigs following acute and chronic lipopolysaccharide challenge.

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Abstract

Weaned pigs have to overcome several stress factors following weaning, which demand an adequate immune response. Beyond nutrition, minerals play a role in immune system function. However, bioavailability of minerals varies according to mineral source. The objective of this research was to evaluate dietary sources and concentrations of copper, manganese, and zinc on growth performance and the immune response of nursery pigs following an acute and chronic immune challenge. Two experiments with two hundred and eighty weaned pigs each were performed. Following a 7-d adaptation period, 10 pigs per pen were randomly allotted according to body weight (BW) and gender to one of four dietary treatments with 7 replicates in each experiment. The dietary

treatments consisted of: **LS** (low sulfates: 5 ppm of Cu from copper sulfate, 16 ppm of Mn from manganese sulfate, and 50 ppm of Zn from zinc sulfate), **HS** (high sulfates: 10 ppm of Cu from copper sulfate, 32 ppm of Mn from manganese sulfate, and 100 ppm of Zn from zinc sulfate), **LC** (low chlorides: 5 ppm of Cu from copper chloride, 16 ppm of Mn from manganese chloride, and 50 ppm of Zn from zinc chloride), and **HC** (high chlorides: 10 ppm of Cu from copper chloride, 32 ppm of Mn from manganese chloride, and 100 ppm of Zn from zinc chloride). On d 21 and 22 of each experiment, 2 pigs per pen received a single intraperitoneal injection of LPS (25 µg/ kg of BW, serotype *Escherichia coli* O111:B4, Sigma-Aldrich, Co., St. Louis, MO) to stimulate an acute immune response. Body weight, body temperature and blood samples were taken prior to the injection and 3 h afterwards. In the second experiment, pigs from 12 selected pens were also subjected to multiple LPS injections (10 µg/ kg of BW, serotype *E. coli* O55:B5, Sigma-Aldrich, Co., St. Louis, MO) on d 28, 30, 32 and 34 of the experiment to stimulate a chronic immune response. Blood samples were taken prior to the first LPS injection on d 28 and 3 h following the last injection on d 34. Among the remaining 16 pens, 8 pens were subjected to saline solution injection and 8 pens served as the control (no injection). Serum was analyzed for TNF-α and IL-1 β by enzyme-linked immunosorbent assay (Quantikine® ELISA, R&D Systems) and also for total-SOD activity by colorimetric assay (DetectX®, Arbor Assays). The data were analyzed as a 2x2 factorial arrangement (concentration, source and concentration x source interaction). Overall, BW, ADG, ADFI, and G:F ratio were not different ($P > 0.10$) among dietary treatment groups. At h 0 of

the acute immune challenge, the least square means for TNF- α (pg/mL) and IL-1 β (pg/mL) were respectively: **LS** – 145.7, 66.7; **HS** – 128.8, 67.1; **LC** – 130.7, 68.3; **HC** – 140.9, 68.8. Pigs fed chlorides had lower TNF- α 3 h post-injection, compared with pigs fed sulfates (**LC** - 7297.2; **HC** - 6180.5 vs **LS** - 9063.7; **HS** - 7563.0 pg/mL; $P = 0.031$). Interleukin 1 β was also lower in pigs fed chlorides compared with pigs fed sulfates (416.1 and 363.3 vs 639.3 and 420.3 pg/mL; $P = 0.020$). Both chloride sources had lower BW loss 3 h following the single LPS injection ($P = 0.043$). A concentration effect was also observed, with higher dietary concentrations producing less TNF- α ($P = 0.071$) and IL-1 β ($P = 0.024$). Total-SOD activity decreased ($P > 0.10$) among all dietary treatment groups. During the multiple LPS injections, growth performance was negatively affected ($P < 0.005$); however, no differences ($P > 0.10$) were observed among dietary treatment groups. Numerically, TNF- α concentration was lower ($P=0.196$) and total-SOD activity was greater ($P=0.190$) in pigs fed chloride sources. Interleukin-1 β production was not affected ($P > 0.10$) by dietary treatment group during the chronic immune challenge. Chloride sources are able to alleviate the acute immune response by decreasing the proinflammatory cytokine production regardless the dietary concentration, suggesting a higher bioavailability over sulfate sources. Besides that, high dietary concentration of copper, manganese, and zinc presented more promising results, indicating that the immune response may have a greater requirement than that needed for growth. Additionally, following a chronic immune challenge, pigs fed chloride sources had a numerical advantage for immune response over pigs fed sulfate sources, suggesting less

stimulation of the inflammatory response and potential enhanced antioxidant activity. More replicates are needed to confirm the effects of chloride sources following a chronic immune challenge in nursery pigs.

Introduction

Weaned pigs need to cope with several stress factors following weaning. Nutritionally, there is a significant change in the diet, which increases the susceptibility to intestinal diseases, especially diarrhea caused by enterotoxigenic *Escherichia coli* (Lalles et al., 2007; Pluske et al., 1997). In addition, a transient growth check is observed due to changes in intestinal function and decreased feed intake (Lalles et al., 2004). Therefore, weaned pigs demand an adequate immune response and mechanisms to combat the oxidative stress following weaning (Kick et al., 2012; Zhu et al., 2012). The biological alterations in metabolism, immune system, and intestinal functions last several days and have short and long-term effects on subsequent pig growth and health (Boudry et al., 2004). In this way, the adaptation to all stress factors is essential to avoid poor performance and mortality during the nursery phase (Campbell et al., 2013).

To alleviate the weaning transition and minimize enteric diseases, different nutritional approaches have been studied in the past decade (Lalles et al., 2007). Among them, feeding trace minerals in pharmacological concentrations, such as zinc oxide and copper sulfate, have been broadly used to improve growth performance and to control diarrhea in nursery pigs (Hill et al., 2001). However,

this practice has been restricted or limited in many countries due to the environmental impact of excess excretion of these minerals (Hill et al., 2000; Jondreville et al., 2003).

Trace minerals, such as copper, manganese, and zinc are nutritionally important as they are cofactors of several metalloenzymes involved in protein, carbohydrate, lipid metabolism, and also oxidative enzymes (Suttle, 2010). Immunologically, these minerals play a role in a large number of biological processes vital to the immune response (Chandra, 1997; Wintergerst et al., 2007). However, some authors (Johnson, 1998; Kidd, 2004; Van Heugten et al., 2003) suggested that nutritional concentrations of minerals are not able to meet the immune system requirements to obtain an adequate response under immune challenges. Moreover, since the bioavailability is influenced by the mineral source (Klasing, 2013; NRC, 2012), the mineral function, stability, performance results, and immune response are influenced as well (Cohen, 2014; Zhang & Guo, 2007).

Thus, this research aimed to evaluate the effect of dietary sources and concentrations of copper, manganese, and zinc on growth performance and immune response of weaned pigs following acute and chronic lipopolysaccharide (LPS) challenge.

Materials and Methods

Animal Care and Feeding

Two experiments utilizing 280 crossbred (PIC®) pigs (140 barrows and 140 gilts) each were performed for a period of 6 wk in each experiment. Pigs were weaned at average of 20 d of age and allotted randomly to one of four dietary treatments with 7 replicates per treatment within each experiment. The pigs were handled and cared for according to the guidelines established by the Oklahoma State University Institutional Animal Care and Use Committee.

In each experiment, pigs were blocked randomly by body weight (BW), gender, and housed in pens in a proportion of 5 barrows to 5 gilts, totaling 10 pigs per pen. Pigs were housed in a nursery facility with control of environmental temperature and ventilation.

During the first seven days of the experiment, all pigs received the same diet (**N1**) (Table III.1 and III.2). This common diet was formulated to 5 ppm of added Cu from copper sulfate, 16 ppm of added Mn from manganese sulfate, and 50 ppm of added Zn from zinc sulfate. All remaining nutrients in the diet were added at or above the requirements listed in the NRC (2012).

On d 7, the treatment diets were allotted to pens: **LS** (5 ppm of added Cu from copper sulfate, 16 ppm of added Mn from manganese sulfate, and 50 ppm of added Zn from zinc sulfate), **HS** (10 ppm of added Cu from copper sulfate, 32 ppm of added Mn from manganese sulfate, and 100 ppm of added Zn from zinc sulfate), **LC** (5 ppm of added Cu from copper chloride, 16 ppm of added Mn from manganese chloride, and 50 ppm of added Zn from zinc chloride), and **HC** (10 ppm of added Cu from copper chloride, 32 ppm of added Mn from manganese chloride, and 100 ppm of added Zn from zinc chloride). To properly meet the

nutritional requirements of all remaining nutrients according to the NRC (2012), the treatment diets were formulated in two different phases: **N2** and **N3** (Table III.1 and III.2). The **N2** diet was fed from d 7 to 21, and the **N3** diet was fed from d 21 to 42. Both diets were formulated as basal diets, and each treatment mineral premix was added subsequently during the mixing process.

During the entire experiment, pigs were allowed to consume feed and water *ad libitum*. Water was provided via water nipple and each pen had a multiple-hole stainless steel feeder. The feed provided was recorded for each pen at every feeding time according to the treatment, and the feed intake was calculated weekly after weighing feeders and subtracting the remaining feed. Additionally, body weight (BW) was also measured on a weekly basis, starting on d 0, followed by d 7, 14, 21, 28, 35 and 42 of the experiment. Average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (G:F) were then calculated.

Mineral Premix and Diet Analysis

The mineral premixes were analyzed by Cumberland Valley Analytical Services (Hagerstown, MD) through Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for Cu, Mn, and Zn. Briefly, this technique combines a high temperature ICP (Inductively Coupled Plasma) source with a mass spectrometer. The ICP source converts the atoms of the elements in the sample to ions. These ions are then separated and detected by the mass spectrometer (Wolf, 2005).

All diets were analyzed for moisture, dry matter, crude protein, crude fiber, crude fat, ash, net energy (NE), digestible energy (DE), metabolizable energy (ME), calcium, phosphorus, magnesium, potassium, sulfur, sodium, iron, zinc, manganese and copper. All analyzes were performed by Servitech (Dodge City, KS).

Acute Escherichia coli Lipopolysaccharide Challenge

On d 21 and 22 of both experiments, 1 barrow and 1 gilt per pen were subjected to a single lipopolysaccharide (LPS) challenge. To study the immune response, the *Escherichia coli* LPS O111:B4 (Sigma-Aldrich, Co., St. Louis, MO) was suspended in 9 g/L of sterile saline solution for a final dosage of 25 µg of LPS/kg of body weight (Bible, 2013; Mandali et al., 2000). The suspension was kept in cold storage.

Prior to the injection, the selected pigs were ear tagged, their rectal temperature and body weight were recorded and blood samples were taken. Then, the injection was performed in the lower abdomen in the intraperitoneal cavity with the weight-dependent LPS suspension. Besides the h 0, rectal temperatures and body weight were recorded at 3 and 6 h post-injection. Blood was drawn at 3 h post-injection. The time for sampling after LPS injection was chosen based on the maximal peak obtained for concentrations of tumor necrosis factor- α (TNF- α) and interleukin 1 β (IL-1 β) (Mandali et al., 2000; Warren et al., 1997; Webel et al., 1997). Changes in rectal temperature and percentage of body weight were calculated using h 0 as a baseline.

Blood Collection

Blood was taken from the anterior vena cava (jugular) in the supine position using a 20 gauge 3.8 cm vacutainer needle with a 10 mL sterile serum tube (BD, Franklin Lakes, NJ) followed by a 3 mL sterile plasma tube (BD, Franklin Lakes, NJ). The h 0 sampling was used as the baseline. The blood samples were placed on ice after collection and stored at 2-5°C overnight. The samples were centrifuged for 10 minutes at 2,000 x g to separate the serum or plasma, following the manufacturer's instructions. Afterwards, the serum or plasma was collected using a plastic disposable transfer pipet and dispensed into appropriately labeled micro centrifuge tubes. The tubes were stored at -20°C until further analysis.

Blood Analysis

Serum samples from h 0 and 3 were analyzed in duplicate for TNF- α , IL-1 β and total-SOD activity. An enzyme-linked immunosorbent assay (ELISA) kit was used to measure the concentration of TNF- α and IL-1 β (R&D Systems, Inc., Minneapolis, MN), while total-SOD activity was measured by a colorimetric activity assay (Arbor Assays, Ann Arbor, MI). All samples were analyzed following the manufacturer's instructions according to the specific assay. All the supplies needed for the analysis were purchased from VWR (Radnor, PA).

The enzyme-linked immunosorbent assay (ELISA) employs the quantitative sandwich enzyme immunoassay technique. Standard, control, and samples were added to the wells with a pre-coated monoclonal antibody specific

for each porcine cytokine. After incubation for 2 h, the unbound substances were washed away, and an enzyme-linked monoclonal antibody specific for the measured cytokine was added to the wells to bind the cytokine immobilized during the first incubation. A further 2 h of incubation was followed by a wash to remove any unbound antibody-enzyme reagent, and then a substrate solution was added to the wells. Color developed in proportion to the amount of the cytokine bound in the initial step. The color development was stopped by adding the stop solution, and the intensity of the color was measured at 450 nm with the correction wavelength set at 570 nm. The sample values were then read off the standard curve. In the TNF- α ELISA assay, the h 3 samples were diluted 10-fold.

The colorimetric activity assay for SOD utilizes one reagent to generate superoxide in the presence of oxygen in the samples, which converts a colorless substrate into a yellow colored product. The colored product is read at 450 nm. Increasing levels of SOD in the samples causes a decrease in superoxide concentration and a reduction in yellow product. The results are expressed in terms of units of SOD activity per mL. A bovine erythrocyte SOD standard is provided to generate a standard curve for the assay and all samples should be read off of the standard curve.

Chronic Escherichia coli Lipopolysaccharide Challenge

On d 28, 30, 32 and 34 of Experiment 2, pigs from 12 selected pens were subjected to lipopolysaccharide (LPS) challenge. There were 3 replications of each dietary treatment among these 12 pens. The remaining 16 pens were

divided evenly across two treatments: 8 pens were subjected to saline solution injection and 8 pens served as the control (no injection) (Table III.3).

The *Escherichia coli* O55:B5 lipopolysaccharide (Sigma-Aldrich, Co., St. Louis, MO) was chosen to perform the chronic immune challenge according to Rakhshandeh and de Lange (2012), who studied the chronic stimulation of the immune system of growing pigs following multiple LPS injections. The dose was chosen after a Pilot Study performed in the Swine Research Facility (Oklahoma State University), which can be accessed in this thesis (Appendix 3). The LPS was suspended in a 9 g/L of sterile saline solution for a final dosage of 10 µg of LPS /kg of body weight. The suspension was kept in cold storage.

Prior to the injection, all 28 pens and feeders were individually weighed. From each of the 12 pens determined to be challenged with LPS, 2 ear tagged pigs per pen were bled. Then, the LPS injection was performed on all pigs intramuscularly in the neck just behind and below the ear according to the average pen weight. Saline solution was administered intramuscularly in all pigs from the remaining 8 pens to evaluate whether the stress from the injection and handling would impact growth performance. The amount injected was 1 ml per pig. The injection procedures were repeated on d 30, 32 and 34, and blood was drawn from the same 2 ear tagged pigs per pen at 3 h following the last injection on d 34.

On d 35 of the trial, all 28 pens and feeders were weighed individually to calculate ADG, ADFI and G:F ratio. Changes in blood parameters and body weight were calculated using h 0 from d 28 as a baseline.

Blood collection and analysis were similar to that described previously. In the TNF- α ELISA assay, the h 3 samples were diluted 10-fold.

For both experiments, the inter-assay CV for TNF- α , IL-1 β and SOD were 3.3%, 3.4%, and 3.6%, while the intra-assay CV were 4.0%, 6.6%, and 15%, respectively.

Statistical Analysis

Initially, data within each experiment were analyzed as a randomized complete block design with body weight as the blocking effect. Growth performance as well as body weight, rectal temperature, and blood analysis following acute and chronic LPS challenges were analyzed using a GLM procedure (SAS Institute, version 9.2), in a 2 x 2 factorial design (concentration, source and concentration x source interaction). The LPS data were sorted by hour before analysis.

Because both experiments were similar, data from Experiment 1 and 2 relative to growth performance and acute immune challenge measurements were combined. The combined data were analyzed as a randomized complete block design with the model including the effects of experiment, treatment, block and the interactions. Treatment effects were tested as 2 x 2 factorial design using orthogonal contrasts. The main effects of dietary source of minerals and concentrations, and their interaction were tested.

Pen served as the experimental unit. The treatment means are presented as least square means. Differences were considered significant at the $P < 0.05$ level and a trend at $0.05 < P < 0.10$.

Results

There were no experiment by treatment interactions ($P > 0.209$) noted for any response criteria; thus, the pooled data are presented.

Growth performance

The growth performance data are presented in Table III.4. Overall, there were no differences ($P > 0.10$) in growth performance among the dietary treatment groups. The initial body weight (BW) was not different ($P > 0.10$), with an average of 7.04 kg on d 7. On d 21, prior to the LPS challenge, no differences were observed ($P > 0.10$) in average BW, ADG, ADFI and G:F ratio. Following the LPS challenge, between d 21 and 42 of the experiment, average BW, ADG, ADFI and G:F ratio were also not affected ($P > 0.10$) by dietary treatment. There was a tendency ($P=0.085$) for pigs fed the sulfate sources to have better G:F ratio between d 7 and 42. There were no source x dietary concentration interactions ($P > 0.412$) for any growth performance data.

There were no experiment by treatment interactions for growth performance; however, differences between experiments existed. Pigs in Exp. 1 had poorer performance ($P<0.0001$) compared with pigs in Exp. 2. The initial average BW on d 7 was 6.8 kg (Exp. 1) vs 7.3 kg (Exp. 2), resulting in a 0.5 kg

difference. Prior to the LPS challenge, on d 21, the average BW was 10.9 kg (Exp. 1) vs 12.5 kg (Exp. 2), resulting in 1.6 kg difference. On d 42, average BW was 20.7 kg (Exp. 1) vs 24.2 kg (Exp. 2), a difference of 3.5 kg of BW.

Acute LPS challenge – Body weight and rectal temperature changes

The changes in body weight observed 3 h post-injection were affected by dietary treatment (Figure III.1). Pigs fed chloride sources had lower ($P = 0.043$) BW loss than pigs fed sulfate sources (Table III.5). Between 3 and 6 h post-injection, pigs fed high dietary concentrations of sulfates and chlorides had lower body weight change ($P = 0.028$).

Overall, pigs fed high dietary concentrations of both sources (**HS** and **HC**) had lower ($P = 0.002$) change in body weight 6 h following the immune challenge. There were no source x dietary concentration interactions ($P > 0.164$) for any immune response data.

The combined data demonstrated an increase in rectal temperature following the LPS challenge among all treatment groups, with the peak occurring within 3 h post-injection (Table III.5). Numerically, pigs fed the high dietary concentrations (**HS** and **HC**) had the lowest ($P = 0.129$) increase in body temperature 3 h following the immune challenge (Figure III.2). Between 3 and 6 h, no differences were observed among dietary treatment groups. Overall, no differences ($P > 0.10$) were observed for rectal temperature change between h 0 and 6 of the acute immune challenge, and no source x dietary concentration interactions ($P = 0.853$) were noted. However, there was a difference for body

weight change ($P < 0.05$) and rectal temperature change ($P < 0.01$) between experiments. Experiment 1 was conducted during the summer season while Experiment 2 was conducted at the end of the fall season. The greater change in BW and rectal temperature observed in Experiment 1 could have been related to heat stress.

Acute LPS challenge – Blood analytes

The blood data are presented in Table III.6. At h 0, pigs fed low sulfates (**LS**) had the highest TNF- α concentration ($P = 0.031$). There was a source effect ($P = 0.031$) for TNF- α concentration 3 h post-injection (Figure III.3). Pigs fed chloride sources had lower production of this proinflammatory cytokine. Also, a tendency for a concentration effect ($P = 0.071$) was observed, with pigs fed high dietary concentrations having lower TNF- α compared with those fed the lower dietary concentrations. No interaction between source and concentration ($P = 0.788$) was observed for this blood analyte, although numerically, pigs fed the high dietary concentration of chlorides had the lowest TNF- α . The TNF- α fold change for pigs fed chlorides had a tendency to be lower ($P = 0.077$) than for pigs fed sulfates (Figure III.4).

For IL-1 β concentrations, there was a tendency for a source effect ($P = 0.091$) prior to the LPS injection (Table III.6). Pigs fed the chloride sources had the highest IL-1 β concentrations in their serum. Following the immune challenge, there was a source effect ($P = 0.018$) for IL-1 β concentrations 3 h post LPS injection (Figure III.5). Pigs fed the chloride sources (**LC** and **HC**) produced less

IL-1 β than those fed sulfates (**LS** and **HS**). Similarly to TNF- α , there was also a concentration effect ($P = 0.023$) for IL-1 β concentrations with pigs fed high dietary concentrations producing lower IL-1 β amounts. Even though no interaction of source and concentration ($P=0.159$) was observed for IL-1 β , numerically, pigs fed the high dietary concentrations of chlorides produced the lowest amount of IL-1 β (Table III.6). The same was observed for the IL-1 β fold change, with a source effect ($P=0.010$) showing that pigs fed chlorides had lower IL-1 β fold change than pigs fed sulfate (Figure III.6). Additionally, there was a concentration effect ($P=0.021$) for IL-1 β fold change, with high dietary concentrations having lower IL-1 β change. Again, no interaction of source and concentration ($P=0.158$) was observed for IL-1 β fold change, although numerically, pigs fed high dietary concentrations of chlorides produced the lowest amount of IL-1 β .

The decrease in total-SOD activity was not affected ($P > 0.10$) by source or dietary concentration of copper, manganese, and zinc following the acute immune challenge (Figure III.7).

There was a difference for TNF- α ($P < 0.0001$) and IL-1 β ($P<0.01$) between experiments. Overall, pigs from Experiment 2 produced higher amounts of both proinflammatory cytokines following the acute immune challenge. However, no source x dietary concentration interaction ($P > 0.158$) was noted.

Growth performance following chronic LPS challenge

The multiple LPS injections affected ($P < 0.005$) growth performance compared to the groups that received saline solution or no injection (Table III.7). The effects of the chronic immune challenge lasted throughout the study (d 28 to 42). The stress related to the injection and handling did not affect ($P > 0.10$) growth performance parameters.

The growth performance results presented in Table III.8 refer to the 12 pens subjected to multiple LPS injections between d 28 and 34 of the Experiment 2. There was no difference ($P > 0.10$) in BW, ADG, ADFI, and G:F ratio among dietary treatment groups at the end of the chronic immune challenge (d 35). No differences ($P > 0.10$) were observed during the subsequent weeks (d 35 to 42) of the immune challenge as well.

Chronic LPS challenge – Blood analytes

The data for blood measurements are presented in Table III.9. At the end of the chronic immune challenge, the concentration of TNF- α was numerically ($P = 0.196$) lower in both dietary concentrations of chloride sources (Figure III.8 and 9). The IL-1 β concentration was not different ($P > 0.10$) among dietary treatment groups at the end of the chronic immune challenge, although numerically ($P = 0.305$) pigs fed high dietary concentrations had lower production of this proinflammatory cytokine (Figure III.10 and 11).

Moreover, pigs fed chlorides also had numerically ($P = 0.190$) higher total-SOD activity compared to pigs fed sulfate sources (Figure III.12 and 13).

Discussion

According to the Nutrient Requirements of Swine (NRC, 2012), the recommended concentrations of copper, manganese, and zinc in nursery diets are 5-6 ppm, 3-4 ppm, and 80-100 ppm, respectively. These concentrations were defined based on review of scientific literature to guarantee optimum growth performance and metabolic functions. The NRC (2012) states that requirements include contributions of all dietary ingredients, even though micro mineral contents and bioavailability estimates are variable and largely unknown. Consequently, it is common to supplement micro minerals in excess of requirements established in the NRC (Gowanlock et al., 2013).

During the entire experiment, all 4 dietary treatment diets met properly the nutritional requirements of copper, manganese, and zinc regardless of their source and concentration (Table III.1 and III.2). The dietary amount provided in each diet exceeded all recommendations listed in the NRC (2012) for these minerals. For this reason, no differences in growth performance were observed throughout the experiment. Previously, some authors have observed the same result. In a study to evaluate growth performance, Mahan et al. (2014) fed weaned pigs during 35 d with corn-soybean meal basal diets with or without addition of copper, manganese, zinc, and iron from organic or inorganic sources at 0%, 25%, 50% and 100% of the concentrations established in the NRC (1998). The results showed no difference on growth performance during the initial 21 d post-weaning. The last 7 d of the study, there was a numerical increase in ADG in the 25% NRC treatment group, but no improvement in growth performance

was observed in higher concentrations of micro minerals. They concluded that during the initial period post-weaning, the carry-over from the sow or the bioavailability of the innate minerals from the basal diet was enough to meet the performance needs of the weaned pigs. In a similar study, after supplementation of organic minerals in nursery diets, Martin et al. (2011) observed no differences in ADG and ADFI when copper, manganese, and zinc were added to the diets at 100% or 150% of the nutritional requirements listed in the NRC (1998). In a previous study, Van Heugten et al. (2003) fed pigs with 104 ppm total zinc (control diet) or control diet added with 80 ppm or 160 ppm of ZnSO₄, Zinc-methionine or Zinc-lysine. They also reported no effect on ADG and ADFI after supplementing nursery pigs with different sources and nutritional concentrations of zinc. In grower-finisher pigs, supplementation of copper, manganese, and zinc at 0%, 50% or 100% of the nutritional requirements (NRC, 2012) did not affect growth performance. In this study, Gowanlock et al. (2013) concluded that there was sufficient amount of innate micro minerals in the corn-soybean meal diet to meet the grower-finisher pig's requirements for growth, although possible other factors, such as disease and environmental stressors, may increase the need for these minerals.

Growth performance would be expected to increase in case of addition of higher amounts of zinc and copper (Hill et al., 2001; Lalles et al., 2004; Shelton et al., 2011), however it would result in pharmacological effects rather than nutritional effects (Cho et al., 2015). Namkung et al. (2006) reported an improvement in ADG during weeks 1 and week 2 post-weaning when pigs were

fed high dietary zinc oxide (3000 ppm) or high dietary copper sulfate (250 ppm) respectively, compared with the control, which was fed 150 ppm of zinc oxide and 15 ppm of copper sulfate. Additionally, these effects would be reached exclusively by using higher concentrations of zinc (2000 to 3000 ppm) from zinc oxide (ZnO) and copper (250 ppm) from copper sulfate (CuSO₄), since both sources have been stated to be safe when added in pharmacological concentrations in nursery diets (Fry et al., 2012; Hahn & Baker, 1993; NRC, 2012).

To study the acute immune response, a single LPS challenge was performed in experiment 1 and 2. Following the LPS injection, pigs from all dietary treatment groups presented sickness behaviors, such as lethargy, vomiting, diarrhea, and reduction of feed intake. Moreover, the rectal temperature was increased among all dietary treatments, with the peak of hyperthermia occurring within 3 h post-injection. All these observations were in accordance with previous studies (Bible et al., 2013; Frank et al., 2005; Johnson & Von Borell, 1994; Mandali et al., 2000; Webel et al., 1997; Wright et al., 2000), demonstrating that the immune system was activated. Furthermore, the proinflammatory cytokines measured increased among all 4 dietary treatments, evidence that activation of the immune system following the LPS injection. Previous studies (Arango Duque & Descoteaux, 2014; Johnson & Von Borell, 1994; Wyns et al., 2015) have reported that many of the behavioral and physiological effects of LPS are attributed to the production of proinflammatory cytokines.

Even though feed intake was not measured following the acute LPS challenge, the reduction in feed consumption was easily noticed. According to Johnson and Von Borell (1994), reduction in intake following a LPS challenge is dose-dependent in intensity and duration, which is typically alleviated after 24 to 48 h. Warren et al. (1997) reported that pigs challenged intraperitoneally (IP) with 50 µg of LPS/kg of BW (*E. coli* serotype K-235) returned to the same feed intake levels of control pigs at 24 h following the challenge, while Wright et al. (2000) after challenging nursery pigs with 100 µg of LPS/kg of BW by IP route (*E. coli* serotype B55:O5) observed that the feed intake returned to levels of control pigs between 24 and 48 h after injection. Li et al. (2008) reported 65% reduction of ADFI in pigs challenged with 100 µg of LPS/kg of BW (*E. coli* serotype B55:O5) over the 48 h of the immune challenge. According to Frank et al. (2005), the differences among these studies may be related to the route of administration, genotype and body weight of the pigs, LPS serotype, and to prior exposure to environmental pathogens. As reported in previous studies (Johnson, 1997; Warren et al., 1997; Wright et al., 2000), the suppression of feed intake by pigs observed in the 24 h following the LPS challenge is consistent with the anorectic effects that accompany immune challenges.

Little research has been published in regards to feeding different dietary concentrations and sources of copper, manganese, and zinc and their effects on the immune response of weaned pigs. Indeed, there are some data available demonstrating that pharmacological concentrations, especially of copper and zinc, impact positively the immune system response. Namkung et al. (2006)

reported that pigs supplemented with 3000 ppm of zinc oxide or 250 ppm of copper sulfate subjected to LPS challenge (*E. coli*, 055:B5, 75 ug LPS/ kg BW) had reduced plasma cortisol levels as compared with pigs on the control diet. Sunder et al. (2006) reported that supplementation of manganese at 100 ppm was essential for enhanced immune response affecting both humoral and cell mediated immune responses in broiler chickens.

In the present research, pigs fed high dietary concentration of sulfates (**HS**) and pigs fed high dietary concentration of chlorides (**HC**) had lower body weight change and faster recovery of the initial body weight post single LPS injection. The recovery was probably due to return to water intake, alleviating the dehydration process and consequently the changes in the BW. These results suggest that the immune system when activated has a higher demand for copper, manganese, and zinc than that needed for growth performance. According to Sun et al. (2009), the changes in metabolism during an immunological challenge modify the nutritional requirements mainly because the nutrients are redistributed away from the growth process to support the immune system function.

The increase in rectal temperature following the acute LPS challenge was not different among the dietary treatment groups, although numerically, some differences in body temperature change were observed between h 0 and 3. Overall, the results are promising for high dietary concentrations of chlorides (**HC**), however more replicates are needed to confirm this.

Pigs fed both dietary concentrations of chloride source (**LC** and **HC**) presented a more attenuated immune response following an acute immune challenge. Both dietary treatment groups produced less TNF- α and IL-1 β , having a numerical advantage over sulfates sources (**LS** and **HS**). It suggests that chloride sources may have a higher bioavailability than sulfate sources, which agrees with previous studies (Ammerman et al., 1995; Miles et al., 1998; Zhang & Guo, 2007). Overall, TNF- α and IL-1 β concentration were lower in pigs fed high dietary concentration of chlorides (**HC**), which may explain the numerical lower increase in the rectal temperature 3 h following the acute immune challenge for this group of pigs. Once again, these results suggest an increased demand of the immune system for nutrients to promote an adequate response when it is activated (Johnson, 1998).

The lower production of proinflammatory cytokines indicates that the immune system was less stimulated by infectious or inflammatory process. Consequently, the increase in catabolic processes and the impairment in growth performance will be less pronounced (Elsasser et al., 2008; Williams et al., 2009), which is very important especially during chronic health challenges.

The total-SOD activity decreased in all dietary treatment groups, which agrees with studies that reported an increase in the oxidative stress during an immune response (Ibrahim et al., 2000; Li & Zhou, 2011; Perry et al., 2010). Superoxide dismutase (SOD) protects cells from reactive oxygen species by catalyzing the excess of superoxide radicals into molecular oxygen and hydrogen peroxide (Fridovich, 1995; Perry et al., 2010). The elimination of superoxide

radicals by SOD can, therefore, be considered an anti-inflammatory activity (Li & Zhou, 2011). Following an acute immune challenge, no differences were observed by feeding different sources and dietary concentrations of copper, manganese, and zinc in the total-SOD activity.

A chronic immune challenge was also performed in experiment 2. Pigs from 12 pens subjected to multiple LPS injections between d 28 and 34 of the experiment decreased their feed intake and presented sickness behaviors, such as lethargy, diarrhea, vomiting and shivering right after the LPS injections, which agree with previous studies (Johnson, 1997; Webel et al., 1997; Wright et al., 2000; Wyns et al., 2015). Although these observations were not different among dietary treatment groups, they did contrast with pens that were not subjected to LPS injection, suggesting an effective immune stimulation. Moreover, the performance worsening occurred exclusively due to LPS injection, since there were no differences in growth performance between groups that were injected with saline solution and groups that were not injected.

It has been shown that metabolic changes associated with infectious diseases or inflammatory processes can result in decreases in feed intake, weight gain and feed efficiency (Johnson, 1997; Johnson, 1998). As observed by Liu et al. (2003), the poor performance of the challenged pigs compared to non-challenged pigs indicates that there was a partitioning of nutrients away from growth towards the immune system, which decreased the efficiency of nutrient utilization for growth (Elsasser et al., 2008). This scenario contributes to the modification of nutritional requirements following an immune challenge, which

increases the demand for nutrients (Sun et al., 2009), including copper, manganese, and zinc.

The immune parameters evaluated after the chronic immune challenge suggest that both dietary concentrations of chloride sources have advantage over sulfate sources, especially regarding the production of TNF- α and total-SOD activity. Differently from what happened following the acute immune challenge, the total-SOD activity increased in both dietary concentrations of chlorides. According to Fridovich (1995), following an immune challenge, macrophages are activated and the oxygen consumption increases markedly, which results in increased production of ROS and increased demand for SOD. Increases in SOD activity does not necessarily mean a change in the oxidative balance of the cell, but it may affect the potential of the cell to deal with increased superoxide radical production, which is important to regulate metabolism to maintain a normal steady state (Marikovsky et al., 2003). The higher bioavailability of chloride sources (Ammerman et al., 1995; Miles et al., 1998; Zhang & Guo, 2007) may have contributed to this numerical increase in total-SOD activity. Although there were not statistical differences, the results are promising and more replicates are needed to better evaluate the effects of chloride sources following a chronic immune challenge.

Conclusion

Chloride sources are able to alleviate the acute immune response by decreasing the proinflammatory cytokine production regardless of the dietary

concentration, suggesting a higher bioavailability over sulfate sources. Besides that, pigs fed high dietary concentrations of copper, manganese, and zinc showed more promising results, indicating that the immune response may have a greater requirement than that needed for growth.

Table III.1. Nutrient composition of the diets

Ingredients, %	N1	N2	N3
Corn, yellow dent	32.60	45.99	59.26
Soybean meal, 47.5% CP	15.00	22.00	34.30
Whey, dried	25.00	15.00	0.00
Lactose	7.00	0.00	0.00
Fish meal, menhaden	6.00	3.00	0.00
Plasma spray-dried	6.00	3.00	0.00
Soybean Oil	4.00	4.00	3.00
Soy protein concentrate	2.21	2.69	0.00
Dicalcium phosphate 18.5%	0.67	1.39	1.58
Blood cell spray-dried	0.00	1.25	0.00
Salt	0.50	0.50	0.50
Limestone	0.45	0.49	0.74
L-lysine HCl	0.17	0.24	0.25
DL-methionine	0.18	0.20	0.11
L-threonine	0.07	0.10	0.09
Vitamin Premix	0.05	0.05	0.05
Trace Mineral Premix ^a	0.10	0.10	0.10
Total	100.00	100.00	100.00

^aTrace Mineral Premix:

Low Sulfates (Treatment A): 20000 ppm Cu as Copper Sulfate; 295100 ppm Fe as Ferrous Sulfate Monohydrate; 500 ppm Ca as Calcium Iodate; 50000 ppm Mn as Manganese Sulfate; 10000 ppm Se as Selenium Premix; 140800 ppm Zn as Zinc sulfate; 483600 ppm Carrier.

High Sulfates (Treatment B): 40000 ppm Cu as Copper Sulfate; 295100 ppm Fe as Ferrous Sulfate Monohydrate; 500 ppm Ca as Calcium Iodate; 100000 ppm Mn as Manganese Sulfate; 10000 ppm Se as Selenium Premix; 281600 ppm Zn as Zinc sulfate; 272800 ppm Carrier.

Low Chlorides (Treatment C): 8600 ppm Cu as Copper Chloride; 295100 ppm Fe as Ferrous Sulfate Monohydrate; 500 ppm Ca as Calcium Iodate; 36400 ppm Mn as Manganese Chloride; 10000 ppm Se as Selenium Premix; 90900 ppm Zn as Zinc Chloride; 558600 ppm Carrier.

High Chlorides (Treatment D): 17200 ppm Cu as Copper Chloride; 295100 ppm Fe as Ferrous Sulfate Monohydrate; 500 ppm Ca as Calcium Iodate; 72800 ppm Mn as Manganese Chloride; 10000 ppm Se as Selenium Premix; 181800 ppm Zn as Zinc Chloride; 422700 ppm Carrier.

Table III.2. Chemical Composition of the basal diets

Analysis	N1	N2	N3
Met. Energy (Kcal/kg)	3,600	3,495	3,420
Dry Matter, %	92.5	91.1	89.5
Crude Protein, %	23	23.6	21.6
Crude Fiber, %	1.34	1.88	2.51
Crude Fat, %	6.18	6.34	5.49
Ash, %	9.45	9.62	2.02
Lactose, %	33.6	14.6	0
Lysine, %	1.66	1.64	1.36
Manganese (ppm)	17	35.4	27
Copper (ppm)	4	4.4	6.5
Zinc (ppm)	38	29	31.5

Analyzed levels:

N1: LS (9 ppm Cu; 33 ppm Mn; 88 ppm Zn); HS (9 ppm Cu; 33 ppm Mn; 88 ppm Zn); LC (9 ppm Cu; 33 ppm Mn; 88 ppm Zn); HC (9 ppm Cu; 33 ppm Mn; 88 ppm Zn).

N2: LS (8.7 ppm Cu; 52.3 ppm Mn; 78.3 ppm Zn); HS (12 ppm Cu; 60.5 ppm Mn; 122.7 ppm Zn); LC (11 ppm Cu; 54.7 ppm Mn; 89.3 ppm Zn); HC (16 ppm Cu; 70 ppm Mn; 126 ppm Zn).

N3: LS (10 ppm Cu; 45.7 ppm Mn; 78 ppm Zn); HS (16.3 ppm Cu; 44.7 ppm Mn; 141 ppm Zn); LC (11.3 ppm Cu; 45.3 ppm Mn; 83 ppm Zn); HC (15.3 ppm Cu; 76 ppm Mn; 133.3 ppm Zn).

Table III.3. Pens allotment for chronic LPS challenge^a

Block	Pen	Dietary treatment	Treatment
1	5	A	LPS
1	10	B	LPS
1	21	C	LPS
1	22	D	LPS
2	6	A	Saline
2	28	B	Saline
2	16	C	Saline
2	25	D	Saline
3	26	A	Control
3	13	B	Control
3	24	C	Control
3	9	D	Control
4	15	A	LPS
4	11	B	LPS
4	12	C	LPS
4	18	D	LPS
5	1	A	Saline
5	2	B	Saline
5	27	C	Saline
5	3	D	Saline
6	17	A	Control
6	23	B	Control
6	7	C	Control
6	8	D	Control
7	19	A	LPS
7	20	B	LPS
7	4	C	LPS
7	14	D	LPS

^aLPS *Escherichia coli* O55:B5, 10 µg/kg of BW.

Table III.4. Effect of source and dietary concentrations of copper, manganese, and zinc on growth performance of nursery pigs^a

	Source and dietary concentration ^b					P value =				
	LS	HS	LC	HC	SE	Exp	Exp*Trt	Source	Concentration	SxC
d 7-21										
Init BW, kg	7.0	7.0	7.0	7.0	0.11	<.0001	0.998	0.963	0.939	0.939
Final BW, kg	11.7	11.9	11.6	11.8	0.20	<.0001	0.529	0.448	0.369	0.874
ADG, kg	0.337	0.347	0.330	0.339	0.01	<.0001	0.339	0.442	0.340	0.973
ADFI, kg	0.419	0.425	0.411	0.416	0.01	<.0001	0.217	0.402	0.567	0.989
G:F	0.799	0.809	0.798	0.805	0.02	<.0001	0.982	0.827	0.441	0.882
d 21-42										
Init BW, kg	11.8	11.9	11.6	11.8	0.18	<.0001	0.385	0.378	0.484	0.683
Final BW, kg	22.5	22.8	22.2	22.4	0.33	<.0001	0.562	0.200	0.244	0.808
ADG, kg	0.520	0.533	0.514	0.520	0.01	<.0001	0.912	0.336	0.366	0.757
ADFI, kg	0.775	0.787	0.777	0.775	0.01	<.0001	0.786	0.709	0.734	0.619
G:F	0.670	0.676	0.660	0.671	0.01	0.260	0.567	0.199	0.160	0.594
d 7-42										
Init BW, kg	7.0	7.0	7.0	7.0	0.11	<.0001	0.998	0.963	0.939	0.939
Final BW, kg	22.5	22.8	22.2	22.4	0.32	<.0001	0.743	0.307	0.369	0.962
ADG, kg	0.449	0.457	0.439	0.447	0.01	<.0001	0.595	0.220	0.293	1.000
ADFI, kg	0.621	0.631	0.622	0.623	0.01	<.0001	0.685	0.764	0.626	0.677
G:F	0.720	0.723	0.704	0.717	0.01	<.0001	0.949	0.085	0.218	0.412

^aLeast square means for 14 pens/ treatment (exp. 1 and 2).

^bLS: low sulfates; HS: high sulfates; LC: low chlorides; HC: high chlorides.

Table III.5. Effect of source and dietary concentrations of copper, manganese, and zinc on body weight and rectal temperature of nursery pigs^a following an acute LPS challenge^b

Source and dietary concentration ^c								P value =		
	LS	HS	LC	HC	SE	Exp	Exp*Trt	Source	Concentration	SxC
Wt, kg										
H0	13.1	13.0	13.0	12.9	0.25	0.055	0.488	0.724	0.776	0.851
H3	12.6	12.5	12.6	12.6	0.24	0.013	0.452	0.915	0.958	0.907
H6	12.3	12.7	12.5	12.5	0.26	0.088	0.526	0.827	0.520	0.546
Temp, °C										
H0	39.5	39.6	39.5	39.6	0.05	0.013	0.481	0.516	0.038	0.933
H3	41.0	41.0	40.9	40.9	0.07	<.0001	0.653	0.209	0.806	0.760
H6	40.3	40.4	40.3	40.4	0.10	<.0001	0.780	0.966	0.365	0.668

^aLeast square means for 14 pens/ treatment (exp. 1 and 2).

^bLPS *Escherichia coli* O111:B4, 25 µg/kg of BW.

^cLS: low sulfates; HS: high sulfates; LC: low chlorides; HC: high chlorides.

Table III.6. Effect of source and dietary concentrations of copper, manganese, and zinc on blood analytes and total-SOD activity of nursery pigs^a following an acute LPS challenge^b

		Source and dietary concentration ^c				SE	Exp	Exp*Trt	P value =	
		LS	HS	LC	HC				Source	Concentration
TNF, pg/mL^d										
	H0	145.7	128.8	130.7	140.9	6.08	<.0001	0.588	0.815	0.586
	H3	9064	7563	7297	6181	708	<.0001	0.625	0.031	0.071
IL-1β, pg/mL^e										
	H0	66.7	67.1	68.3	68.8	0.95	0.118	0.343	0.091	0.605
	H3	639.3	420.3	416.1	363.3	58.1	0.010	0.533	0.020	0.024
Total-SOD^f activity, U/ml										
	H0	1.210	1.194	1.258	1.224	0.04	0.087	0.987	0.368	0.561
	H3	0.989	0.973	1.031	1.015	0.04	0.926	0.999	0.340	0.713

^aLeast square means for 14 pens/ treatment (exp. 1 and 2).

^bLPS *Escherichia coli* O111:B4, 25 μ g/kg of BW.

^cLS: low sulfates; HS: high sulfates; LC: low chlorides; HC: high chlorides.

^dTNF- α = tumor necrosis factor- α .

^eIL-1 β = interleukin 1 β .

^fTotal-SOD activity = Cu/Zn and Mn superoxide dismutase.

Table III.7. Effect of multiple intramuscular injections of LPS or saline solution on growth performance of nursery pigs^a

	Control	Saline	LPS	SE	P value
d 28-35					
Init BW, kg	15.3 ^b	15.4 ^b	15.3 ^b	0.21	0.864
Final BW, kg	19.9 ^b	19.9 ^b	19.0 ^c	0.25	0.052
ADG, kg	0.649 ^b	0.634 ^b	0.532 ^c	0.01	<.0001
ADFI, kg	0.868 ^b	0.867 ^b	0.753 ^c	0.02	<.0001
G:F	0.748 ^b	0.731 ^b	0.707 ^c	0.01	0.005
d 35-42					
Init BW, kg	19.9 ^b	19.9 ^b	19.0 ^c	0.25	0.052
Final BW, kg	24.7 ^b	24.7 ^b	23.8 ^c	0.29	0.092
ADG, kg	0.801 ^b	0.795 ^b	0.798 ^b	0.01	0.953
ADFI, kg	1.206 ^b	1.211 ^b	1.176 ^b	0.02	0.477
G:F	0.664 ^b	0.656 ^b	0.679 ^b	0.02	0.144
d 28-42					
Init BW, kg	15.3 ^b	15.4 ^b	15.3 ^b	0.21	0.864
Final BW, kg	24.7 ^b	24.7 ^b	23.8 ^c	0.29	0.092
ADG, kg	0.719 ^b	0.708 ^b	0.655 ^c	0.01	0.001
ADFI, kg	1.024 ^b	1.026 ^b	0.947 ^c	0.02	0.003
G:F	0.702 ^b	0.691 ^b	0.692 ^c	0.01	0.204

^aFor pigs administered chronic LPS (3 pens/trt), saline solution (2 pens/trt) or no injections (2 pens/trt) on d 28, 30, 32, and 34.

^{b,c}Means that have no superscript in common are significantly different from each other.

Table III.8. Effect of source and dietary concentrations of copper, manganese, and zinc on growth performance of nursery pigs^a subjected to multiple LPS challenges^b

	Source and dietary concentration ^c					P value =		
	LS	HS	LC	HC	SE	Source	Concentration	SxC
d 28-35								
Init BW, kg	15.5	15.5	14.9	15.2	0.33	0.171	0.649	0.690
Final BW, kg	19.3	19.2	18.6	18.9	0.42	0.233	0.758	0.689
ADG, kg	0.539	0.531	0.529	0.530	0.03	0.877	0.918	0.918
ADFI, kg	0.776	0.759	0.724	0.752	0.04	0.456	0.894	0.558
G:F	0.692	0.699	0.728	0.705	0.04	0.302	0.696	0.441
d 35-42								
Init BW, kg	19.3	19.2	18.6	18.9	0.42	0.233	0.758	0.689
Final BW, kg	24.2	23.8	23.4	23.6	0.50	0.350	0.881	0.531
ADG, kg	0.825	0.763	0.807	0.797	0.03	0.765	0.234	0.370
ADFI, kg	1.234	1.120	1.181	1.167	0.05	0.951	0.216	0.322
G:F	0.667	0.681	0.683	0.683	0.04	0.646	0.729	0.718
d 28-42								
Init BW, kg	15.5	15.5	14.9	15.2	0.33	0.171	0.649	0.690
Final BW, kg	24.2	23.8	23.4	23.6	0.50	0.350	0.881	0.531
ADG, kg	0.671	0.638	0.657	0.654	0.02	0.972	0.451	0.542
ADFI, kg	0.983	0.925	0.934	0.943	0.04	0.670	0.522	0.382
G:F	0.682	0.690	0.703	0.693	0.02	0.335	0.948	0.490

^aLeast square means for 3 pens/ treatment subjected to multiple LPS injections.

^bLPS *Escherichia coli* O55:B5, 10 µg/kg of BW.

^cLS: low sulfates; HS: high sulfates; LC: low chlorides; HC: high chlorides.

Table III.9. Effect of source and dietary concentrations of copper, manganese, and zinc on blood analytes and total-SOD activity of nursery pigs^a following a chronic LPS challenge^b

Source and dietary concentration ^c						P value =		
	LS	HS	LC	HC	SE	Source	Concentration	SxC
TNF, pg/mL^d								
H0	221.4	210.6	176.6	187.7	35.1	0.372	0.997	0.766
H3	1116.6	971.0	833.0	581.8	231.4	0.196	0.424	0.827
IL-1β, pg/mL^e								
H0	56.4	51.0	52.8	53.7	2.03	0.832	0.310	0.175
H3	125.4	97.4	102.8	94.1	16.4	0.460	0.305	0.578
Total-SOD^f activity, U/ml								
H0	0.983	1.079	0.900	1.045	0.10	0.571	0.260	0.811
H3	0.920	0.976	0.986	1.091	0.08	0.303	0.355	0.766

^aLeast square means for 3 pens/ treatment.

^bLPS *Escherichia coli* O55:B5, 10 μ g/kg of BW.

^cLS: low sulfates; HS: high sulfates; LC: low chlorides; HC: high chlorides.

^dTNF- α = tumor necrosis factor- α .

^eIL-1 β = interleukin 1 β .

^fTotal-SOD activity = Cu/Zn and Mn superoxide dismutase.

BW change following acute immune challenge

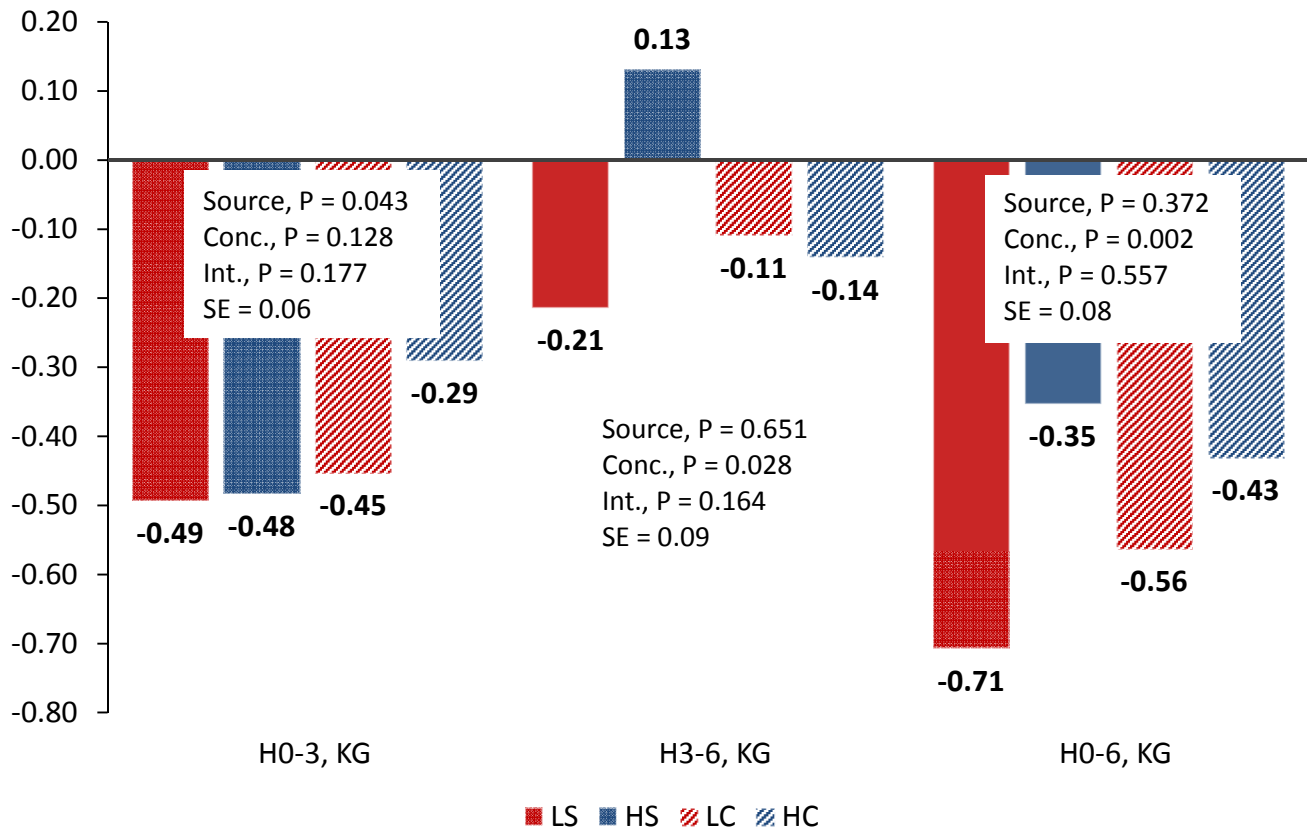


Figure III.1. Effect of dietary source and concentrations of copper, manganese, and zinc on body weight of nursery pigs following acute lipopolysaccharide challenge. The serotype of LPS was *Escherichia coli* O111:B4, 25 µg/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

Rectal temperature change following acute immune challenge

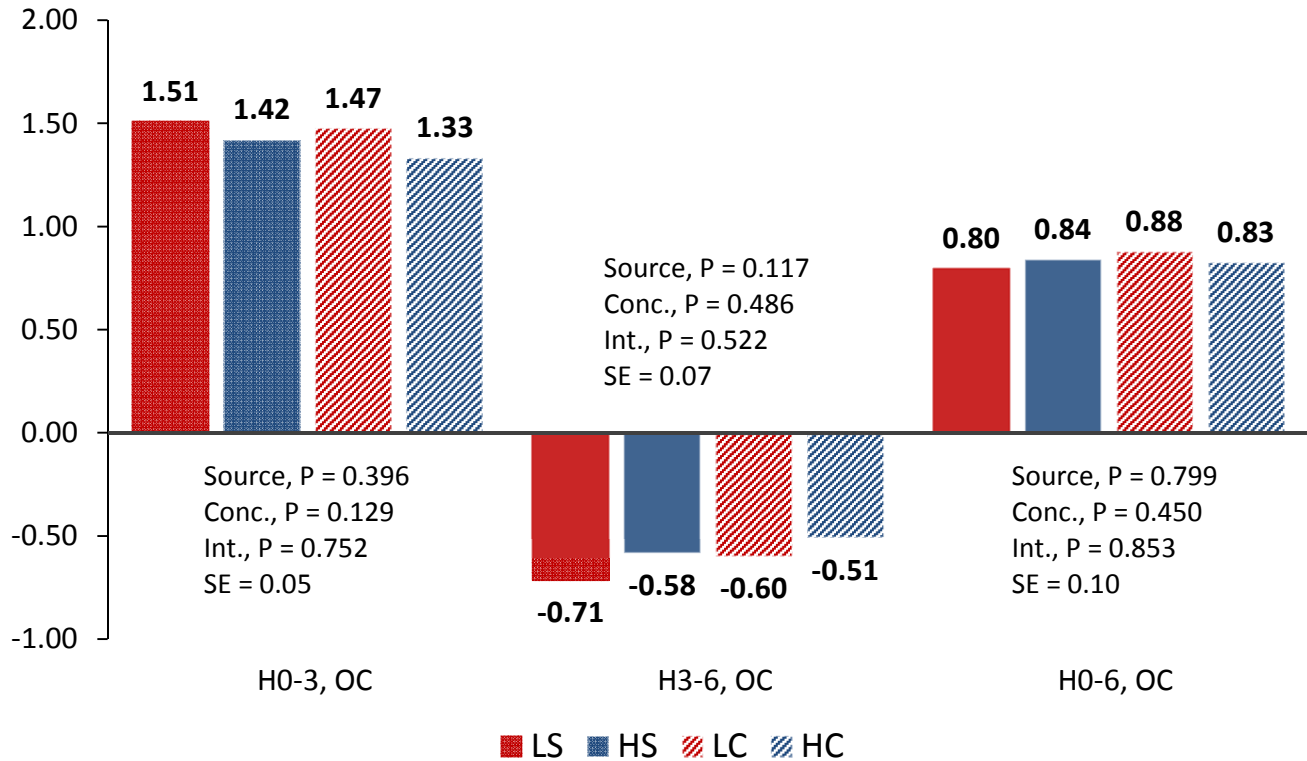


Figure III.2. Effect of dietary source and concentrations of copper, manganese, and zinc on rectal temperature of nursery pigs following acute lipopolysaccharide challenge. The serotype of LPS was *Escherichia coli* O111:B4, 25 µg/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

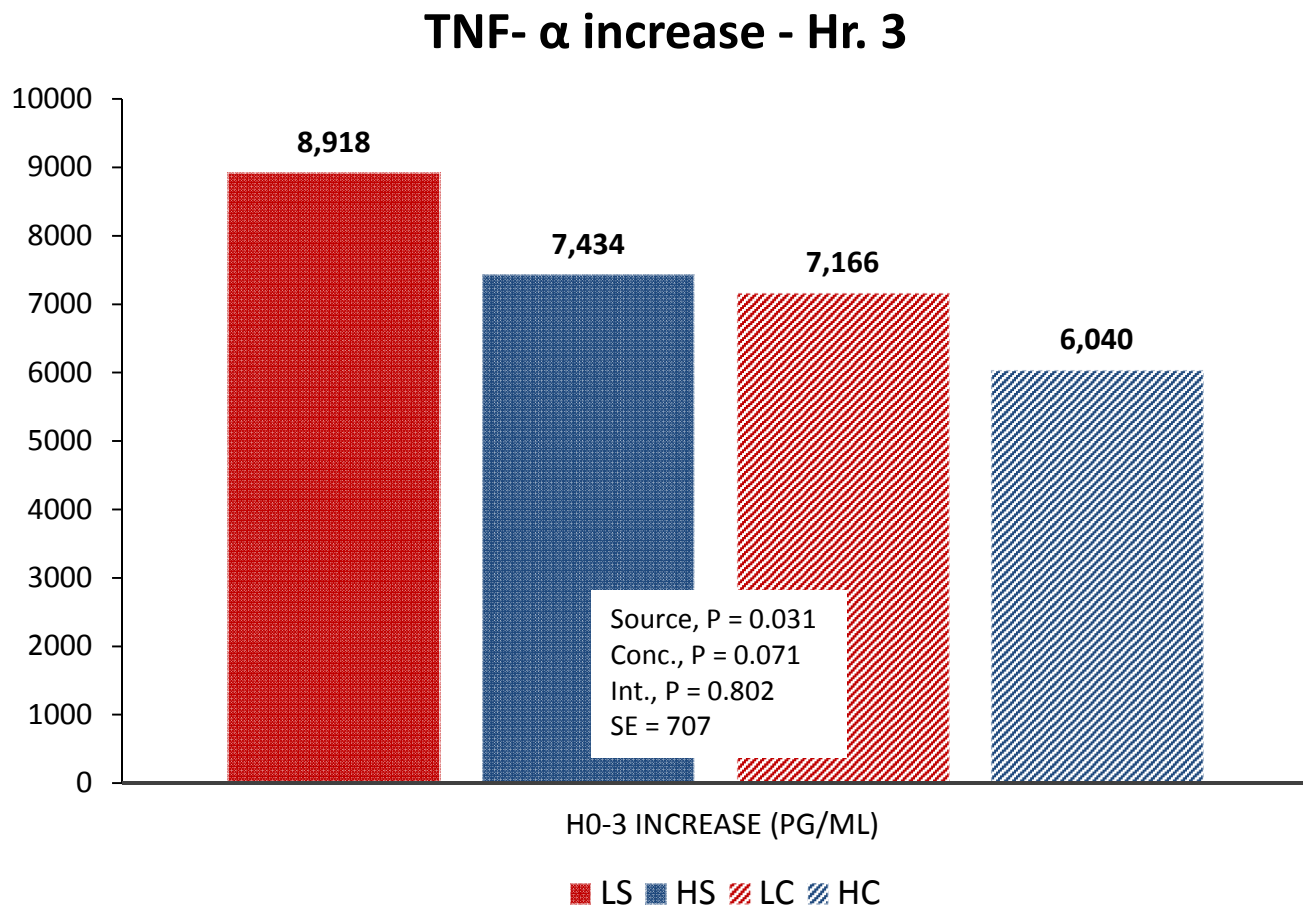


Figure III.3. Effect of dietary source and concentrations of copper, manganese, and zinc on serum tumor necrosis factor- α (TNF- α) of nursery pigs 3 hours following acute lipopolysaccharide challenge. The serotype of LPS was *Escherichia coli* O111:B4, 25 μ g/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

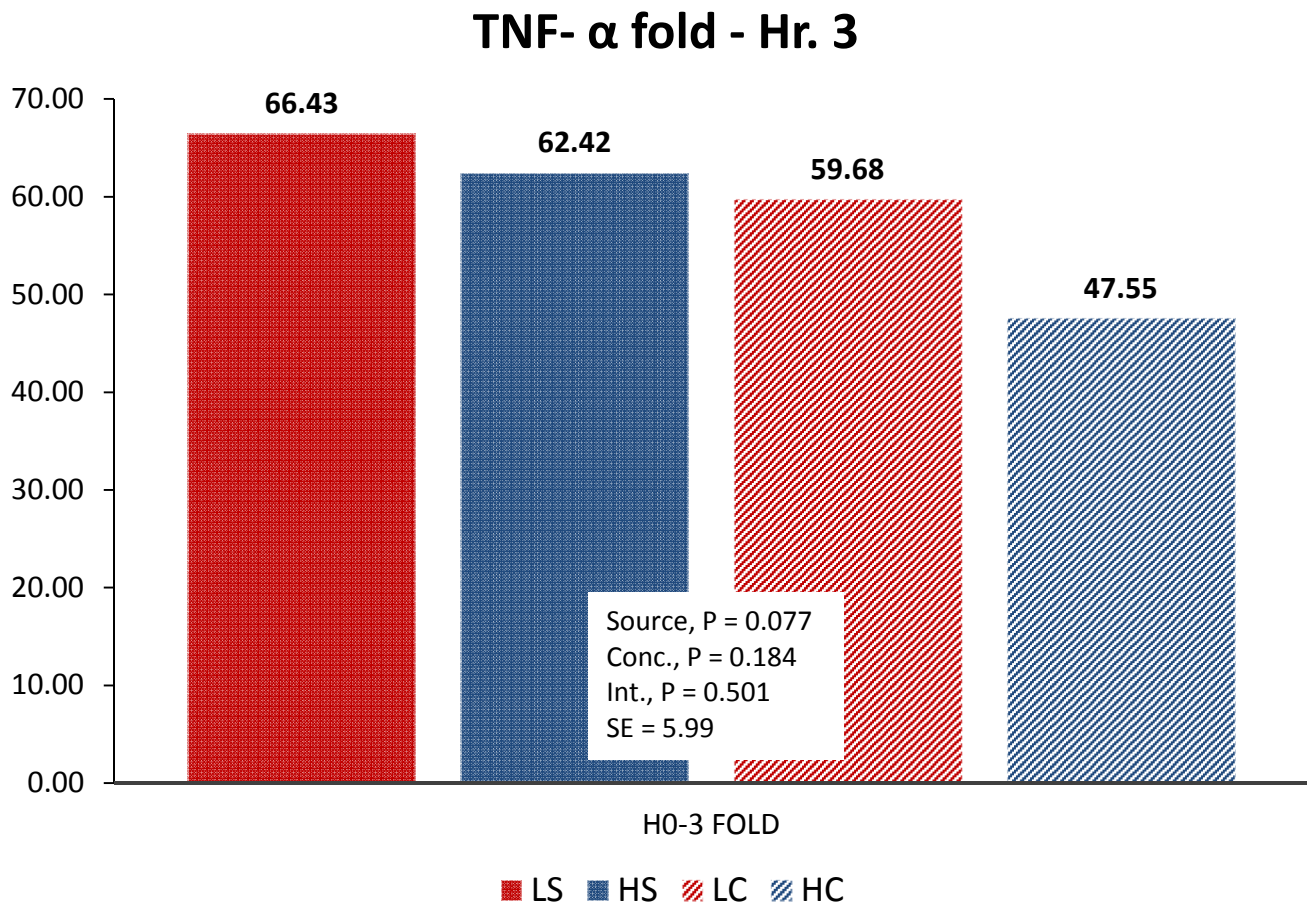


Figure III.4. Effect of dietary source and concentrations of copper, manganese, and zinc on serum tumor necrosis factor- α (TNF- α) fold of nursery pigs 3 hours following acute lipopolysaccharide challenge. The serotype of LPS was *Escherichia coli* O111:B4, 25 μ g/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

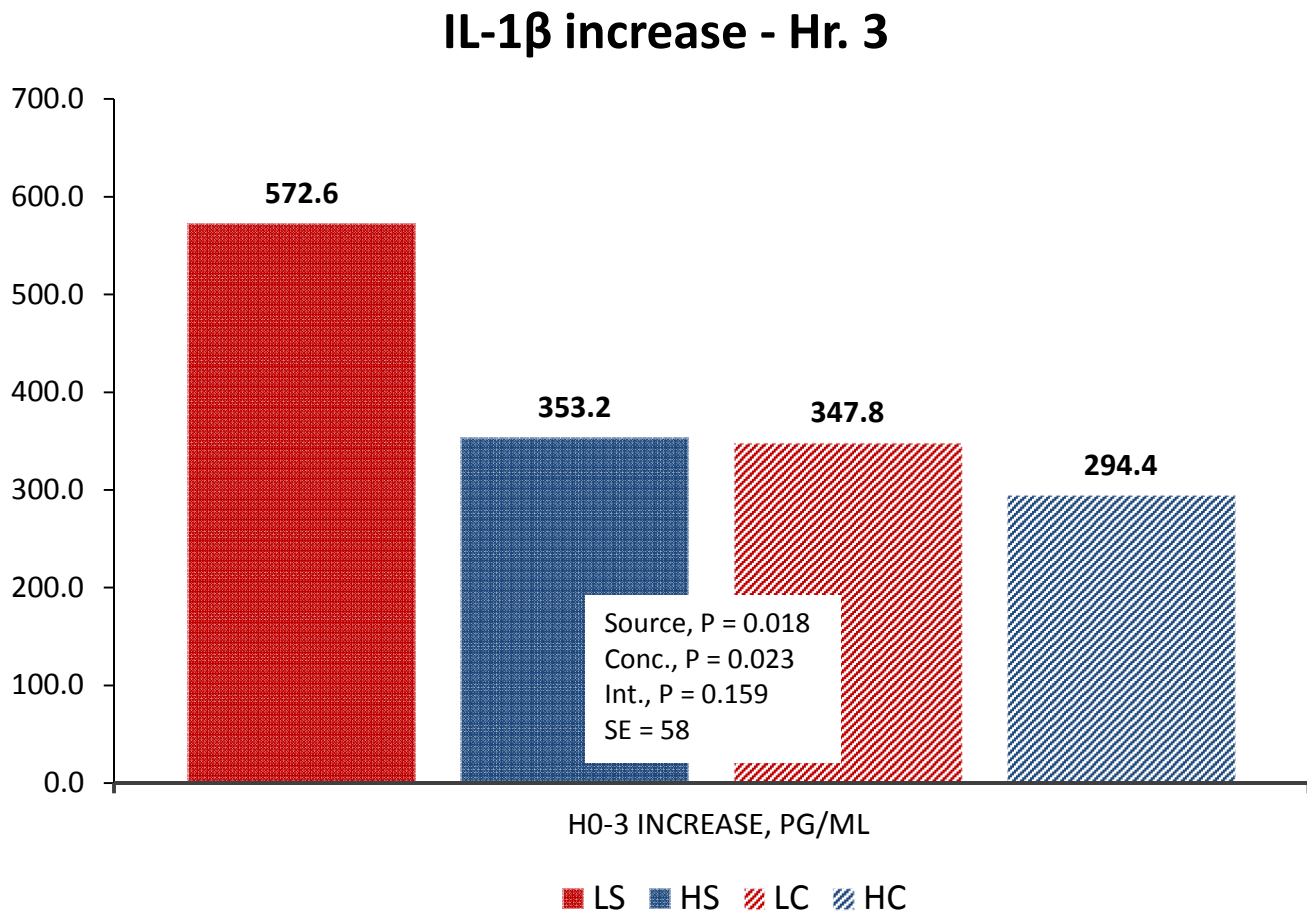


Figure III.5. Effect of dietary source and concentrations of copper, manganese, and zinc on serum interleukin-1 β (IL-1 β) of nursery pigs 3 hours following acute lipopolysaccharide challenge. The serotype of LPS was *Escherichia coli* O111:B4, 25 μ g/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

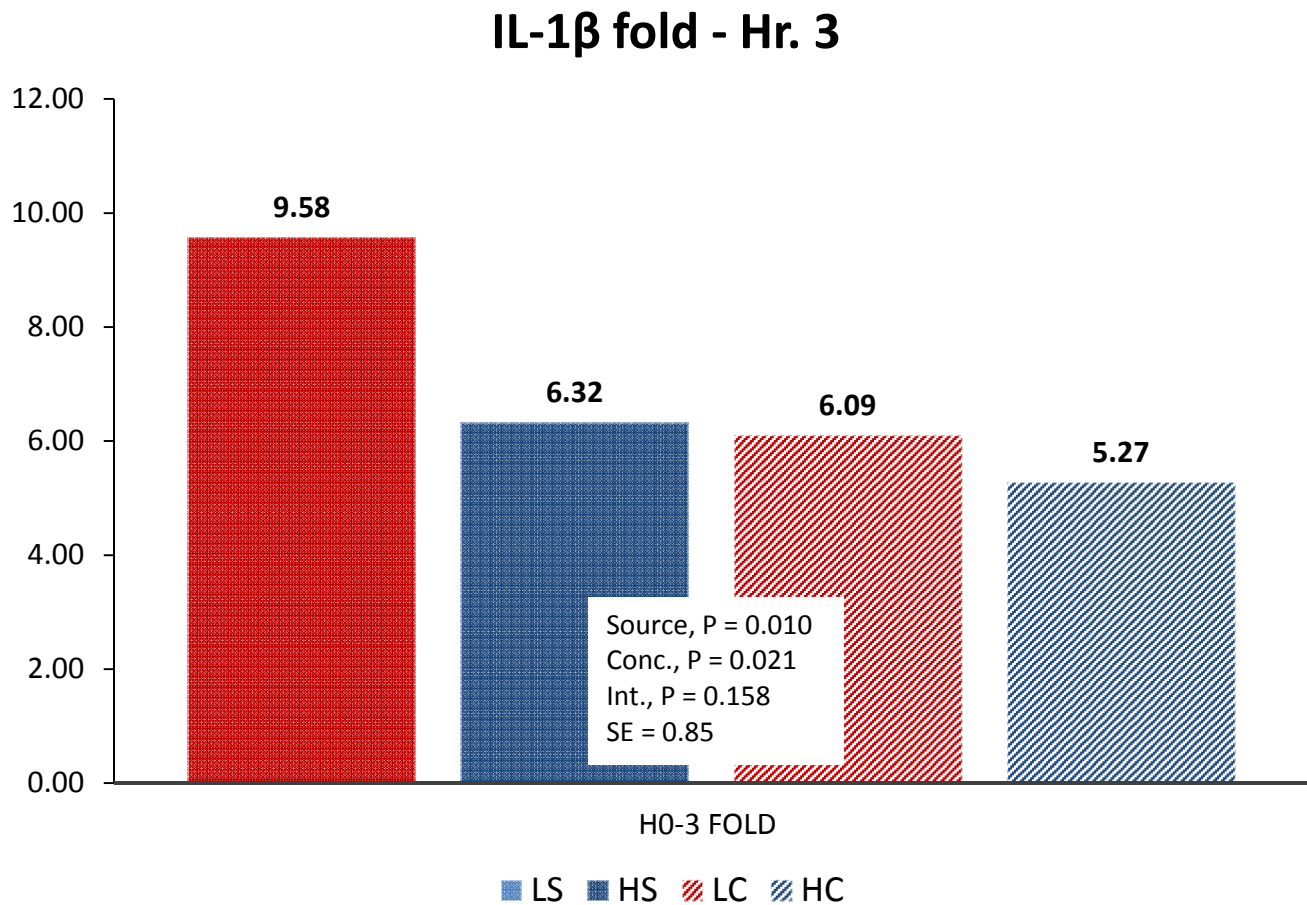


Figure III.6. Effect of dietary source and concentrations of copper, manganese, and zinc on serum interleukin-1 β (IL-1 β) fold of nursery pigs 3 hours following acute lipopolysaccharide challenge. The serotype of LPS was *Escherichia coli* O111:B4, 25 μ g/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

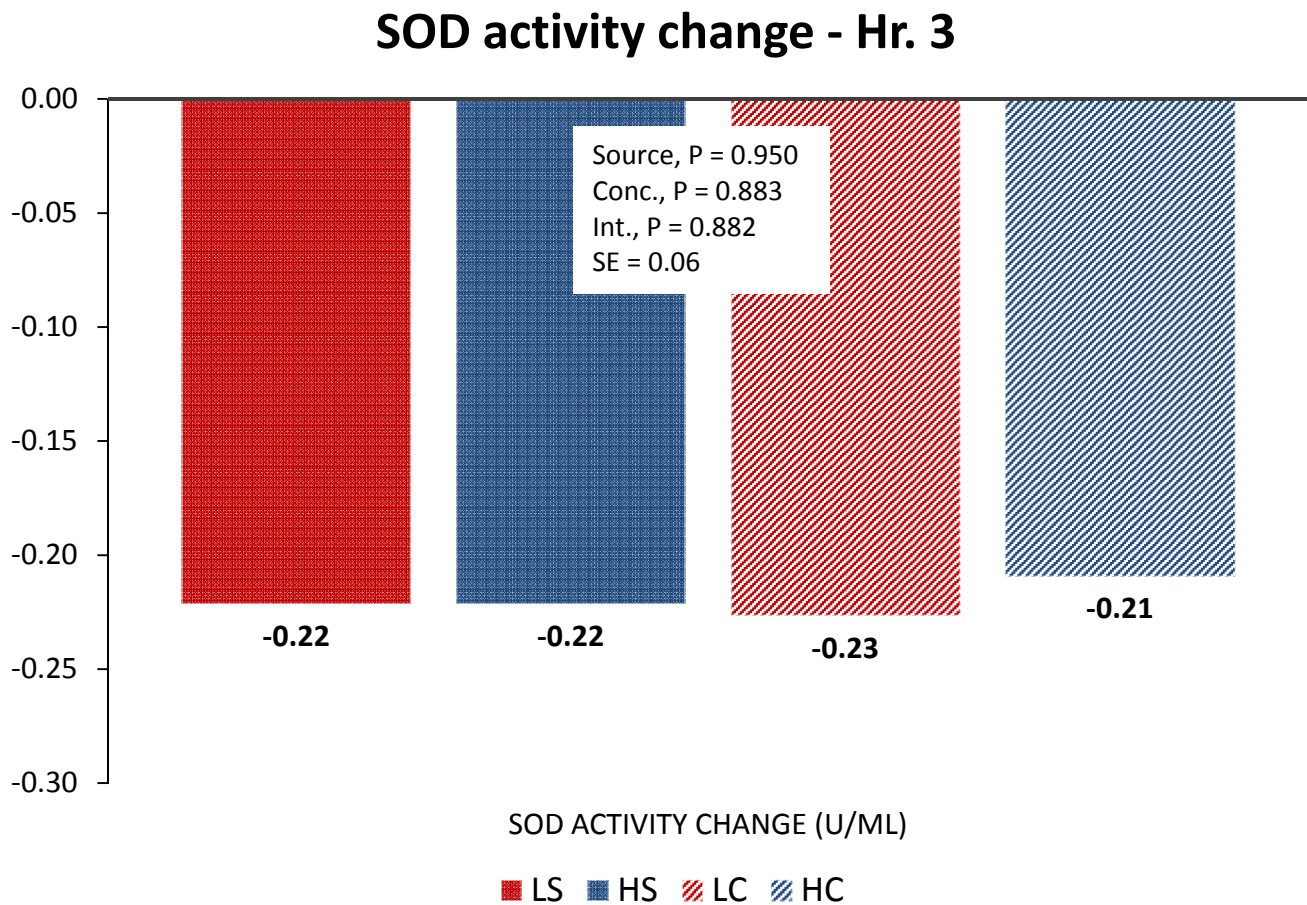


Figure III.7. Effect of dietary source and concentrations of copper, manganese, and zinc on serum total superoxide dismutase (total-SOD) activity of nursery pigs 3 hours following acute lipopolysaccharide challenge. The serotype of LPS was *Escherichia coli* O111:B4, 25 µg/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

TNF- α increase following chronic immune challenge

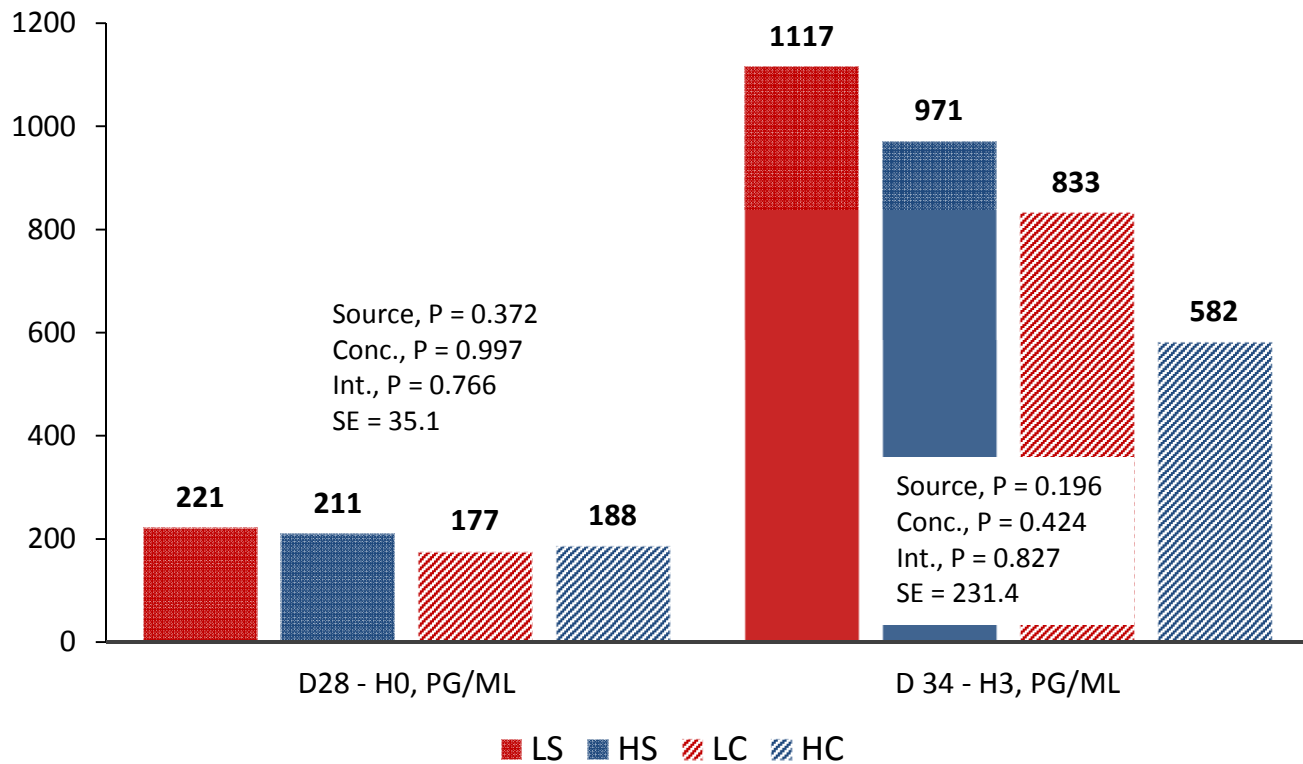


Figure III.8. Effect of dietary source and concentrations of copper, manganese, and zinc on serum tumor necrosis factor- α (TNF- α) of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34. The serotype of LPS was *Escherichia coli* O55:B5, 10 μ g/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

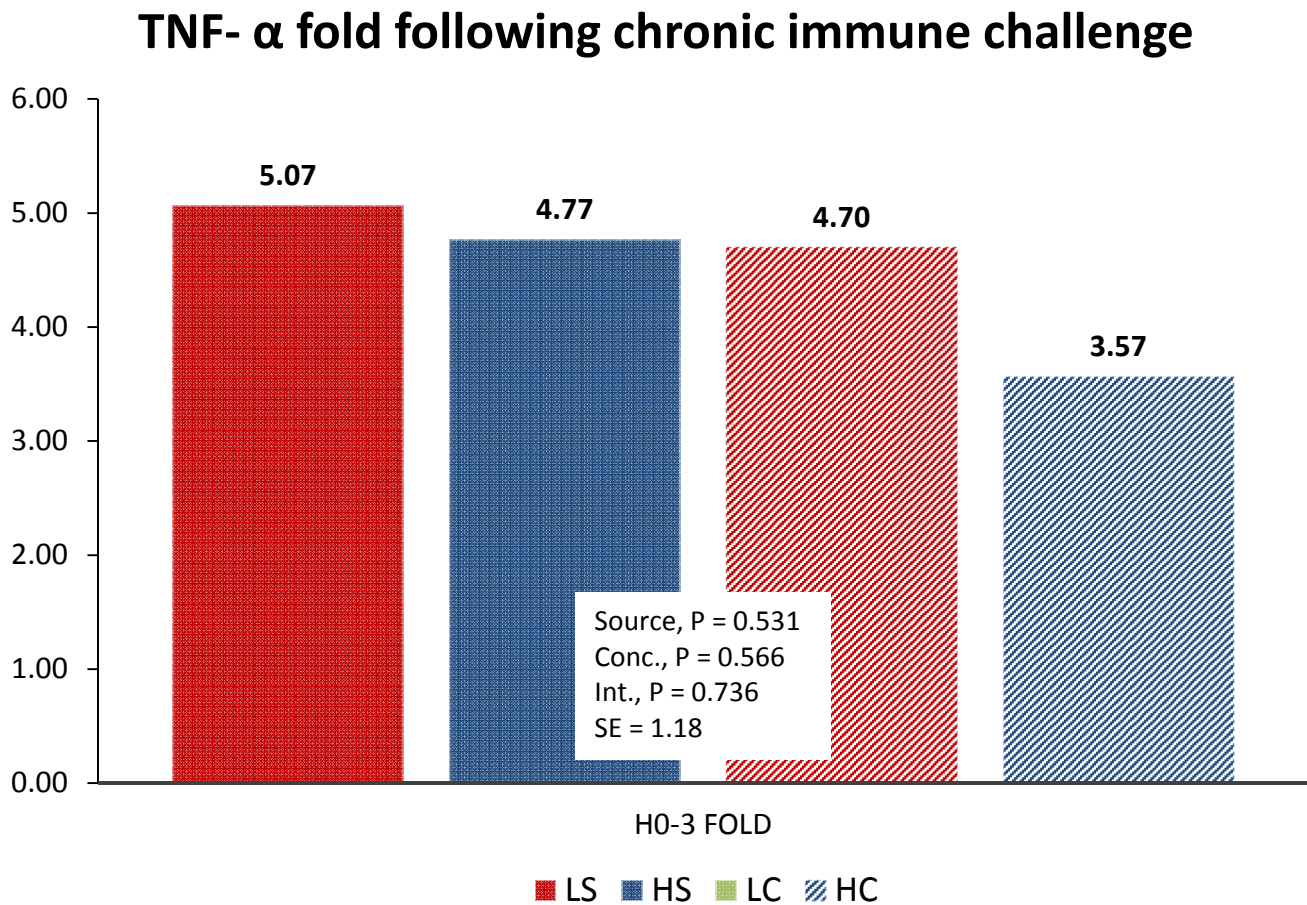


Figure III.9. Effect of dietary sources and concentrations of copper, manganese, and zinc on serum tumor necrosis factor- α (TNF- α) fold of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34. The serotype of LPS was *Escherichia coli* O55:B5, 10 μ g/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

IL-1 β increase following chronic immune challenge

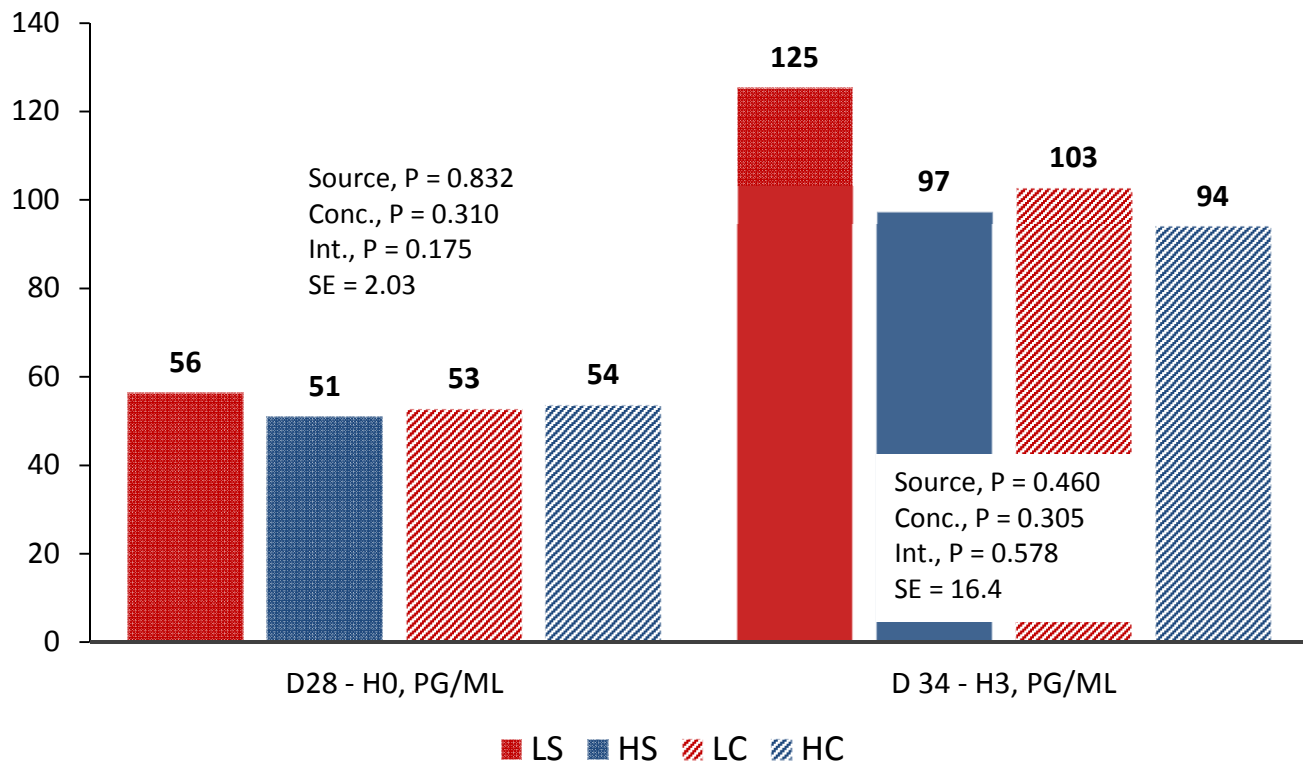


Figure III.10. Effect of dietary source and concentrations of copper, manganese, and zinc on serum interleukin-1 β (IL-1 β) of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34. The serotype of LPS was *Escherichia coli* O55:B5, 10 μ g/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

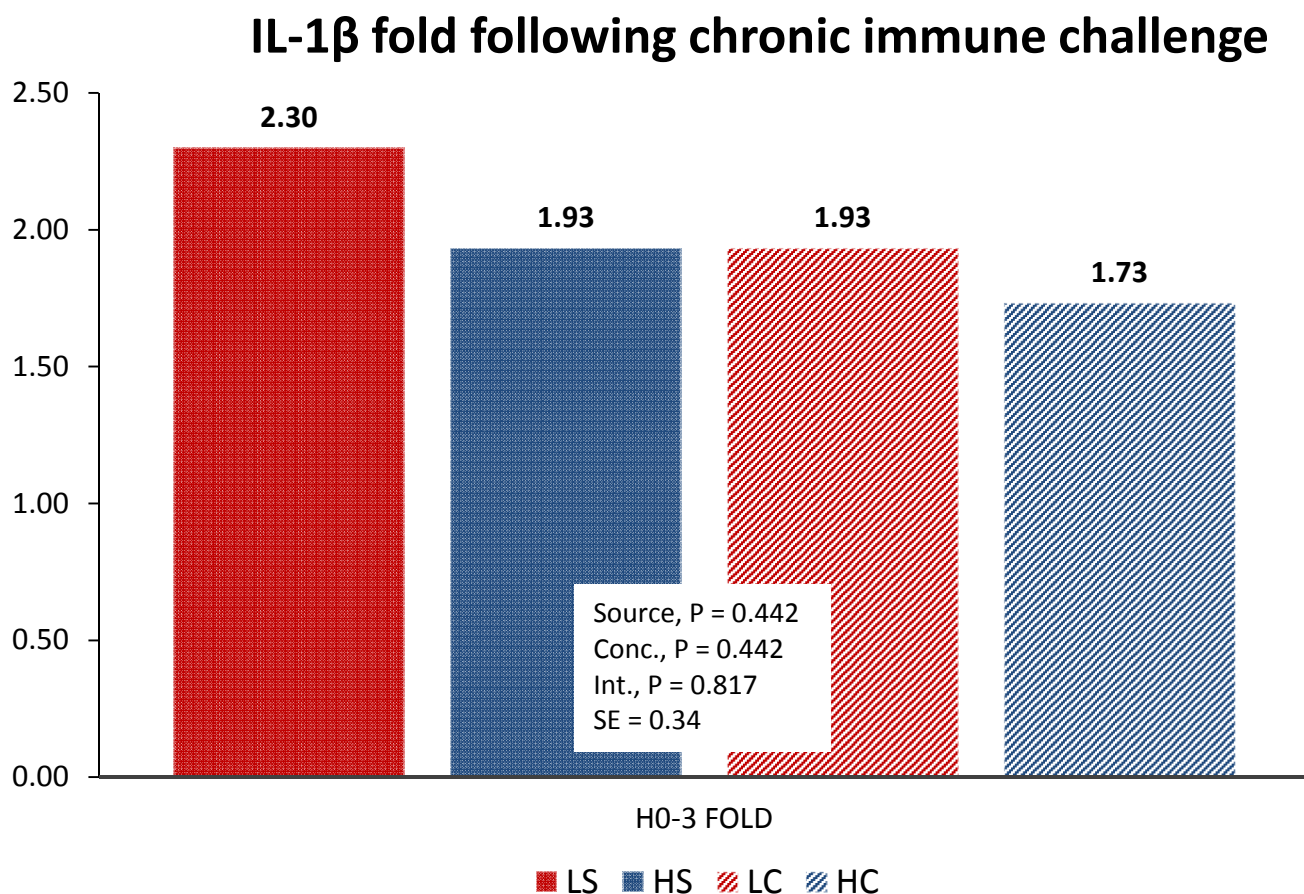


Figure III.11. Effect of dietary source and concentrations of copper, manganese, and zinc on serum interleukin-1 β (IL-1 β) fold of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34. The serotype of LPS was *Escherichia coli* O55:B5, 10 μ g/kg of BW. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

Total-SOD activity following chronic immune challenge

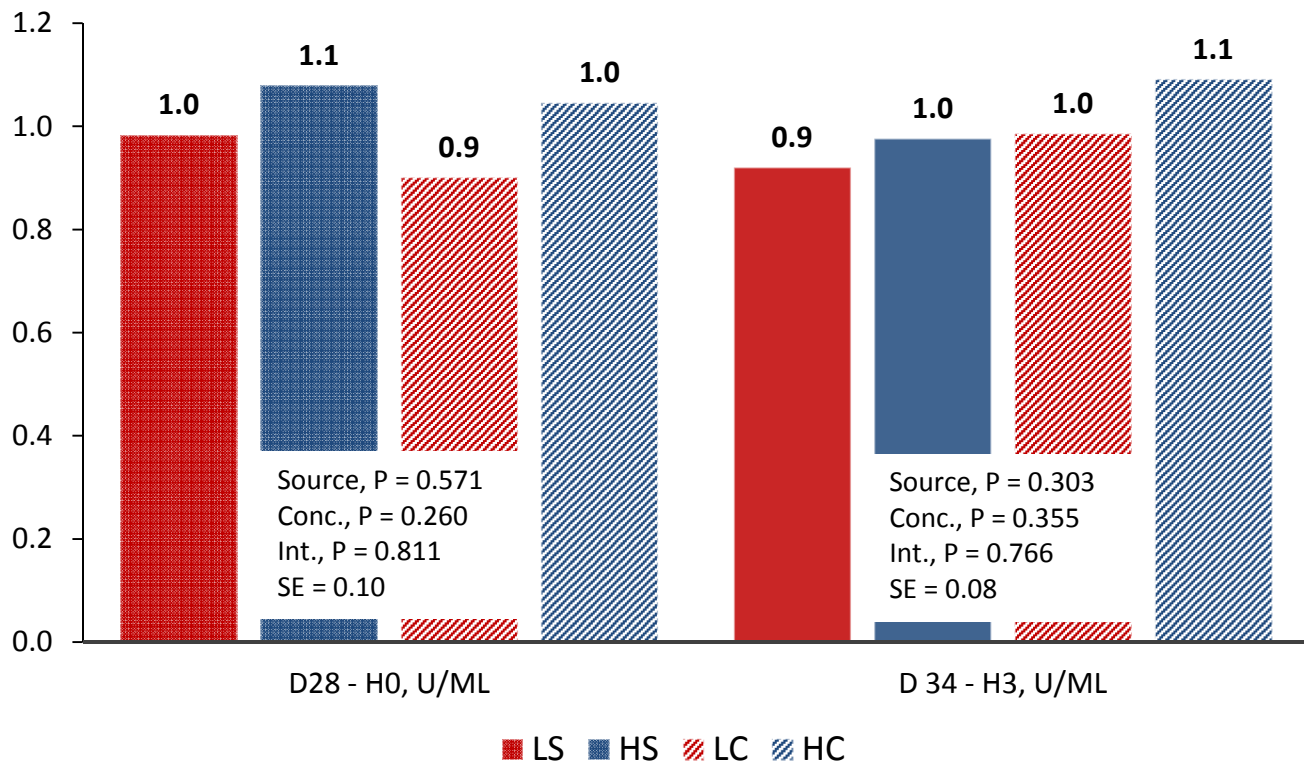


Figure III.12. Effect of dietary source and concentrations of copper, manganese, and zinc on serum total superoxide dismutase (SOD) activity of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34, 10 $\mu\text{g/kg}$ of BW. The serotype of LPS was *Escherichia coli* O55:B5. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

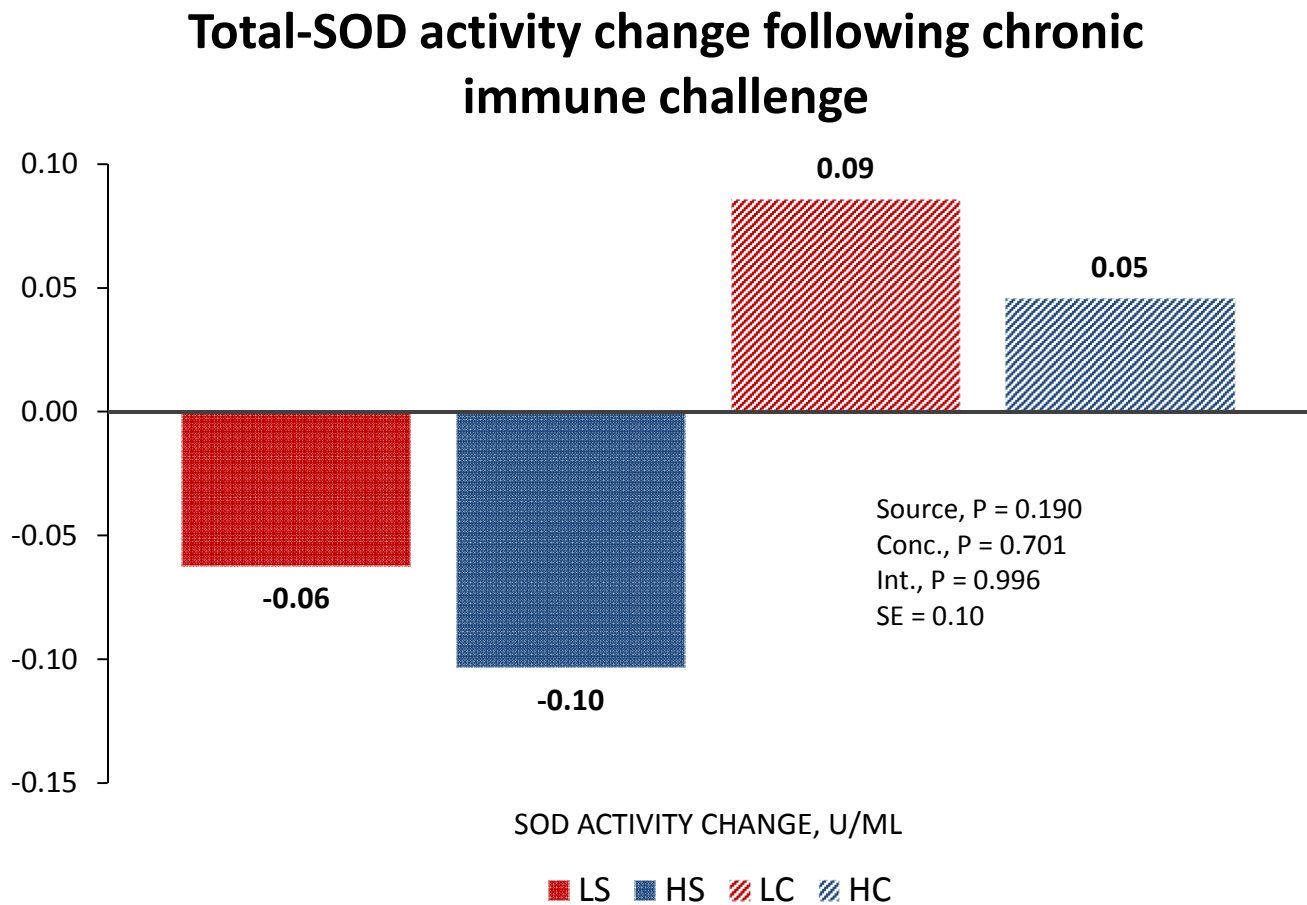


Figure III.13. Effect of dietary source and concentrations of copper, manganese, and zinc on changes in serum total superoxide dismutase (SOD) activity of nursery pigs subjected to chronic lipopolysaccharide challenge between days 28 and 34, 10 µg/kg of BW. The serotype of LPS was *Escherichia coli* O55:B5. LS = low sulfates; HS = high sulfates; LC = low chlorides; HC = high chlorides.

CHAPTER IV

Summary

The study of the interaction between nutrition and immune system has increased in recent years, especially in humans. In swine nutrition, little research has been done and for this reason, there are a lot of opportunities to improve the knowledge in this area. The present research demonstrated that nutritional dietary concentrations of copper, manganese, and zinc are adequate to support normal metabolism and immune response, although the immune system seems to require higher amounts of these trace minerals while it is activated. Moreover, this research suggests that the bioavailability of sulfate sources and chloride sources might be different, which influence the immune response as well.

In swine production, an adequate immune response during the nursery phase contributes to the growth performance results. Therefore, the study of minerals and their impact on the immune response is a relevant nutritional strategy, and more studies in this area are essential to better understand this interaction.

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APPENDICES

Appendix 1

Experiment 1

Appendix 1. Table 1. Pen means for body weight and average daily gain

Block	Treatment	Pen	BW, kg			ADG, g		
			D7	D21	D42	D7-21	D21-42	D7-42
1	A	11	7.5	11.7	22.1	298	497	417
1	B	21	7.4	12.3	23.7	353	542	466
1	C	10	7.4	12.3	21.9	347	461	416
1	D	3	7.4	11.6	21.3	302	462	398
2	A	23	7.1	11.2	20.9	289	463	393
2	B	4	7.1	11.7	21.3	328	460	407
2	C	18	7.0	11.5	22.0	321	501	429
2	D	6	7.2	10.9	20.6	266	460	382
3	A	2	6.9	11.2	20.2	305	428	379
3	B	28	7.0	12.3	21.9	379	458	427
3	C	16	6.9	10.9	21.0	285	481	403
3	D	15	6.9	11.6	22.2	337	506	438
4	A	20	6.8	10.5	19.4	266	422	359
4	B	9	6.8	10.9	20.8	292	469	398
4	C	14	6.9	10.7	20.1	272	448	377
4	D	12	6.8	10.4	19.1	256	415	352
5	A	7	6.7	11.0	21.1	308	482	412
5	B	19	6.7	10.6	21.1	279	497	410
5	C	8	6.7	10.7	20.3	289	458	390
5	D	1	6.6	10.4	19.7	276	443	376
6	A	17	6.3	10.5	20.5	305	474	406
6	B	13	6.6	10.9	21.4	308	500	423
6	C	5	6.3	10.2	19.5	276	443	376
6	D	25	6.6	10.6	20.5	285	472	397
7	A	24	6.2	9.9	18.9	263	430	363
7	B	26	6.0	9.2	17.9	224	415	339
7	C	22	6.2	10.8	19.0	328	393	367
7	D	27	6.2	10.3	20.6	295	492	413

Appendix 1. Table 2. Pen means for average daily feed intake and gain to feed ratio

Block	Treatment	Pen	ADFI, g			G:F		
			D7-21	D21-42	D7-42	D7-21	D21-42	D7-42
1	A	11	426	726	603	0.700	0.684	0.692
1	B	21	470	793	656	0.752	0.683	0.711
1	C	10	427	714	596	0.812	0.646	0.697
1	D	3	376	690	556	0.802	0.670	0.715
2	A	23	372	690	562	0.777	0.671	0.699
2	B	4	391	668	550	0.838	0.689	0.740
2	C	18	433	745	617	0.741	0.672	0.695
2	D	6	357	653	527	0.745	0.705	0.725
3	A	2	417	690	567	0.730	0.620	0.669
3	B	28	460	740	628	0.824	0.619	0.679
3	C	16	400	745	594	0.714	0.646	0.679
3	D	15	450	795	649	0.749	0.636	0.675
4	A	20	348	620	511	0.764	0.680	0.703
4	B	9	389	688	553	0.750	0.683	0.721
4	C	14	357	671	546	0.763	0.666	0.692
4	D	12	353	605	501	0.725	0.686	0.701
5	A	7	379	697	559	0.813	0.692	0.738
5	B	19	372	735	590	0.750	0.677	0.695
5	C	8	393	713	581	0.735	0.643	0.672
5	D	1	359	674	548	0.769	0.658	0.687
6	A	17	408	721	588	0.747	0.657	0.690
6	B	13	395	702	569	0.780	0.713	0.744
6	C	5	376	657	534	0.732	0.673	0.703
6	D	25	409	723	594	0.697	0.653	0.669
7	A	24	354	613	510	0.741	0.701	0.712
7	B	26	329	631	510	0.680	0.658	0.664
7	C	22	432	650	562	0.758	0.605	0.653
7	D	27	359	718	570	0.822	0.685	0.725

Appendix 1. Table 3. Pen mortality on day 7, 21 and 42

Block	Treatment	Pen	D7	D21	D42
1	A	11	0	1	1
1	B	21	0	2	2
1	C	10	0	1	1
1	D	3	0	2	2
2	A	23	0	0	0
2	B	4	0	2	2
2	C	18	0	1	1
2	D	6	0	2	2
3	A	2	0	2	2
3	B	28	0	0	0
3	C	16	0	2	2
3	D	15	0	1	1
4	A	20	0	0	0
4	B	9	0	3	3
4	C	14	0	0	0
4	D	12	0	1	1
5	A	7	0	2	2
5	B	19	0	0	0
5	C	8	0	1	1
5	D	1	0	0	0
6	A	17	0	1	1
6	B	13	0	3	3
6	C	5	0	2	2
6	D	25	0	1	1
7	A	24	0	0	0
7	B	26	0	0	0
7	C	22	0	1	1
7	D	27	0	1	1

Appendix 1. Table 4. Pen means for body weight and changes in body weight following acute LPS challenge^a

Block	Treatment	Pen	BW, kg			BW change, g		
			H0	H3	H6	H0 - 3	H0 - 6	H3 - 6
1	A	11	13.1	12.6	12.0	-431	-1022	-590
1	B	21	13.9	13.6	14.2	-329	269	598
1	C	10	12.9	12.6	12.9	-306	34	341
1	D	3	13.7	13.2	13.2	-488	-458	30
2	A	23	12.4	11.9	12.0	-465	-420	45
2	B	4	13.2	12.7	12.3	-420	-821	-401
2	C	18	13.2	12.7	11.8	-533	-1370	-836
2	D	6	12.4	11.9	11.7	-443	-632	-189
3	A	2	14.5	13.9	13.6	-613	-919	-306
3	B	28	13.6	13.1	13.2	-556	-390	166
3	C	16	14.0	13.3	13.1	-670	-874	-204
3	D	15	13.8	13.5	13.2	-284	-579	-295
4	A	20	12.9	12.3	12.4	-545	-477	68
4	B	9	13.7	13.1	13.0	-579	-636	-57
4	C	14	12.3	11.9	11.5	-431	-855	-424
4	D	12	12.3	12.2	12.5	-170	204	375
5	A	7	12.4	11.9	11.9	-511	-530	-19
5	B	19	12.4	11.9	12.0	-499	-435	64
5	C	8	12.9	12.4	13.0	-488	129	617
5	D	1	12.2	11.7	11.8	-465	-367	98
6	A	17	12.3	11.7	11.5	-636	-802	-166
6	B	13	13.4	11.8	13.3	-1612	-106	1506
6	C	5	11.9	11.4	11.3	-465	-624	-159
6	D	25	11.1	10.6	10.8	-465	-314	151
7	A	24	11.3	10.9	10.1	-375	-1214	-840
7	B	26	10.6	10.4	10.7	-250	38	288
7	C	22	13.5	13.1	13.1	-454	-397	57
7	D	27	11.2	10.9	11.0	-284	-178	106

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 µg/kg BW)

Appendix 1. Table 5. Pen means for rectal temperature and changes in rectal temperature following acute LPS challenge^a

Block	Treatment	Pen	Rectal Temperature, °C			Changes in Rectal Temperature		
			H0	H3	H6	H0 - 3	H0 - 6	H3 - 6
1	A	11	39.4	41.0	40.6	1.58	1.17	-0.42
1	B	21	39.6	41.2	40.7	1.54	1.06	-0.48
1	C	10	39.8	41.5	40.6	1.71	0.77	-0.94
1	D	3	39.5	41.3	40.9	1.76	1.35	-0.42
2	A	23	39.4	41.6	40.2	2.17	0.85	-1.31
2	B	4	39.5	41.2	41.0	1.69	1.52	-0.18
2	C	18	39.5	41.2	40.9	1.61	1.33	-0.28
2	D	6	39.8	40.8	40.5	1.03	0.66	-0.37
3	A	2	39.8	41.1	40.2	1.28	0.32	-0.96
3	B	28	39.7	41.3	40.9	1.61	1.14	-0.47
3	C	16	39.7	41.2	40.4	1.58	0.75	-0.83
3	D	15	39.5	40.9	40.4	1.36	0.87	-0.49
4	A	20	39.4	41.2	40.8	1.82	1.44	-0.38
4	B	9	39.9	41.6	40.5	1.67	0.65	-1.02
4	C	14	39.3	41.1	40.2	1.79	0.91	-0.88
4	D	12	39.3	40.6	40.6	1.29	1.26	-0.03
5	A	7	39.3	41.1	40.9	1.82	1.64	-0.18
5	B	19	39.7	41.5	41.5	1.79	1.74	-0.05
5	C	8	39.7	41.0	40.6	1.35	0.90	-0.45
5	D	1	39.7	41.1	41.0	1.35	1.29	-0.06
6	A	17	39.7	41.1	40.8	1.38	1.06	-0.32
6	B	13	39.8	41.2	40.8	1.44	1.05	-0.39
6	C	5	39.4	41.3	40.9	1.89	1.50	-0.39
6	D	25	39.5	41.4	40.6	1.87	1.11	-0.76
7	A	24	39.5	41.2	40.9	1.71	1.44	-0.26
7	B	26	39.5	40.7	40.0	1.24	0.50	-0.74
7	C	22	39.6	41.1	40.4	1.58	0.85	-0.73
7	D	27	39.9	41.3	40.4	1.38	0.45	-0.92

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 µg/kg BW)

Appendix 1. Table 6. Pen means for TNF- α and changes in TNF- α following acute LPS challenge^a

Block	Treatment	Pen	TNF- α , pg/mL		Changes in TNF- α	
			H0	H3	H0 - 3	Fold
1	A	11	147.3	4090.2	3942.96	27.77
1	B	21	92.3	4195.5	4103.16	45.43
1	C	10	163.8	5051.2	4887.48	30.85
1	D	3	139.6	5135.1	4995.50	36.79
2	A	23	155.7	8321.2	8165.55	53.45
2	B	4	172.1	7107.7	6935.54	41.29
2	C	18	170.3	3524.0	3353.68	20.70
2	D	6	141.2	5829.1	5687.86	41.28
3	A	2	180.9	8657.2	8476.36	47.87
3	B	28	146.3	4000.6	3854.37	27.35
3	C	16	151.6	9986.2	9834.60	65.86
3	D	15	173.7	7459.0	7285.23	42.93
4	A	20	165.1	7692.7	7527.65	46.61
4	B	9	119.7	5031.2	4911.43	42.02
4	C	14	149.3	6312.5	6163.24	42.29
4	D	12	155.8	3684.7	3528.91	23.65
5	A	7	158.6	3999.9	3841.32	25.23
5	B	19	157.3	4155.8	3998.49	26.42
5	C	8	153.1	4054.6	3901.50	26.48
5	D	1	179.0	3468.6	3289.55	19.37
6	A	17	170.0	11306.4	11136.40	66.52
6	B	13	155.6	9379.5	9223.91	60.28
6	C	5	154.0	6990.9	6836.86	45.38
6	D	25	166.3	5589.9	5423.61	33.62
7	A	24	213.8	8443.3	8229.49	39.49
7	B	26	146.3	1913.6	1767.28	13.08
7	C	22	105.7	4736.8	4631.05	44.80
7	D	27	167.9	3786.2	3618.27	22.55

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 μ g/kg BW)

Appendix 1. Table 7. Pen means for IL-1 β and changes in IL-1 β following acute LPS challenge^a

Block	Treatment	Pen	IL-1 β , pg/mL		Changes in IL-1 β	
			H0	H3	H0 - 3	Fold
1	A	11	69.6	339.1	269.47	4.87
1	B	21	70.3	158.4	88.05	2.25
1	C	10	68.2	216.2	148.02	3.17
1	D	3	66.5	216.9	150.33	3.26
2	A	23	66.2	359.4	293.16	5.43
2	B	4	64.6	270.2	205.55	4.18
2	C	18	72.0	338.7	266.74	4.71
2	D	6	69.6	421.7	352.08	6.06
3	A	2	70.3	1106.9	1036.61	15.75
3	B	28	70.8	275.5	204.69	3.89
3	C	16	70.4	1012.1	941.71	14.38
3	D	15	67.5	485.3	417.86	7.19
4	A	20	60.9	350.5	289.66	5.76
4	B	9	67.8	161.3	93.52	2.38
4	C	14	68.8	337.5	268.70	4.90
4	D	12	72.2	253.0	180.77	3.50
5	A	7	69.1	446.3	377.24	6.46
5	B	19	65.0	227.0	162.00	3.49
5	C	8	64.1	226.0	161.90	3.53
5	D	1	75.9	281.1	205.17	3.70
6	A	17	63.1	787.6	724.46	12.48
6	B	13	76.3	569.6	493.29	7.47
6	C	5	67.7	372.1	304.45	5.50
6	D	25	74.4	338.1	263.71	4.55
7	A	24	65.8	318.6	252.80	4.84
7	B	26	69.6	367.9	298.27	5.28
7	C	22	68.7	206.8	138.12	3.01
7	D	27	62.5	259.1	196.56	4.14

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 μ g/kg BW)

Appendix 1. Table 8. Pen means for SOD activity and changes in SOD activity following acute LPS challenge^a

Block	Treatment	Pen	SOD activity, U/mL		Changes in SOD activity
			H0	H3	H0 - 3
1	A	11	1.178	0.954	-0.224
1	B	21	1.050	1.201	0.150
1	C	10	1.079	0.911	-0.168
1	D	3	1.377	0.968	-0.409
2	A	23	1.155	1.233	0.077
2	B	4	1.389	0.855	-0.534
2	C	18	1.700	1.240	-0.461
2	D	6	1.291	1.215	-0.076
3	A	2	1.289	1.011	-0.278
3	B	28	1.058	0.873	-0.184
3	C	16	1.167	1.078	-0.089
3	D	15	1.108	0.954	-0.154
4	A	20	1.321	0.843	-0.478
4	B	9	1.166	1.021	-0.145
4	C	14	1.190	1.290	0.100
4	D	12	1.199	1.145	-0.054
5	A	7	0.952	0.932	-0.020
5	B	19	1.102	1.038	-0.064
5	C	8	1.182	0.982	-0.200
5	D	1	1.199	0.775	-0.424
6	A	17	1.152	0.983	-0.170
6	B	13	1.204	1.036	-0.169
6	C	5	1.170	1.044	-0.126
6	D	25	1.105	0.935	-0.170
7	A	24	1.136	0.916	-0.220
7	B	26	1.223	0.800	-0.423
7	C	22	1.050	0.679	-0.371
7	D	27	0.976	1.088	0.112

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 µg/kg BW)

Appendix 2

Experiment 2

Appendix 2. Table 1. Pen means for body weight and average daily gain

Block	Treatment	Pen	BW, kg			ADG, g		
			D7	D21	D42	D7-21	D21-42	D7-42
1	A	5	7.7	13.3	24.2	399	545	485
1	B	10	7.7	13.2	24.1	391	547	483
1	C	21	7.8	12.8	25.0	357	611	506
1	D	22	7.7	13.5	25.2	412	588	515
2	A	6	7.6	13.6	25.8	425	611	534
2	B	28	7.6	13.1	25.9	397	640	540
2	C	16	7.5	12.0	22.7	324	536	449
2	D	25	7.6	13.7	25.9	438	611	540
3	A	26	7.4	13.3	23.8	420	523	480
3	B	13	7.4	13.3	25.7	418	623	538
3	C	24	7.5	12.3	24.2	345	593	491
3	D	9	7.4	13.3	24.9	422	577	513
4	A	15	7.2	12.7	24.2	392	575	500
4	B	11	7.4	12.8	23.4	386	530	471
4	C	12	7.2	11.8	22.4	331	528	447
4	D	18	7.4	11.4	23.5	284	585	474
5	A	1	7.2	12.3	24.2	363	599	502
5	B	2	7.1	12.4	24.2	381	586	502
5	C	27	7.2	11.0	21.1	.	.	.
5	D	3	7.1	12.6	23.8	392	558	490
6	A	17	7.0	11.8	25.1	341	637	532
6	B	23	7.0	11.9	23.6	350	586	489
6	C	7	7.0	12.8	25.5	412	638	545
6	D	8	6.9	12.7	24.6	412	592	518
7	A	19	6.6	11.5	24.3	350	602	519
7	B	20	6.8	12.0	24.0	370	601	506
7	C	4	6.8	12.1	22.7	379	531	469
7	D	14	6.6	11.8	22.2	373	517	458

Appendix 2. Table 2. Pen means for average daily feed intake and gain to feed ratio

Block	Treatment	Pen	ADFI, g			G:F		
			D7-21	D21-42	D7-42	D7-21	D21-42	D7-42
1	A	5	497	860	710	0.803	0.634	0.682
1	B	10	461	802	662	0.848	0.682	0.729
1	C	21	428	911	700	0.833	0.671	0.723
1	D	22	477	886	718	0.863	0.663	0.718
2	A	6	492	914	740	0.864	0.668	0.722
2	B	28	470	936	733	0.845	0.684	0.737
2	C	16	392	817	642	0.827	0.656	0.699
2	D	25	489	903	711	0.896	0.677	0.759
3	A	26	470	808	660	0.894	0.647	0.728
3	B	13	477	912	710	0.877	0.683	0.758
3	C	24	411	891	682	0.841	0.665	0.720
3	D	9	492	862	709	0.857	0.669	0.723
4	A	15	464	837	649	0.845	0.687	0.770
4	B	11	436	772	625	0.885	0.687	0.753
4	C	12	401	768	606	0.825	0.688	0.738
4	D	18	371	852	642	0.765	0.687	0.739
5	A	1	424	876	690	0.858	0.684	0.728
5	B	2	447	895	700	0.853	0.655	0.717
5	C	27
5	D	3	431	861	668	0.908	0.649	0.733
6	A	17	398	918	675	0.855	0.694	0.789
6	B	23	413	862	666	0.848	0.679	0.734
6	C	7	441	943	729	0.934	0.676	0.748
6	D	8	488	871	714	0.843	0.680	0.726
7	A	19	412	886	665	0.849	0.679	0.781
7	B	20	433	887	675	0.853	0.677	0.749
7	C	4	439	799	651	0.864	0.664	0.720
7	D	14	413	757	610	0.902	0.683	0.750

Appendix 2. Table 3. Pen mortality on day 7, 21 and 42

Block	Treatment	Pen	D7	D21	D42
1	A	5	0	0	0
1	B	10	0	0	0
1	C	21	0	1	1
1	D	22	0	0	0
2	A	6	0	0	0
2	B	28	0	1	1
2	C	16	0	0	0
2	D	25	0	2	2
3	A	26	0	1	1
3	B	13	0	2	2
3	C	24	0	1	1
3	D	9	0	0	0
4	A	15	0	4	4
4	B	11	0	1	1
4	C	12	0	2	2
4	D	18	0	0	1
5	A	1	0	0	0
5	B	2	0	1	1
5	C	27	0	0	1
5	D	3	1	1	2
6	A	17	0	1	2
6	B	23	0	1	1
6	C	7	1	0	1
6	D	8	0	0	0
7	A	19	0	1	2
7	B	20	0	2	2
7	C	4	0	0	0
7	D	14	0	1	1

Appendix 2. Table 4. Pen means for body weight and changes in body weight following acute LPS challenge^a

Block	Treatment	Pen	BW, kg			BW change, g		
			H0	H3	H6	H0 - 3	H0 - 6	H3 - 6
1	A	5	14.0	13.6	13.5	-386	-511	-125
1	B	10	14.6	14.2	14.1	-420	-477	-57
1	C	21	12.9	12.6	12.8	-363	-159	204
1	D	22	12.5	12.0	12.0	-409	-488	-79
2	A	6	15.2	14.5	14.3	-670	-863	-193
2	B	28	12.3	12.0	12.0	-295	-269	26
2	C	16	11.6	11.3	11.1	-306	-443	-136
2	D	25	13.5	13.3	11.9	-261	-1646	-1385
3	A	26	13.5	13.1	13.2	-386	-306	79
3	B	13	13.4	13.1	13.2	-397	-216	182
3	C	24	13.2	12.7	12.1	-477	-1105	-628
3	D	9	14.1	14.0	13.9	-68	-125	-57
4	A	15	13.1	12.7	11.7	-409	-1396	-987
4	B	11	13.3	13.0	13.8	-306	496	802
4	C	12	14.1	13.7	13.7	-363	-375	-11
4	D	18	13.7	13.3	13.3	-420	-431	-11
5	A	1	13.9	13.1	13.1	-783	-738	45
5	B	2	13.4	13.0	12.0	-375	-1358	-984
5	C	27	12.6	12.2	12.1	-363	-522	-159
5	D	3	13.4	13.0	12.6	-363	-768	-405
6	A	17	12.1	11.9	12.0	-227	-174	53
6	B	23	12.9	12.5	12.2	-420	-628	-208
6	C	7	13.3	12.8	12.6	-556	-760	-204
6	D	8	14.3	14.2	13.8	-148	-477	-329
7	A	19	12.1	11.6	11.5	-465	-522	-57
7	B	20	11.7	11.4	11.3	-306	-397	-91
7	C	4	13.9	13.3	13.3	-568	-556	11
7	D	14	12.5	12.7	12.7	204	227	23

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 µg/kg BW)

Appendix 2. Table 5. Pen means for rectal temperature and changes in rectal temperature following acute LPS challenge^a

Block	Treatment	Pen	Rectal Temperature, °C			Changes in Rectal Temperature		
			H0	H3	H6	H0 - 3	H0 - 6	H3 - 6
1	A	5	39.8	40.8	40.4	0.99	0.63	-0.36
1	B	10	39.5	41.0	40.3	1.50	0.71	-0.79
1	C	21	39.3	40.5	40.2	1.17	0.88	-0.29
1	D	22	39.1	40.7	40.1	1.64	1.00	-0.64
2	A	6	39.4	41.3	40.5	1.89	1.14	-0.75
2	B	28	39.3	41.1	40.4	1.78	1.06	-0.72
2	C	16	39.3	40.6	40.2	1.25	0.89	-0.36
2	D	25	39.7	40.8	40.6	1.10	0.94	-0.15
3	A	26	39.3	41.0	39.5	1.64	0.17	-1.47
3	B	13	39.6	40.5	39.4	0.88	-0.17	-1.04
3	C	24	39.3	40.7	39.9	1.35	0.64	-0.71
3	D	9	39.6	41.2	40.6	1.61	0.96	-0.65
4	A	15	39.4	40.3	39.0	0.97	-0.39	-1.36
4	B	11	39.7	41.1	40.5	1.37	0.81	-0.57
4	C	12	39.4	40.6	40.0	1.14	0.60	-0.54
4	D	18	39.4	41.0	40.2	1.56	0.76	-0.79
5	A	1	39.5	40.8	40.0	1.26	0.47	-0.79
5	B	2	39.5	40.8	40.4	1.28	0.91	-0.37
5	C	27	39.3	41.2	40.2	1.83	0.89	-0.94
5	D	3	39.9	40.9	40.4	0.98	0.46	-0.52
6	A	17	39.6	40.9	40.4	1.35	0.77	-0.58
6	B	23	39.4	40.4	39.9	1.00	0.46	-0.54
6	C	7	39.3	40.9	40.3	1.57	1.01	-0.56
6	D	8	39.5	40.6	40.3	1.10	0.79	-0.31
7	A	19	39.5	40.8	40.0	1.29	0.44	-0.85
7	B	20	39.5	40.6	39.8	1.07	0.32	-0.75
7	C	4	39.6	40.4	39.9	0.82	0.39	-0.43
7	D	14	39.4	40.1	39.1	0.63	-0.36	-0.99

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 µg/kg BW)

Appendix 2. Table 6. Pen means for TNF- α and changes in TNF- α following acute LPS challenge^a

Block	Treatment	Pen	TNF- α , pg/mL		Changes in TNF- α	
			H0	H3	H0 - 3	Fold
1	A	5	123.8	6966.8	6842.99	56.29
1	B	10	130.9	9044.6	8913.71	69.10
1	C	21	111.9	8861.4	8749.51	79.21
1	D	22	92.9	3506.4	3413.43	37.73
2	A	6	103.3	7007.7	6904.37	67.81
2	B	28	100.0	9731.4	9631.48	97.34
2	C	16	101.3	7647.9	7546.64	75.51
2	D	25	107.7	13876.5	13768.72	128.80
3	A	26	108.9	6895.9	6787.02	63.32
3	B	13	112.8	11704.9	11592.08	103.77
3	C	24	96.2	12149.3	12053.09	126.32
3	D	9	86.5	6326.0	6239.53	73.14
4	A	15	118.0	15288.9	15170.89	129.55
4	B	11	132.5	12938.6	12806.03	97.63
4	C	12	101.3	8831.8	8730.55	87.19
4	D	18	150.9	4506.7	4355.75	29.86
5	A	1	107.5	11283.2	11175.71	104.98
5	B	2	81.0	8405.9	8324.95	103.80
5	C	27	123.4	4379.5	4256.08	35.48
5	D	3	169.7	8468.0	8298.28	49.90
6	A	17	110.4	14295.4	14185.07	129.54
6	B	23	109.4	9458.5	9349.11	86.50
6	C	7	113.0	7102.7	6989.71	62.87
6	D	8	129.8	5862.0	5732.21	45.16
7	A	19	176.4	12643.5	12467.13	71.67
7	B	20	147.2	8814.3	8667.14	59.89
7	C	4	135.3	12532.2	12396.99	92.66
7	D	14	111.6	9028.7	8917.11	80.88

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 μ g/kg BW)

Appendix 2. Table 7. Pen means for IL-1 β and changes in IL-1 β following acute LPS challenge^a

Block	Treatment	Pen	IL-1 β , pg/mL		Changes in IL-1 β	
			H0	H3	H0 - 3	Fold
1	A	5	68.7	669.6	600.96	9.75
1	B	10	65.2	467.3	402.16	7.17
1	C	21	63.3	511.1	447.73	8.07
1	D	22	62.6	172.2	109.61	2.75
2	A	6	71.4	374.5	303.10	5.25
2	B	28	61.5	382.3	320.81	6.21
2	C	16	72.5	437.3	364.80	6.03
2	D	25	67.5	411.0	343.49	6.09
3	A	26	63.4	448.2	384.79	7.07
3	B	13	68.7	491.5	422.87	7.16
3	C	24	66.4	601.1	534.62	9.05
3	D	9	71.5	288.5	217.06	4.04
4	A	15	67.7	1139.2	1071.45	16.82
4	B	11	60.8	867.6	806.88	14.28
4	C	12	69.0	301.8	232.75	4.37
4	D	18	72.5	289.0	216.57	3.99
5	A	1	65.7	761.7	695.96	11.59
5	B	2	68.3	384.9	316.61	5.63
5	C	27	65.9	412.5	346.62	6.26
5	D	3	70.9	724.7	653.86	10.23
6	A	17	66.8	985.0	918.24	14.75
6	B	23	64.4	398.3	333.82	6.18
6	C	7	72.2	433.8	361.62	6.01
6	D	8	67.6	652.3	584.70	9.64
7	A	19	65.2	863.5	798.27	13.24
7	B	20	66.7	862.8	796.16	12.94
7	C	4	66.7	417.9	351.19	6.26
7	D	14	62.6	292.8	230.25	4.68

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 μ g/kg BW)

Appendix 2. Table 8. Pen means for SOD activity and changes in SOD activity following acute LPS challenge^a

Block	Treatment	Pen	SOD activity, U/mL		Changes in SOD activity
			H0	H3	H0 - 3
1	A	5	1.482	0.916	-0.566
1	B	10	1.198	1.124	-0.075
1	C	21	1.082	0.926	-0.155
1	D	22	1.334	0.992	-0.341
2	A	6	1.335	1.209	-0.126
2	B	28	1.288	0.878	-0.410
2	C	16	1.867	1.127	-0.740
2	D	25	1.210	1.239	0.029
3	A	26	1.221	1.037	-0.184
3	B	13	1.129	0.859	-0.270
3	C	24	1.137	1.055	-0.081
3	D	9	1.340	0.911	-0.429
4	A	15	1.338	0.899	-0.439
4	B	11	1.216	1.080	-0.136
4	C	12	1.226	1.385	0.159
4	D	18	1.214	1.243	0.030
5	A	1	1.111	0.900	-0.211
5	B	2	1.261	0.995	-0.266
5	C	27	1.127	1.010	-0.117
5	D	3	1.399	0.725	-0.674
6	A	17	1.137	1.036	-0.101
6	B	23	1.159	1.052	-0.107
6	C	7	1.405	1.017	-0.387
6	D	8	1.266	0.849	-0.417
7	A	19	1.138	0.979	-0.159
7	B	20	1.278	0.811	-0.467
7	C	4	1.223	0.690	-0.534
7	D	14	1.118	1.170	0.051

^a2 males and 2 females subjected to intraperitoneal LPS injection (O111:B4, 25 µg/kg BW)

Appendix 2. Table 9. Pen means for body weight and average daily gain subjected to multiple LPS injection, saline solution injection and no injection^a

Block	Trt	Pen	Injection	BW, kg				ADG,g				
				D21	D28	D35	D42	D21- 28	D28- 35	D35- 42	D28- 42	D21 -42
1	A	5	LPS	13.3	15.6	19.5	24.2	324	558	787	664	545
1	B	10	LPS	13.2	15.9	19.6	24.1	396	525	749	629	547
1	C	21	LPS	12.8	15.6	19.7	25.0	405	584	883	722	611
1	D	22	LPS	13.5	16.7	20.5	25.2	460	538	795	657	588
2	A	6	Saline	13.6	16.4	21.0	25.8	409	655	795	719	611
2	B	28	Saline	13.1	16.1	20.8	25.9	432	663	858	753	640
2	C	16	Saline	12.0	14.3	18.2	22.7	331	545	764	646	536
2	D	25	Saline	13.7	16.3	20.8	25.9	368	649	851	742	611
3	A	26	No injection	13.3	15.1	19.1	23.8	254	577	773	667	523
3	B	13	No injection	13.3	16.2	20.8	25.7	425	657	813	729	623
3	C	24	No injection	12.3	14.8	19.3	24.2	361	634	816	718	593
3	D	9	No injection	13.3	15.9	20.2	24.9	363	623	772	691	577
4	A	15	LPS	12.7	15.7	18.9	24.2	426	460	883	655	575
4	B	11	LPS	12.8	15.3	18.7	23.4	368	483	773	617	530
4	C	12	LPS	11.8	14.4	17.8	22.4	363	490	766	617	528
4	D	18	LPS	11.8	14.6	18.7	23.5	404	577	807	683	585
5	A	1	Saline	12.3	14.9	19.5	24.2	376	655	795	719	599
5	B	2	Saline	12.4	14.9	19.5	24.2	356	656	773	710	586
5	C	27	Saline	11.6	13.6	17.3	21.1
5	D	3	Saline	12.6	15.1	19.4	23.8	355	616	728	668	558
6	A	17	No injection	12.4	15.3	20.1	25.1	416	689	832	755	637
6	B	23	No injection	11.9	14.4	18.8	23.6	362	620	807	706	586
6	C	7	No injection	12.8	15.6	20.5	25.5	411	699	832	761	638
6	D	8	No injection	12.7	15.1	20.0	24.6	344	694	764	726	592
7	A	19	LPS	12.3	15.3	19.5	24.3	430	600	804	694	602
7	B	20	LPS	12.0	15.3	19.4	24.0	477	584	766	668	601
7	C	4	LPS	12.1	14.5	18.1	22.7	344	512	772	632	531
7	D	14	LPS	11.8	14.1	17.5	22.2	324	476	790	621	517

^aPens subjected to intramuscular LPS injections (O55:B5, 10 µg/kg BW), saline solution injections or no injections on day 28, 30, 32 and 34.

Appendix 2. Table 10. Pen means for average daily feed intake and gain to feed ratio subjected to multiple LPS injection, saline solution injection and no injection^a

Block	Trt	Pen	Injection	ADFI, g					G:F				
				D21-28	D28-35	D35-42	D28-42	D21-42	D21-28	D28-35	D35-42	D28-42	D21-42
1	A	5	LPS	607	791	1235	996	860	0.534	0.705	0.637	0.666	0.634
1	B	10	LPS	605	762	1080	909	802	0.654	0.689	0.694	0.692	0.682
1	C	21	LPS	738	749	1306	1006	911	0.549	0.780	0.676	0.718	0.671
1	D	22	LPS	715	769	1223	979	886	0.644	0.700	0.650	0.671	0.663
2	A	6	Saline	662	879	1248	1049	914	0.617	0.745	0.637	0.686	0.668
2	B	28	Saline	660	901	1304	1087	936	0.654	0.736	0.658	0.693	0.684
2	C	16	Saline	591	765	1140	938	817	0.560	0.712	0.671	0.689	0.656
2	D	25	Saline	626	900	1240	1057	903	0.588	0.721	0.686	0.702	0.677
3	A	26	No injection	546	771	1158	950	808	0.466	0.748	0.668	0.703	0.647
3	B	13	No injection	676	908	1201	1043	912	0.628	0.723	0.677	0.699	0.683
3	C	24	No injection	642	891	1186	1028	891	0.562	0.711	0.687	0.699	0.665
3	D	9	No injection	623	826	1182	990	862	0.583	0.754	0.653	0.698	0.669
4	A	15	LPS	674	699	1265	947	837	0.633	0.659	0.698	0.692	0.687
4	B	11	LPS	598	688	1072	865	772	0.616	0.702	0.722	0.713	0.687
4	C	12	LPS	610	672	1116	873	768	0.595	0.729	0.686	0.707	0.688
4	D	18	LPS	610	808	1186	983	852	0.661	0.714	0.680	0.695	0.687
5	A	1	Saline	606	870	1198	1022	876	0.620	0.753	0.663	0.704	0.684
5	B	2	Saline	646	884	1203	1031	895	0.551	0.742	0.643	0.688	0.655
5	C	27	Saline
5	D	3	Saline	616	867	1144	995	861	0.576	0.710	0.637	0.671	0.649
6	A	17	No injection	629	898	1277	1073	918	0.662	0.767	0.652	0.704	0.694

6	B	23	No injection	616	850	1168	996	862	0.587	0.729	0.691	0.709	0.679
6	C	7	No injection	656	906	1322	1098	943	0.626	0.772	0.629	0.693	0.676
6	D	8	No injection	603	896	1155	1016	871	0.570	0.775	0.661	0.715	0.680
7	A	19	LPS	662	840	1203	1008	886	0.650	0.714	0.668	0.689	0.679
7	B	20	LPS	674	825	1209	1002	887	0.708	0.707	0.634	0.666	0.677
7	C	4	LPS	570	752	1122	923	799	0.603	0.681	0.688	0.685	0.664
7	D	14	LPS	569	680	1093	869	757	0.570	0.701	0.723	0.715	0.683

^aPens subjected to intramuscular LPS injections (O55:B5, 10 µg/kg BW), saline solution injections or no injections on day 28, 30, 32 and 34.

Appendix 2. Table 11. Pen means for average daily gain and gain to feed ratio subjected to multiple LPS injection, saline solution injection and no injection without dead pig weights^a

Block	Trt	Pen	Injection	ADG without dead wts, g				G:F without dead wts			
				D21-28	D28-35	D28-42	D21-42	D21-28	D28-35	D28-42	D21-42
1	A	5	LPS	324	558	664	545	0.534	0.705	0.666	0.634
1	B	10	LPS	396	525	629	547	0.654	0.689	0.692	0.682
1	C	21	LPS	364	584	722	550	0.494	0.780	0.718	0.603
1	D	22	LPS	460	538	657	588	0.644	0.700	0.671	0.663
2	A	6	Saline	409	655	719	611	0.617	0.745	0.686	0.668
2	B	28	Saline	388	663	753	576	0.589	0.736	0.693	0.616
2	C	16	Saline	331	545	646	536	0.560	0.712	0.689	0.656
2	D	25	Saline	294	649	742	489	0.470	0.721	0.702	0.542
3	A	26	No injection	229	577	667	471	0.420	0.748	0.703	0.582
3	B	13	No injection	340	657	729	498	0.503	0.723	0.699	0.546
3	C	24	No injection	325	634	718	534	0.506	0.711	0.699	0.599
3	D	9	No injection	363	623	691	577	0.583	0.754	0.698	0.669
4	A	15	LPS	298	394	562	360	0.443	0.659	0.593	0.430
4	B	11	LPS	331	483	617	477	0.554	0.702	0.713	0.618
4	C	12	LPS	363	392	494	448	0.595	0.729	0.566	0.583
4	D	18	LPS	404	577	683	585	0.661	0.714	0.695	0.687
5	A	1	Saline	376	655	719	599	0.620	0.753	0.704	0.684
5	B	2	Saline	320	656	710	528	0.496	0.742	0.688	0.589
5	C	27	Saline
5	D	3	Saline	315	616	668	496	0.512	0.710	0.671	0.577
6	A	17	No injection	370	689	755	566	0.588	0.767	0.704	0.617
6	B	23	No injection	326	620	706	527	0.528	0.729	0.709	0.611
6	C	7	No injection	411	699	761	638	0.626	0.772	0.693	0.676
6	D	8	No injection	344	694	726	592	0.570	0.775	0.715	0.680
7	A	19	LPS	382	600	694	535	0.577	0.714	0.689	0.603
7	B	20	LPS	381	584	668	481	0.566	0.707	0.666	0.542
7	C	4	LPS	344	512	632	531	0.603	0.681	0.685	0.664
7	D	14	LPS	324	429	559	477	0.570	0.701	0.644	0.630

^aPens subjected to intramuscular LPS injections (O55:B5, 10 µg/kg BW), saline solution injections or no injections on day 28, 30, 32 and 34.

Appendix 2. Table 12. Pen means for average daily gain and gain to feed ratio subjected to multiple LPS injection, saline solution injection and no injection with dead pig weights^a

Block	Trt	Pen	Injection	ADG with dead wts, g				G:F with dead wts			
				D21-28	D28-35	D28-42	D21-42	D21-28	D28-35	D28-42	D21-42
1	A	5	LPS	324	558	664	545	0.534	0.705	0.666	0.634
1	B	10	LPS	396	525	629	547	0.654	0.689	0.692	0.682
1	C	21	LPS	382	584	722	602	0.518	0.780	0.718	0.661
1	D	22	LPS	460	538	657	588	0.644	0.700	0.671	0.663
2	A	6	Saline	409	655	719	611	0.617	0.745	0.686	0.668
2	B	28	Saline	406	663	753	630	0.615	0.736	0.693	0.673
2	C	16	Saline	331	545	646	536	0.560	0.712	0.689	0.656
2	D	25	Saline	385	649	742	607	0.615	0.721	0.702	0.681
3	A	26	No injection	261	577	667	525	0.478	0.748	0.703	0.650
3	B	13	No injection	403	657	729	612	0.596	0.723	0.699	0.671
3	C	24	No injection	393	634	718	603	0.612	0.711	0.699	0.677
3	D	9	No injection	363	623	691	577	0.583	0.754	0.698	0.669
4	A	15	LPS	442	459	645	501	0.656	0.657	0.681	0.673
4	B	11	LPS	357	483	617	526	0.596	0.702	0.713	0.681
4	C	12	LPS	363	440	588	457	0.595	0.655	0.673	0.649
4	D	18	LPS	404	577	683	585	0.661	0.714	0.695	0.687
5	A	1	Saline	376	655	719	599	0.620	0.753	0.704	0.684
5	B	2	Saline	406	656	710	602	0.628	0.742	0.688	0.673
5	C	27	Saline
5	D	3	Saline	383	616	668	567	0.622	0.710	0.671	0.659
6	A	17	No injection	392	689	755	628	0.624	0.767	0.704	0.685
6	B	23	No injection	377	620	706	590	0.611	0.729	0.709	0.684
6	C	7	No injection	411	699	761	638	0.626	0.772	0.693	0.676
6	D	8	No injection	344	694	726	592	0.570	0.775	0.715	0.680
7	A	19	LPS	424	600	694	600	0.641	0.714	0.689	0.676
7	B	20	LPS	464	584	668	596	0.688	0.707	0.666	0.672
7	C	4	LPS	344	512	632	531	0.603	0.681	0.685	0.664
7	D	14	LPS	324	497	631	496	0.570	0.732	0.727	0.683

^aPens subjected to intramuscular LPS injections (O55:B5, 10 µg/kg BW), saline solution injections or no injections on day 28, 30, 32 and 34.

Appendix 2. Table 13. Mortality in pens subjected to multiple LPS injection, saline solution injection and no injection^a

Block	Treatment	Pen	Injection	D21-28	D28-35	D35-42	D28-42	D21-42
1	A	5	LPS	0	0	0	0	0
1	B	10	LPS	0	0	0	0	0
1	C	21	LPS	1	0	0	0	1
1	D	22	LPS	0	0	0	0	0
2	A	6	Saline	0	0	0	0	0
2	B	28	Saline	1	0	0	0	1
2	C	16	Saline	0	0	0	0	0
2	D	25	Saline	2	0	0	0	2
3	A	26	No injection	1	0	0	0	1
3	B	13	No injection	2	0	0	0	2
3	C	24	No injection	1	0	0	0	1
3	D	9	No injection	0	0	0	0	0
4	A	15	LPS	3	1	0	1	4
4	B	11	LPS	1	0	0	0	1
4	C	12	LPS	0	2	0	2	2
4	D	18	LPS	0	0	0	0	0
5	A	1	Saline	0	0	0	0	0
5	B	2	Saline	1	0	0	0	1
5	C	27	Saline
5	D	3	Saline	1	0	0	0	1
6	A	17	No injection	1	0	0	0	1
6	B	23	No injection	1	0	0	0	1
6	C	7	No injection	0	0	0	0	0
6	D	8	No injection	0	0	0	0	0
7	A	19	LPS	1	0	0	0	1
7	B	20	LPS	2	0	0	0	2
7	C	4	LPS	0	0	0	0	0
7	D	14	LPS	0	1	0	1	1

^aPens subjected to intramuscular LPS injections (O55:B5, 10 µg/kg BW), saline solution injections or no injections on day 28, 30, 32 and 34.

Appendix 2. Table 14. Pen means for TNF- α and changes in TNF- α following chronic immune challenge^a

Block	Treatment	Pen	TNF- α , pg/ mL		Changes in TNF- α
			Hr. 0	Hr. 3	Fold
1	A	5	296.2	1605.9	5.42
1	B	10	151.1	891.1	5.90
1	C	21	172.4	917.9	5.32
1	D	22	220.0	220.0	0.00
4	A	15	234.6	1023.3	4.36
4	B	11	236.5	640.1	2.71
4	C	12	163.6	703.3	4.30
4	D	18	139.9	944.8	6.75
7	A	19	133.4	720.6	5.40
7	B	20	244.2	1381.7	5.66
7	C	4	193.8	877.7	4.53
7	D	14	203.2	580.7	2.86

^aBlood collected from 2 pigs per pen subjected to IM LPS injection (O55:B5, 10 μ g/kg BW) on days 28, 30, 32 and 34.

Appendix 2. Table 15. Pen means for IL-1 β and changes in IL-1 β following chronic immune challenge^a

Block	Treatment	Pen	IL-1 β , pg/ mL		Changes in IL-1 β
			H0	H3	Fold
1	A	5	60.9	132.2	2.17
1	B	10	49.6	79.8	1.61
1	C	21	53.0	139.3	2.63
1	D	22	56.7	111.4	1.97
4	A	15	59.2	89.1	1.50
4	B	11	52.2	76.1	1.46
4	C	12	51.6	83.4	1.62
4	D	18	53.9	93.6	1.74
7	A	19	49.1	154.8	3.15
7	B	20	51.3	136.2	2.65
7	C	4	53.9	85.7	1.59
7	D	14	50.5	77.2	1.53

^aBlood collected from 2 pigs per pen subjected to IM LPS injection (O55:B5, 10 μ g/kg BW) on days 28, 30, 32 and 34.

Appendix 2. Table 16. Pen means for SOD activity and changes in SOD activity following chronic immune challenge^a

Block	Treatment	Pen	SOD activity, U/mL		Changes in SOD activity
			H0	H3	H0 - 3
1	A	5	0.950	0.971	0.021
1	B	10	0.853	0.770	-0.083
1	C	21	0.936	0.985	0.049
1	D	22	1.109	1.036	-0.073
4	A	15	0.998	0.834	-0.164
4	B	11	1.467	1.118	-0.349
4	C	12	0.935	1.017	0.083
4	D	18	1.048	1.301	0.254
7	A	19	1.000	0.956	-0.044
7	B	20	0.918	1.039	0.122
7	C	4	0.829	0.955	0.126
7	D	14	0.979	0.936	-0.043

^aBlood collected from 2 pigs per pen subjected to IM LPS injection (O55:B5, 10 µg/kg BW) on days 28, 30, 32 and 34.

Appendix 3

Pilot Study

Pilot Study: Multiple intramuscular lipopolysaccharide injections and the effect on body weight, body temperature, and survival rate of nursery pigs.

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Abstract

One experiment was conducted to evaluate the effect of multiple intramuscular injections of lipopolysaccharide on body weight, body temperature, and survival rate of nursery pigs. A total of 10 pigs (average of 20 d old) were allotted in 1 of 2 treatments: LPS injection or control. On d 28, 30, 32 and 34, 5 pigs were injected intramuscularly with LPS *Escherichia coli* O55:B5 (Sigma-Aldrich, Co., St. Louis, MO). After the first injection on d 28, the amount of LPS was increased by 12% at each injection to avoid resistance. Previously to each injection, BW and rectal temperature were recorded. Body temperature was also recorded 3 h following each LPS administration. Pigs were fed the same diet and allowed to consume feed and water *ad libitum* during the study. The results demonstrated BW loss following the first LPS injection and decreased ADG during the subsequent days of LPS injection. Rectal temperatures increased substantially following each of the injections indicating that the immune system

was consistently activated. No mortality was observed during the study.

Therefore, multiple intramuscular injections of 10 µg of LPS/ kg of body weight is an adequate amount to stimulate the immune system without compromising survival rate in nursery pigs. Additionally, increasing amounts of LPS (12% at each injection) is able to avoid resistance.

Introduction

Single injections of LPS has been demonstrated to be a safe method to stimulate the acute immune response in pigs (Wyns et al., 2015). To study the relationship between chronic immune system stimulation and nutrient level, a model that represents chronic stimulation is needed. This model can be used to evaluate potential nutritional strategies to alleviate the impact of immune system stimulation on animals (Escribano et al., 2014; Rakhshandeh & de Lange, 2012). However, few studies have used multiple injections of LPS to evaluate chronic immune response in pigs (Rakhshandeh & de Lange, 2012). More than that, there is no published data related to this methodology in nursery pigs. Therefore, the objective of this pilot study was to evaluate the effect of multiple intramuscular injections of lipopolysaccharide on body weight, body temperature, and survival rate of nursery pigs.

Materials and Methods

Animal Care and Feeding

A total of ten crossbred (PIC®) pigs (6 barrows and 4 gilts) were weaned at average of 20 d of age and allotted randomly to one of two treatments. The pigs were handled and cared according to the guidelines established by the Oklahoma State University Institutional Animal Care and Use Committee.

Pigs were blocked randomly by body weight (BW) and gender and housed in pens in a proportion of 3 barrows to 2 gilts, totalizing 5 pigs per pen. Pigs were housed at the wean-to-finish facility with the environment temperature and ventilation controlled and checked twice a day. During the trial, all pigs received the same diet formulated to exceed the requirements listed in the NRC (2012). The pigs were allowed to consume feed and water *ad libitum*. Water was provided via water nipple and each pen had a multiple-hole stainless steel feeder. Body weight (BW) and average daily gain (ADG) were calculated in this study.

Escherichia coli Lipopolysaccharide Challenge

On d 28, 30, 32 and 34 of the experiment, 5 pigs were subjected to intramuscular lipopolysaccharide (LPS) injection, while the remaining 5 pigs served as the control. The *Escherichia coli* O55:B5 LPS (Sigma-Aldrich, Co., St. Louis, MO) was suspended in a 9 g/L of sterile saline for a final dosage of 10 µg of LPS/ kg of body weight. Following the first injection on d 28, the subsequent amount of LPS was increased by 12% to avoid resistance (Rakhshandeh & de Lange, 2012). The suspension was kept in cold storage.

Prior to each injection, pigs were weighed, and their temperature was recorded. The injections were performed intramuscularly in the neck with the weight-dependent LPS suspension. Rectal temperatures was recorded again 3 h post injection. Body weight was also recorded on d 35 of the study. Changes in rectal temperature and percentage of body weight were calculated using h 0 from d 28 as a baseline.

Statistical Analysis

No statistical analysis were performed for this study. The results were interpreted numerically according to observations for each pig subjected to LPS treatment or control.

Results

The results are presented in Table.1 and 2. Pigs injected with LPS lost 0.43 kg 48 h following the first LPS injection, while the control group gained 0.53 kg. The subsequent injections did not result in BW loss, although pigs subjected to multiple LPS injections had decreased ADG compared to the control group. On d 35, 24 hours after the last LPS injection, pigs that received LPS gained 2.88 kg while control pigs gained 3.77 kg, resulting in a numerical difference of 0.128 g in ADG between treatment groups. Rectal temperatures increased in LPS injected pigs following each of the injections compared to the control. Although sickness behaviors were not evaluated, lethargy and shivering were observed right after the LPS injection. There was no mortality during the study.

Discussion

The BW loss and the increment in body temperature agrees with observations made in previous studies (Bible, 2013; Johnson & Von Borell, 1994; Rakhshandeh & de Lange, 2012; Wright et al., 2000), demonstrating that the protocol established in this study, in fact, stimulated the immune system without compromising the survival rate.

The worsening in the ADG compared to the control group can be explained by the action of the LPS. According to (Johnson et al., 1996; Warren et al., 1997) lipopolysaccharide activates the hypothalamus-pituitary-adrenal (HPA) axis via proinflammatory cytokine stimulation, resulting in increased secretions of glucocorticoids and reduced response to growth hormone (Luo & Murphy, 1989). Moreover, glucocorticoids increase energy expenditure, muscle protein degradation and loss of body nitrogen (Williams et al., 2009). Therefore, the activation of the immune system redirect the nutrients to support its function rather than to growth (Spurlock, 1997), which impairs nutrient utilization and performance (Johnson, 1997; Liu et al., 2003).

Conclusion

Multiple intramuscular injections of 10 µg of LPS/ kg of body weight of LPS *Escherichia coli* O55:B5 seems to be an adequate amount to stimulate the immune system without compromising survival rate in nurse piglets. Additionally, increasing amounts of LPS (12% at each injection) is able to avoid resistance.

Appendix 3. Table 1. Effect of multiple LPS injections on body weight of nursery pigs^{a,b,c}

Pig	Trt.	Day 28	Day 30	Day 32	Day 34	Day 35
		Body Weight	Body Weight	BW change	Body Weight	BW change
1	L	15.1	15.0	-0.09	16.5	1.45
2	L	13.8	14.4	0.54	15.6	1.73
3	L	15.3	15.1	-0.27	16.3	0.95
4	L	14.5	13.8	-0.73	15.0	0.50
5	L	19.9	18.3	-1.59	20.4	0.54
Average		15.7	15.3	-0.43	16.8	1.04
6	C	12.1	12.7	0.54	14.4	2.27
7	C	12.6	13.2	0.64	14.9	2.36
8	C	21.9	22.4	0.50	23.4	1.45
9	C	13.2	13.5	0.27	14.7	1.50
10	C	10.7	11.4	0.68	12.4	1.73
Average		14.1	14.6	0.53	16.0	1.86

^aLPS *Escherichia coli* O55:B5, 10 µg/kg of BW.

^bBW variation from day 28.

^cBody weight recorded prior (hour 0) to the LPS injection.

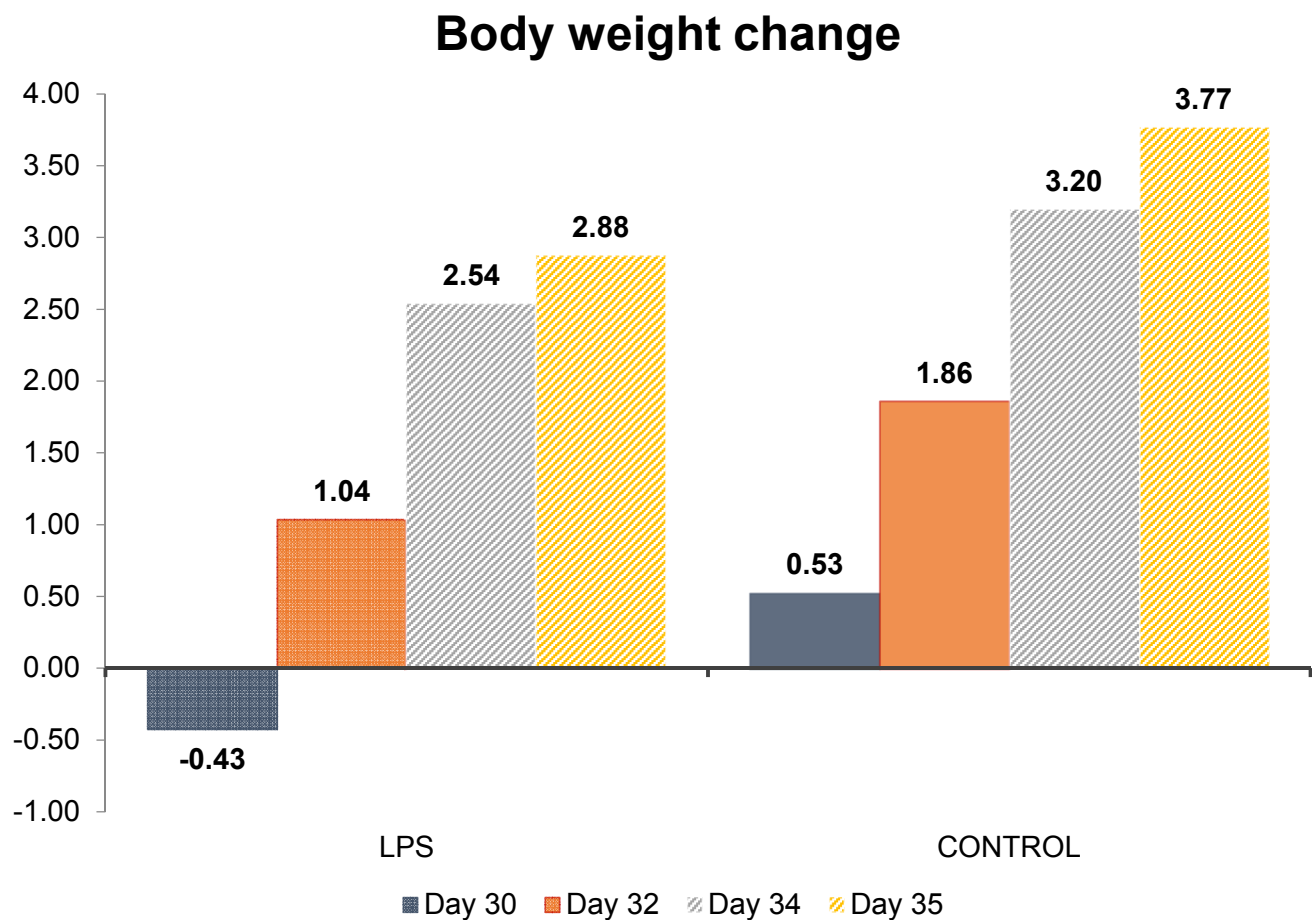
Appendix 3. Table 2. Effect of multiple LPS injections on rectal temperature of nursery pigs^{a,b,c}

Pig	Trt.	Temp.	Day 30 ^c		Temp.	Day 32		Temp.	Day 34	
			Temp. 3h	Temp. change		Temp. 3h	Temp. change		Temp. 3h	Temp. change
1	L	39.2	40.2	0.94	39.6	40.6	1.06	39.4	40.9	1.50
2	L	39.5	40.7	1.22	39.3	40.1	0.83	39.1	39.9	0.89
3	L	39.4	40.8	1.39	38.9	39.8	0.83	39.2	40.1	0.89
4	L	39.5	41.2	1.72	39.6	39.8	0.22	39.2	40.1	0.89
5	L	39.4	40.2	0.78	39.4	40.4	1.00	39.2	39.4	0.17
Average		39.4	40.6	1.21	39.3	40.1	0.79	39.2	40.1	0.87
6	C	0.0	0.0	0.00	39.4	39.6	0.17	39.1	38.8	-0.33
7	C	0.0	0.0	0.00	38.9	39.3	0.39	38.8	39.1	0.28
8	C	0.0	0.0	0.00	39.3	39.7	0.33	39.3	39.1	-0.17
9	C	0.0	0.0	0.00	39.7	39.6	-0.11	39.6	39.2	-0.39
10	C	0.0	0.0	0.00	39.9	40.1	0.17	39.2	39.1	-0.06
Average		0.0	0.0	0.00	39.4	39.6	0.19	39.2	39.1	-0.13

^aLPS *Escherichia coli* O55:B5, 10 µg/kg of BW.

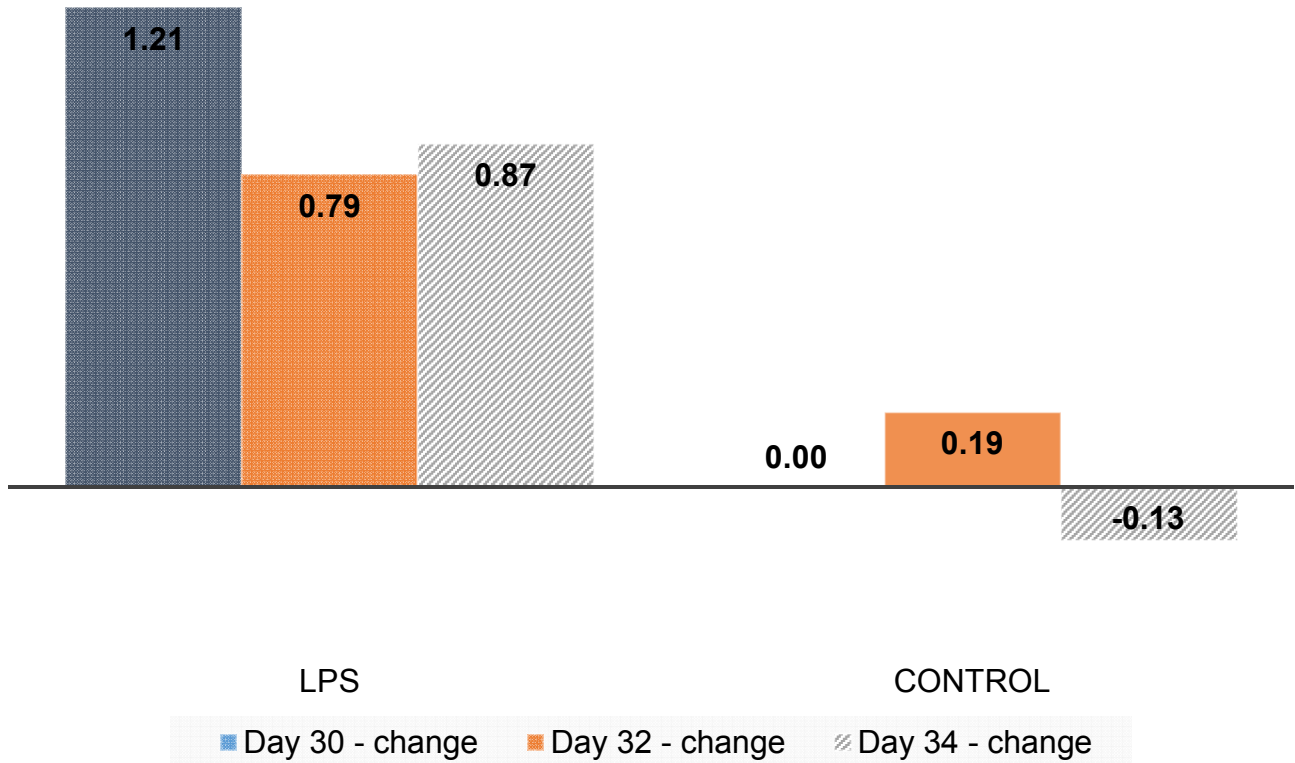
^bRectal temperature recorded prior (hour 0) and 3 hours following the LPS injection.

^cRectal temperature of the control group was not recorded on day 30.



Appendix 3. Figure 1. Effects of multiple lipopolysaccharide injections on body weight change of nursery pigs between days 28 and 35. The serotype of LPS was *Escherichia coli* O55:B5.

Rectal temperature change



Appendix 3. Figure 2. Effects of multiple lipopolysaccharide injections on rectal temperature change of nursery pigs between days 28 and 35. The serotype of LPS was *Escherichia coli* O55:B5.

VITA

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Master of Science

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