January 2015

INVESTIGATION OF ACUTE STRESS IMPACT ON NURSERY PIG GASTROINTESTINAL FUNCTION AND ABILITY OF BIOACTIVE COMPONENTS OF GARLIC TO MITIGATE STRESS-INDUCED PHYSIOLOGICAL CHANGES

Nathan Lane Horn
Purdue University

Graduate School Form 30
Updated 1/15/2015

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Nathan L. Horn

Entitled
Investigation of acute stress impact on nursery pig gastrointestinal function and ability of bioactive components of garlic to mitigate stress-induced physiological changes

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

Olayiwola Adeola
Chair

Kolapo Ajuwon

Frank Ruch

Susan Eicher

To the best of my knowledge and as understood by the student in the Thesis/Dissertation Agreement, Publication Delay, and Certification Disclaimer (Graduate School Form 32), this thesis/dissertation adheres to the provisions of Purdue University's "Policy of Integrity in Research" and the use of copyright material.

Approved by Major Professor(s): Olayiwola Adeola

Approved by: Ryan Cabot 12/4/2015
Head of the Departmental Graduate Program Date

INVESTIGATION OF ACUTE STRESS IMPACT ON NURSERY PIG GASTROINTESTINAL FUNCTION AND ABILITY OF BIOACTIVE COMPONENTS OF GARLIC TO MITIGATE STRESS-INDUCED PHYSIOLOGICAL CHANGES

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Nathan Horn

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

December 2015

Purdue University

West Lafayette, Indiana

For my wife Mandy and daughter Adeline

ACKNOWLEDGEMENTS

The author would like to express appreciation to all students, staff, and faculty at Purdue University who may have assisted with studies presented in this document. Further appreciation is given to all employees at JBS United, Inc. and Biomatrix International that may have contributed to dissertation research efforts.

The author would specifically like to express gratitude to the industry partners that helped make this research possible. First, Dr. Guy Miller for his encouragement, guidance, and friendship over the last several years. Appreciation beyond words is also given to management at JBS United, Inc., specifically, Dr. Ron Moser, Dr. Doug Webel, and John Swisher for their support and encouragement over the last four years.

Gratitude is given to Dr. Layi Adeola, director of this dissertation, for his professional and personal guidance, mentoring, and support throughout the author's academic career. The author would also like to show appreciation to Dr. Kola Ajuwon for opening up his lab and for his mentorship. Appreciation is also extended to other advising committee members Dr. Frank Ruch and Dr. Susan Eicher for their contributions. Although, countless Purdue staff members have helped the author along his journey, a few stand out and include: Pat Jaynes, Jason Fields, Pat Jonas, and Marlene Miller. The author has had the great privilege of working with several undergraduate students, graduate students, and other scholars while at Purdue. Many made contributions

to the research presented within this document, but special appreciation is given to: Dr. Tayo Adedokun, Katherine McCormick, Hang Lu, Hui Yan, and Ali Gautier.

The author also wishes to expression appreciation to his wife, Mandy. Her patience and support allowed for this dissertation to be possible. The kindness and humor offered by the author's daughter Adeline allowed for him to stay focused and kept him motivated.

Most importantly, thanks be to God.

TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

Table Page

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

Horn, Nathan L. Ph.D., Purdue University, December 2015. Investigation of acute stress impact on nursery pig gastrointestinal function and ability of bioactive components of garlic to mitigate stress-induced physiological effects. Major Professor: Olayiwola Adeola.

Experiments were conducted to determine the effect of post-weaning feed and water deprivation on nursery pig growth performance, gastrointestinal function, and ability of garlic-derived diallyl disulfide (DADS) and diallyl trisulfide (DATS) to mitigate deprivation-induced effects. For the first experiment, the effects of a 24-h post-weaning feed, water, or feed + water deprivation event on nursery pig growth and intestinal characteristics were determined. Water deprivation more severely impacted nursery pig growth and intestinal measurements compared to feed deprivation. The water deprivation event resulted in an increase in serum stress markers and altered intestinal morphology and tight junction gene expression during the first week post-weaning. Furthermore, the acute post-weaning deprivation event impacted growth performance throughout the nursery period and resulted in pigs 0.57 kg lighter at 28 d post-weaning. A second experiment examined the interaction between a 24-h post-weaning feed + water deprivation event and a subsequent cyclic heat stress event. The results showed that the feed + water deprivation event reduced growth performance, increased serum stress markers, decreased ileal similar to the first experiment. Growth performance

and intestinal tight junction gene expression were decreased during the heat stress period. Growth performance results showed a stress event interaction in which nursery pig performance was poorest in pigs exposed to both stress events. Next, an in-vitro experiment was conducted to determine if garlic-derived DADS + DATS could mitigate hydrogen peroxide- and LPS-induced oxidant and endotoxin stress, respectively, in porcine epithelial (IPEC-J2) cells. Results showed that the garlic-derived compounds could mitigate oxidative stress by increasing superoxide dismutase and catalase activity. Furthermore, DADS + DATS were immune modulatory and augmented the LPS-induced increase in interleukin 8 (IL-8) secretion. Following the in-vitro evaluation, two in-vivo pilot trials were conducted to identify the optimal dosage of DADS + DATS and to evaluate the effect of graded doses of DADS + DATS on nursery pig and broiler chicken performance and gastrointestinal function. Garlic-derived DADS + DATS were supplemented to pigs and chickens by daily oral gavage for a period of 6 d. The oral gavage of DATS + DATS did not impact nursery pig growth performance, although ileal villus height was increased. Furthermore, there was a linear increase in IL-8 and a decrease in zonula occludens 1 (ZO-1) ileal gene expression due to oral DADS + DATS administration. The optimal dosage of DATS + DATS to maximize ileal villus height was determined to be 1.7 mg per kg BW. For the broiler chicken trial, DADS + DATS supplementation by oral gavage improved BW gain, ileal morphology,

and digestibility of DM, N, and E. The average optimal oral dose of DADS + DATS to optimize BW gain and villus height in broiler chickens was 2.5 mg DADS + DATS per kg BW. In the final experiment, the ability of a daily oral gavage of DADS + DATS to mitigate effects of a post-weaning feed + water deprivation event in nursery pigs was investigated. The post-weaning feed + water deprivation event reduced growth performance, reduced ileal villus height, decreased activity of mucosal superoxide dismutase, and decreased expression of occludin and ZO-1 tight junction genes in the ileum. Oral supplementation of DADS + DATS partially mitigated the effects of the feed + water deprivation event on ileal villus height and superoxide dismutase activity. In conclusion, the overall results from these studies showed that post-weaning stress events have short- and long-term implications on nursery pig growth performance and intestinal characteristics. Additionally, garlic-derived DADS + DATS impact epithelial cell oxidative and immune status, improve pig and chicken ileal morphology, and can partially mitigate effects of an acute post-weaning feed + water deprivation event.

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Following weaning pigs are exposed to environmental, nutritional, and psychological stressors that have short- and long-term implications on gastrointestinal function, health, and growth performance (Lalles, 2004). During the first week post-weaning poor feed intake and lethargy lead to atrophy of intestinal architecture which leads to a drastic decrease in brush border enzyme activity (Pluske et al., 1997). Furthermore, information from the literature shows that ability to produce pancreatic and brush border enzymes is not fully developed until 3 to 4 week of age (Pluske et al., 1997). Post-weaning intestinal damage along with limited intestinal enzyme function in young pigs leads to poor nutrient utilization, which is a causative factor of post-weaning diarrhea. Recent information from the literature shows that nursery pigs may be prone to over-stimulation of the mucosal immune system (Pie et al., 2004) and weaning-associated stress hormone spikes are linked to activation of inflammatory mediators (Moeser et al., 2007; Smith et al., 2010). Additionally, growing and finishing pigs are susceptible to heat stress which also impacts gastrointestinal integrity and growth and may be exacerbated by post-weaning stress events (Hyun et al., 1998).

Recent evidence shows that weaning-associated gastrointestinal dysfunction is related to breakdown of epithelial cell tight junction proteins (Overman et al., 2012). Taken altogether, a nursery pig weaned at 18 to 28 d of age is vulnerable to intestinal barrier breakdown which has long-term implications on health and performance.

Gastrointestinal integrity can be assessed in-vivo by measurement of urinary oligosaccharide excretion, serum endotoxin level, and tight junction gene and protein expression (Lambert, 2009). Furthermore, ex-vivo and in-vitro tools allow assessment of gastrointestinal permeability by determining passage rate of an electric charge (trans-epithelial electrical resistance (TEER)) or fluorescently-labeled oligosaccharide probes such as FITC-dextran. Intestinal porcine epithelial cells (IPEC) serve as a viable cell culture model to study porcine gastrointestinal integrity due to their in-vivo like expression of tight junctions and immune components (Geens and Niewold, 2011). Consequently, IPEC cells serve as a screening tool for nutritional products that may mitigate gastrointestinal dysfunction by altering cellular immunological function or oxidative status (Brosnahan and Brown, 2012).

Nutritional strategies to mitigate post-weaning gastrointestinal dysfunction have been extensively studied and include dietary supplementation of milk-based products, plasma protein, probiotics, and prebiotics, and plant-based compounds (Lalles et al., 2007). Historically, supplementation of plant-based compounds to enhance gastrointestinal dysfunction has been overlooked due to a high variability of efficacy reported in the literature (Windisch et al., 2008). Garlic-derived bioactive compounds have been shown to have antimicrobial, antioxidant, and immune modulatory properties using in-vitro and rodent models (Amagase, 2006). Additionally, recent evidence

shows that crude garlic extracts enhance intestinal morphology, immune function, and nutrient utilization in young pigs and broiler chickens (Tatara et al., 2008; Haung et al., 2011; Olukosi and Dono, 2014). Therefore, supplementation of garlic-derived compounds may be a nutritional option to alleviate post-weaning gastrointestinal dysfunction.

The objective of studies presented in this dissertation include: first, to determine the effect of an acute post-weaning feed and water deprivation event on nursery pig performance, intestinal morphology, and mucosa gene expression. The second objective was to determine if a relationship exists between post-weaning feed and water deprivation and exposure to a subsequent heat stress event. The third objective was to use IPEC-J2 cells as an in-vitro model to determine if garlic-derived diallyl disulfide (DADS) + diallyl trisulfide (DATS) could mitigate oxidant- and endotoxin-induced effects. The fourth objective was to identify an optimal oral dose of DADS + DATS in nursery pigs and broiler chickens based on growth performance, ileal morphology, and mucosa gene expression measurements. Lastly, the fifth objective was to determine if garlic-derived DADS + DATS could mitigate the effects of a post-weaning feed and water deprivation event in pigs.

1.2 Digestive physiology of the weaned pig

The digestive tract is the entryway of nutrients into the body and also serves as a protective barrier. The mouth, pharynx, esophagus, stomach, small and large intestines, cecum, colon, and rectum make up the pig's digestive tract. Furthermore, the accessory

digestive glands (salivary glands, liver, and pancreas) serve a critical role in providing secretions that aid in nutrient digestion and absorption. At weaning age (18 to 28 d of age) drastic changes occur to digestive physiology and there is a critical link between nursery pig digestive physiology, health, and growth efficiency (Yen, 2001).

Starting proximally from the mouth, food enters the oral cavity where it is briefly chewed and mixed with saliva that is mainly secreted from the parotid, mandibular, and sublingual salivary glands (Yen, 2001). Salivary secretions are important for food lubrication and contain amylase, although information in the literature shows that carbohydrate digestion due to salivary amylase is insignificant (Corring ,1980). After quick passage through the esophagus, the chyme enters the stomach which can be divided into four distinct compartments including the esophageal, cardiac, fundic, and pyloric regions. Secretory cells in the fundic region include goblet, parietal, and chief cells, which secrete mucin, hydrochloric acid, and protease zymogens, respectively (Yen, 2001). Gastric hydrochloric acid secretion, which is secreted following neural and physical stimulus, activates gastric pepsinogen to pepsin and thus protein digestion is initiated (Foltman et al., 1995). Subsequently, chyme enters the proximal small intestine where it encounters accessory organ secretions, including bile from the liver and pancreatic juice from the acinar regions of the exocrine pancreas (Yen, 2001). Briefly, the small intestine comprises of the duodenum, jejunum, and ileum which make up approximately 5, 90, and 4 % of the total length of an adult pig, respectively (Yen, 2001). Differentiation between the various segments of the small intestine of an adult pig can be made by morphological examination (villus height increases from the duodenum to the distal jejunum then decreases into the ileum),

vascularization (highest in the jejunum), and presence of peyer's patches (highest concentration in the distal ileum) (Yen, 2001). However, in the post-weaning pig it is difficult to distinguish between the jejunum and ileum based on morphologic differences alone. The wall of the small intestine includes the mucosa, submucosa, muscularis, and serosa layers (Yen, 2001). Furthermore, the mucosa layer can be divided into the muscularis mucosa that includes smooth longitudinal muscle; the lamina propria which contains lymph tissues, blood vessels, and neurons; and the epithelial layer which contains secretory and absorptive cells (Yen, 2001). Specifically, the epithelial layer contains finger-like villus structures which are made up of three types of cells: absorptive enterocytes, goblet cells, and enteroendocrine cells. Goblet cells secrete mucin which is a major constituent of the mucus layer. Mucus acts as a barrier in the gastrointestinal tract, protecting the gut wall from digestive enzymes, pathogens, and acidic chyme present in the gut lumen. In terms of nutrient utilization, mucus lubricates digestive matter, allowing small nutrient particles in close proximity of enterocytes. Cells migrate from the villus crypt to the apical portion of the structure where they are sloughed with cell turnover rate being 3 to 4 d for an adult pig (Yen, 2001). As enterocytes migrate apically, cellular differentiation occurs and most nutrient absorption occurs in enterocytes present in the apical-half of intestinal villi. Secretion of bile and pancreatic juice into the duodenum initiate luminal nutrient digestion. Bile contains bile salts that are conjugated with glycine or taurine and are involved in fat emulsification and bicarbonate which acts to buffer acidic chyme and allows for proper enzyme activity. Briefly, pancreatic juice contains amylase and lipase that are critical for luminal digestion of carbohydrates and lipids, respectively, and protease zymogens

A and B. Trypsinogen is activated by mucosal enterokinase and subsequently trypsin activates the remaining protease zymogens. Following luminal digestion, brush border enzymes digest oligosaccharides to monosaccrides and peptides to di- and tri-peptides and free AA as extensive reviewed by Yen (2001). Various passive, active, and facilitated nutrient transporters exist on the apical and basolateral surface of epithelial cells which allow nutrient transfer from the luminal brush border membrane to vascular systems, tissues, and organs. Furthermore, following emulsification by bile salts, lipids are broken down to monoglycerides and fatty acids and pass through the lumen to the brush border membrane (Argenzio, 1993). Following diffusion across the apical membrane of the enterocytes triglycerides then chymolomicrons are formed and transported into the lymph (Herdt, 1992). The cecum and large intestine make up to 30 to 60 % of the total intestinal tract and intestinal contents reside in the large intestine of an adult pig for about 20 h compared to 2 to 6 h in the small intestine (Low and Zebrowska, 1989). Significant microbial fermentation of carbohydrates and proteins takes place in the large intestine resulting in production of volatile fatty acids (VFA). Although little carbohydrate, lipid, or AA absorption takes place in the large intestine, VFA can be absorbed, and can serve as an energy source. Furthermore, the large intestine serves a critical role in passive and active reabsorption of water and electrolytes, respectively (Yen, 2001).

Furthermore, Hampson et al. (1986) reported that weaning-associated intestinal morphology changes do not start to recover until 8 d post-weaning. In severe cases of weaning-associated anorexia Hall and Byrne (1989) showed that crypt cell proliferation was also decreased that post-weaning nutrient malabsorption is more closely linked

with changes in intestinal architecture rather than age-dependent changes in brush border enzymes. This hypothesis is supported by meta-analysis conducted by Pluske et al. (1997) that showed a direction relationship between villus height and brush border enzyme activity. Weaning-associated stress and nutritional changes have also been shown to induce changes in digestive enzymes. Sanglid et a al. (1994) showed that a weaning-associated corticosteroid spike increase pancreatic amylase and trypsin secretion whereas Makkink et al. (1994) showed protein and fat level and source can increase pancreatic protease and lipase secretion. With that being said, information from the literature shows pig age impacts production of digestive enzymes independent of weaning. Moughan et al. (1992) showed that gastric pepsinogen production increased at 3 to 4 wk of age and Cranwell (1995) showed that pancreatic trypsin and elastase activity increased at 4 to 6 wk of age. Furthermore, Klobasa et al. (1987) showed pancreatic lipase activity was not maximized until 4 week of age and Zhang et al. (1997) showed that little to no sucrase or maltase activity exists until 16 to 21 d of age. Specific to lactase activity, Zhang et al. (1997) showed brush border lactase activity increases from birth to 14 d of age and then decreases with minimal intestinal lactase activity by 40 d of age. Conflicting evidence exists for the impact of age and weaning on mucin dynamics in pigs (Lalles, 2004). Dunsford et al. (1990) reported an increase in goblet cell density from birth to 5 wk of age. Pestova et al. (2000) reported an increase in intestinal mucin although Van der Meulen et al. (2003) was not able to confirm those results. Information in the literature shows that pig age and weaning-associated anorexia can influence physiological factors affecting nutrient utilization that may be related to post-weaning diarrhea, gut dysfunction, and increased susceptibility to enteric

disease (Pluske et al., 1997). Therefore, nutritional and management strategies should be developed to accommodate the aforementioned changes in digestive physiology.

1.3. Mucosal immunity

The mucosal immune system comprises all mucus-lined surfaces and acts as the first point of interaction between the immune system and gut lumen contents. Additionally, proper mucosal immune function plays a critical role in protective immunity, immune tolerance, and gastrointestinal function. In the intestine, the mucosal immune system consists of organized lymphoid tissues (Peyer's patches) and unorganized lymphoid cells that mainly include dendritic cells, macrophages, and cytotoxic T cells present in the intraepithelial space or lamina propria (Murphy, 2012). Although the mucosal immune system contains a unique subset of immune cells, all mucosal lymphoid cells drain through the lymphatic system to mesenteric lymph nodes and thus are in communication with primary and secondary immune tissue (Murphy, 2012). Peyer's patches are organized lymphoid tissues that are present in the lamina propria and increase in concentration distally in the small intestine of swine (Burkey et al., 2009). Peyer's patches form a sub-epithelial dome, which can be distinguished by morphological examination, and contain specialized microfold (M) cells at their apical surface. The M cells survey intestinal luminal contents by pinocytosis and can be morphologically characterized by lack of microvilli and a thin mucus layer (Murphy, 2012). Subsequent to M cell pinocytosis luminal contents are presented to a unique sub-set of dendritic cells that reside at the basolateral surface of M cells. Activation of

dendritic cells by non-pathogenic bacteria leads to activation of B cells to secrete IgA and IL-10 which is hypothesized to suppress activation of inflammatory immune pathways (Burkey et al., 2009; Murphy, 2012). Consequently, commensal bacteria have been shown to play a critical role in IgA secretion into the gut lumen and maintenance of gastrointestinal homeostasis through immune mechanisms (Mcpherson, 2008). Furthermore, another unique sub-set of dendritic cells reside in the lamina propria and intracellular space and are responsible for direct surveillance of gut contents by pinocytosis and play a critical role in tolerance of food antigens and non-pathogenic microflora (Burkey et al., 2009; Murphy, 2012). On the other hand, when dendritic cells perceive antigens, either through direction pattern recognition receptor activation or stimulation from inflammatory cytokines secreted by enterocytes, localized effector cytotoxic T cells and macrophages are activated and recruited (Burkey et al, 2009; Murphy, 2012). Furthermore, in all cases of cellular activation, dendritic cells travel to primary lymphoid tissues and are hypothesized to be responsible for tolerance to food molecules and commensal bacterial (Burkey et al., 2009; Murphy, 2012). Therefore, appropriate development of the mucosal immune system in young animals is critical to both immune protection and gastrointestinal function throughout an animal's lifecycle.

At birth piglets are immune deficient and depend on their mother's colostrum to provide immunological protection (Lalles et al., 2007). Studies based on tolerance to food antigens show that the mucosal immune system starts to develop the first wk of age, but is not fully developed until about 8 week of age (Miller et al., 1994). By about 3 wk of age pigs have a functional yet immature mucosal immune system (Bailey et al., 2005; Lalles et al., 2007). Bailey et al. (2005) showed that before 3 wk of age

intracellular lymphocytes poorly respond to mitogens and subsequently there is poor splenic lymphocyte activation and proliferation. McLamb et al. (2013) showed prior to 18 d of age pigs elicit a poor innate immune response to pathogenic *E.coli*, which subsequently results in enteric disease. This information suggests poor mucosal protective function prior to 3 wk of age and led to the hypothesis that pigs between 3 to 4 wk of age may have an over-active innate immune system and are pre-disposed to chronic gut inflammation. This hypothesis is supported by a report by Pie et al. (2004) in which they showed transient up-regulation of inflammatory cytokines post-weaning. Pie et al. (2004) showed up regulation of cytokines IL-1β, IL-6, and TNF-α in all segments of the small intestine at 2 days post-weaning. With the exception of TNF-α in the ileum, all cytokine levels returned to pre-weaning levels by 5 d post-weaning. Pie et al. (2004) hypothesized that the spike in IL-6 and TNF-α may be to recruit and activate innate immune cells in the mucosa. Furthermore, TNF-α plays a critical role in tight junction regulation, gastrointestinal inflammation, and induction of diarrhea. However, there is scarce information connecting the aforementioned post-weaning cytokine changes to nutritional or environmental stressors during the post-weaning period. Together, these results show that during the post-weaning period nursery pigs may not be able to mount an appropriate adaptive immune response and are prone to overstimulation of the innate immune system which could lead to gastrointestinal dysfunction.

1.4. Intestinal tight junctions

Junction complexes exist between intestinal epithelial cells and play a critical role regulating gastrointestinal integrity (Figure 1-1). Going from the basolateral membrane to the apical surface, the junction complex includes desmosomes, adherens junctions, and tight junctions (Schneedberger and Lynch, 2004). The tight junction proteins appear to be most critically linked to gastrointestinal integrity and are made up of over 20 intracellular or transmembrane proteins (Gonzalez-Mariscal et al., 2003). Tight junction proteins can be broken down into 3 major families: transmembrane claudins (CL), transmembrane occludins (OC), or intracellular zonula occludens (ZO) (Gonzalez-Mariscal et al., 2003; Schneedberger and Lynch, 2004). Occludin proteins are tetra-spanning transmembrane proteins that contain intracellular domains anchored to ZO proteins (Scheedberger and Lynch, 2004). Furthermore, OC are generally regulated by protein kinase C phosphorylation of intracellular Ser and Thr residues and a direct relationship exists between changes in OC protein expression and trans-epithelial cell resistance (TEER) (Scheedberger and Lynch, 2004). Thus, OC proteins are considered to be the belt-like gate-keeper of the tight junction complex. Claudins are also transmembrane proteins and all claudins with the exception of CL-12 are anchored to intracellular ZO (Schneedberger and Lynch, 2004). Claudins are critical for permeable barrier function of the tight junction complex and conformation changes in CL proteins allow for paracellular uptake of sodium, chloride, and calcium (Schneedberger and Lynch, 2004). Zonula Occludens, and to a lesser extent cingulin,

form the link between the spanning tight junction proteins and the cellular cytoskeleton, and therefore are critical for regulation of tight junction structure and function.

Tight junction function and structure are subject to regulation through various mechanisms that impact the myosin-actin cytoskeleton. Toxins, such as *C. difficule* Toxin A can act directly on ZO-1 protein to cause delocalization from actin filaments (Gonzalez-Mariscal, 2003). Furthermore, it is well established that inflammatory cytokines induce changes in tight junction gene and protein expression that are linked to break-down of epithelial barrier function (Cunningham and Turner, 2012). As reviewed by Cunningham and Turner (2012), recent evidence from in-vitro, rodent, and swine models show that inflammatory cytokines TNF-α and IFN-γ cause up-regulation of epithelial cell myosin-light-chain kinase (MLCK). Subsequently, phosphorylation of myosin leads to cytoskeleton and tight junction conformation changes and increased gastrointestinal permeability. These recent studies show the primary link between inflammatory gastrointestinal disease and gut permeability to be related to MLCK up-regulation by cytokines. Furthermore, epithelial barrier function has been shown to be impacted by acute and chronic stress through stress-hormone actions on tight junctions (Kieta and Soderholm, 2010). As reviewed by Kieta and Soderholm (2010) studies in rodents, pigs, and humans show that mast cells are activated and recruited by stress hormones acetocholine and corticotrophin releasing factor (CRF). Activation of intestinal mast cells leads to secretion of inflammatory cytokines, proteases, and histamine. Histamine and the mast cell protease tryptase act directly on OC to decrease epithelial barrier function, whereas TNF-α from mast cell degranulation leads to decreased barrier function by up-regulating MLCK (Kieta and Soderholm, 2010;

Overman et al., 2012). In pigs, weaning stress has been shown to increase serum CRF which was directly connected with intestinal mast cell activation and decreased gastrointestinal integrity (Moeser et al., 2007; Smith et al, 2010). Furthermore, Pearce et al. (2012, 2013a, 2013b, and 2014) showed that heat stress in growing pigs led to up-regulation of MLCK, decreased barrier function, and changes in tight junction gene expression. Pearce et al. (2013a) showed changes in MLCK gene expression were inversely related to ZO-1 gene expression, but not related to OC gene expression. In the specific case of heat stress it is likely that protective mechanisms exists (heat-shock proteins) to protect the fence-like function of the OC portion of the tight junction complex. Information from the literature shows that the tight junction protein complex is critical to maintenance of gut barrier function and subject to regulation by immune-, neurohormone-, or microbial-related mechanisms. An understanding of how environmental or nutritional factors impact tight junction proteins is critical to understanding gastrointestinal function.

1.5. Methods for determining change in epithelial barrier function

Protective barriers in the gut include membranes, the mucus layer, tight junctions, antimicrobial factors, and innate immune cells (Lambert, 2009). These barriers act to control paracellular transfer of nutrients, bacteria, and other gut contents. In cases of compromised intestinal barrier function bacteria, bacteria cell wall components, or food antigens are able to breach the protective epithelial barrier which leads to inflammation, gut dysfunction, and depressed animal performance (Lambert,

2009). Therefore, it is particularly important to be able to understand and accurately measure the impact of physiological and dietary factors on intestinal permeability. In humans and animals, measuring the passage of high molecular oligosaccharides can be used to assess gastrointestinal integrity (Lambert, 2009). For example, urinary sucrose excretion is commonly used as a marker of stomach barrier dysfunction (Lambert, 2009). Sucrose is rapidly broken down by sucrase in the small intestine, therefore changes in urinary sucrose level are indicative of damage to the stomach epithelium. To assess small intestine epithelial damage urinary lactulose excretion can be used as a marker, whereas urinary sucralose can be used a marker to detect damage to the colon epithelium (Lambert, 2009). Briefly, lactulose is subject to microbial degradation in the large intestine, therefore urinary lactulose secretion is indication of small intestine tissue damage, whereas sucralose is generally not subject to microbial degradation in the intestine, and can be indicative of epithelial barrier dysfunction in the small or large intestine. Therefore, examination of the urinary sucralose: lactulose ratio provides insight to where epithelial barrier dysfunction is located (Lambert, 2009). In animal or cell culture models infusion with high molecular weight probes such as fluoroisothiocynate dextran or horseradish peroxidase can be used to assess intestinal integrity (Cameron and Perdue, 2005). Increased transfer of the fluorescently-labelled probes in the serum or media indicate breakdown of barrier function. Furthermore, the presence of the bacterial endotoxin lipopolysaccharide (LPS) in serum has been well documented to be a marker of intestinal leakiness in rodents and pigs (Hall et al., 2001; Pearce et al., 2013a,b). Under normal conditions the level of bacterial endotoxins should be very low in the serum and an increase in serum LPS indicates bacterial cell

wall components have breached the tight junction barrier in the gut. Furthermore, Ussing chambers allow for ex-vivo measurement of paracellular integrity in animals (Lambert, 2009; Wijtten et al., 2011). Briefly, a section of intestinal mucosa is excised and mounted between two fluid filled chambers. An advantage of this ex-vivo technique is that the precise location of intestinal dysfunction can be determined although Ussing chamber techniques must be conducted rapidly to avoid tissue necrosis (Wijtten et al., 2011). Transfer of aforementioned oligosaccharide markers or a decrease in TEER indicate breakdown of paracellular integrity (Wijtten et al., 2011). Likewise, culturing of epithelial cell models in trans-well plate systems and measurement of TEER has been shown to be an effective measurement for assessing bacterial, oxidative, or nutritionally-induced changes in cellular integrity (Geens and Niewold, 2011; Brosnahan and Brown, 2012). Information in the literature shows that oxidative intermediates and inflammatory cytokines reduce intestinal integrity by altering tight junction gene and protein expression (Ivanov et al., 2010). Furthermore, dietary factors such as glucose, zinc, and calcium concentration in the intestinal lumen alter epithelial barrier function by impacting tight junction dynamics (Nusrat et al., 2000). Hu et al. (2013) showed that an early weaning event decreased intestinal barrier function by decreasing expression of tight junction genes occludin, claudin-1, and zonula occludens-1 that was directly related to decreased intestinal TEER and depressed growth performance. Furthermore, Pearce et al. (2013a,b) showed that in heat-stressed pigs there were changes in the expression of tight junction genes that correlated with a spike in serum LPS and depressed growth performance. These data show that measurement of tight-junction dynamics can serve as a marker for changes in intestinal

integrity. However, a degree of caution should be used when interpreting changes in tight junction dynamics because the relationship between tight junction gene expression to protein expression and functionality are variable and still being elucidated.

1.6. IPEC-J2 cells as model for nutritional immunology studies

Intestinal porcine epithelial cells (IPEC-J2) are non-transformed, columnar epithelial cells that were originally isolated by Helen Berschneider in 1989 from the jejunum of a neonatal piglet. Recent information reported in the literature shows that IPEC-J2 cells are a viable model for immunological or microbiological investigations due to robust, in-vivo like expression of immunological signaling mechanisms and markers of cellular integrity (Geens and Niewold, 2011; Brosnahan and Brown, 2012). Currently, Caco-2 cells (human adenocarcinoma cells) are the default epithelial cell culture model, although they lack a functional toll-like receptor (TLR)-4 which limits cellular immunological responsiveness and makes them a poor model for nutritional immunology and microbiology studies (Brosnahan and Brown, 2012). In addition to IPEC-J2 cells two other cell lines, IPI-21 and IPEC-1, are commonly used for in-vitro research (Davin, 2013). Briefly, IPI-21 cells are transformed ileal cells isolated from an adult boar and IPEC-1 cells are non-transformed heterogeneous cells isolated from the small intestine of a neonatal piglet. Information from the literature shows that IPEC-J2 cells are the optimal porcine cell line to use for nutritional immunology studies due to homogeneity and in-vivo-like characteristics in differentiated cells (Davin, 2013). While growing in culture IPEC-J2 cells are typically fed a media mixture containing

standard Dulbecco's Modified Eagle's Medium, with 5% fetal bovine serum, 1 % insulin, transferrin, and selenium, 5 ng epidermal growth factor/mL, and 1% penicillin and streptomycin (Appendices A and B). Furthermore, differentiation is initiated by confluence and nutrient restriction (Appendices A and B). Following differentiation cells form a confluent monolayer, exhibit polarity, and contain microvilli and a glycocalyx (Davin, 2013). As reviewed and summarized by Brosnahan and Brown (2012) previous studies show that differentiated IPEC-J2 cells express tight junction, chemokine, cytokine, toll-like receptor, and other immune marker mRNAs and proteins similar to in-vivo porcine enterocytes. Because IPEC-J2 cells are homogenous enterocytes there is little expression of mucin although they have been demonstrated to express MUC1,2 and 3 messenger RNA (Brosnahan and Brown, 2012). In addition to gene and protein expression markers, mature IPEC-J2 cells can be characterized by TEER and morphological changes (Geens and Niewold, 2011). Based on TEER measurements IPEC-J2 cells seeded on trans-well collagen-coated membranes exhibit the highest degree of paracellular integrity 9 d post-confluence (Geens and Niewold, 2011). Furthermore, TEER significantly decreases starting a 21 d post-confluence suggesting a high degree of apoptosis. Starting at about 9 d post-confluence IPEC-J2 cells exhibit microvilli which continue to enlarge and develop up to 30 d post-confluence (Geens and Niewold, 2011).

Previous studies show that IPEC-J2 cells are a viable model for studying pathogen-enterocyte interactions, intestinal mycotoxicosis, mucosal oxidative stress, and effects of minerals on cellular integrity (Diesing et al., 2011; Brosnahan and Brown, 2012; Cai et al., 2013; Lodemann et al., 2013). As discussed by Geens and Niewold

(2011) wild-type enterotoxigenic K88 positive *E.coli* are able to adhere to IPEC-J2 cells and initiate a cellular immunological response. Other studies show similar IPEC-J2 cell interactions with *Chlamydia* and rotovirus (Liu et al., 2010; Geens and Niewold, 2011). Likewise, Deising et al. (2011) showed that the mycotoxin deoxynivalenol impacts IPEC-J2 integrity as measured by TEER and tight junction gene expression, and Cai et al. (2013) showed the IPEC-J2 cells are a viable model for studying hydrogen peroxide-induced oxidative cellular damage. These studies show that IPEC-J2 cells are a viable model for studying various factors related to porcine intestinal dysfunction.

Recent information from the literature shows that IPEC-J2 cells are a valuable in-vitro model for screening nutritional products aimed to mitigated pathogen infection and gut dysfunction (Liu et al., 2010; Zanello et al., 2011; Cai et al., 2013). Interestingly, Zanello et al. (2011) showed that incubation with live yeast can mitigate enterotoxigenic *E.coli* adherence and activation of inflammatory pathways related to IL-6 and IL-8 secretion, suggesting that certain live yeast strains can mitigate pathogenic *E.coli* infection. Furthermore, Liu et al. (2010) showed that IPEC-J2 cells can be used as a probiotic screening tool. Liu et al. (2010) found that specific strains of *L. reuteri* were able to mitigate LPS activation of TLR-4 through stimulation of regulatory T cells. These aforementioned studies show that IPEC-J2 cells can be used as a screening tool for nutritional products prior to in-vivo validation and are particularly valuable in providing insight in regards to nutritional supplement-immunological interactions. Expression of nutrient transporters and other proteins related to enterocyte absorptive and secretory function have been well documented in Caco-2 cells (Sambuy et al.,

2005). However, to the author's knowledge there are currently no published studies characterizing the expression and activity of nutrient transporters in IPEC-J2 cells.

1.7. Implications of weaning and heat stress on swine physiology

During the pig's lifecycle psychological, environmental, and nutritional stress events are encountered (Lalles, 2004; Lambert, 2009). Common psychological stress events include separation from the sow post-weaning, social mixing, or crowding stress whereas environmental stress events include exposure to excessive heat, cold, or humidity (Lalles, 2004). Post-weaning nursery pigs (6 kg bodyweight) have a thermal neutral zone of approximately 25 to 33 ˚C with the ideal ambient air temperature at 31˚C, which usually requires heat supplementation (Lammers et al., 2007). Conversely, late nursery phase and grow-finish pigs are susceptible to heat stress due to lack of sweat glands and the thermal neutral zone for a 125 kg pig ranges from 12 to 24˚C with an ideal ambient temperature around 16˚C (Lammers et al., 2007). Heat stress is particularly troublesome in grow-finish pigs and sows because of difficulty in reducing ambient air temperatures in modern swine production systems. Additionally, pigs are susceptible to dietary-induced stress particularly at the time of diet phase change, especially at weaning when pigs are abruptly transitioned from a milk-based to a grain-based diet (Lalles, 2004). In order to protect vital organs and attempt to maintain homeostasis, physiological and metabolic changes occur during and following stress events that have implications on gastrointestinal integrity, nutrient metabolism, immune dynamics, and growth performance (Lambert, 2009). Nueroendocrine systems provide

the link between stress perception and physiological or metabolic changes. Activation of the hypothalamic-pituitary axis (HPA) by stress perception leads to central secretion of corticotrophin release factor (CRF) by the paraventricular nucleus of the hypothalamus which subsequently acts on the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), and then glucocorticoids are secreted from the cortical portions of the adrenal glands (Mayer, 2000). Central and peripheral secretion of CRF has been linked to activation of mast cells and subsequent tight-junction mediated changes in gastrointestinal permeability, which causes short- and long-term gastrointestinal dysfunction in swine (Moeser et al, 2007). Furthermore, cortisol-induced metabolic changes that stimulate glycogenlysis and lipolysis and have been shown to decrease glucose absorption by affecting GLUT-2 trafficking in rats and pigs (Shepherd et al., 2004; Pearce et al., 2013a). Stress-induced spikes in cortisol are also linked to general immune suppression (Mayer, 2000). Briefly, circulating cortisol activates glucocorticoid receptors and subsequently the activated receptors prevent the action of immune modulatory transcription factors (Mayer, 2000). On the other hand, acute stress-induced activation of the sympathetic nervous system leads catecholamine secretion from the adrenal medulla which induces the classical "flight or fight" response and has been shown to be immune stimulatory. Briefly, activation of β-adrenergic receptors lead to a complex series of events that ultimately allow for c-AMP-dependent Protein Kinase A activation of transcription factors such as CREB which up-regulate immune-related genes, such as IL-6 (Mayer, 2000). The following paragraphs will provide a literature review specific to the impact of post-weaning stress and heat stress

on nursery and grower pig growth performance, gastrointestinal function, and immune dynamics.

Post-weaning stress events induce neuroendocrine changes that are linked to short- and long-term gastrointestinal dysfunction, disease susceptibility, and reduced growth performance (Lalles 2004; Wijtten et al., 2011). Following weaning, poor feed intake is common and causes the "transient growth check" period which lasts for 2 to 5 d and is associated with significant loss of bodyweight (Lalles, 2004). Brooks et al. (2001) estimated that approximately 50 and 10 % of pigs do not consume feed during the first 24 and 48-h post- weaning, respectively, which obviously restricts energy consumption and is an etiological factor for gastrointestinal dysfunction and over stimulation of the mucosal immune system (Lalles, 2004). Pluske et al. (1997) showed that post-weaning villus atrophy ranged from 45 to 75% although crypt depth was only impacted in cases of severe anorexia. Spreeuwenberg et al. (2001) showed a decrease in villus height to crypt depth ratio correlated to a decrease in aminopeptidase A and N, lactase, maltase, and sucrose activity. This information shows a connection exists between post-weaning anorexia, intestinal morphology, and AA and carbohydrate utilization. Furthermore, review of the information in the literature generally shows a decrease in exocrine pancreatic secretions during the first week following weaning (Lalles, 2004). As discussed in a review by Lalles (2004) weaning anorexia was related to a decrease in intestinal protein mass and DNA synthesis, especially in the small intestine. Although weaning-induced changes in intestinal morphology generally show recovery by 7 to 10 d post-weaning intestinal permeability generally increases from 2 to 15 d post-weaning (Lalles, 2004). This information suggests that weaning-associated

stress has short- and long-term implications on gastrointestinal function that go beyond morphological changes. Early weaning stress has been show to induce villus atrophy and increase intestinal permeability (Hu et al.,2013; McLamb et al., 2013). Information reported by Moeser et al. (2007) and Smith et al. (2010) show that stress-induced changes in central and peripheral stress mediators are largely responsible for changes in gastrointestinal function during the first several weeks following weaning. Specifically, weaning stress induces an increase in central cortisol and central and peripheral CRF. An increase in serum and mucosa CRF is highly correlated with post-weaning inflammation and loss of integrity and recent evidence shows that CRF recruits and activates intestinal mast cells (Smith et al., 2010). Mast-cell release of tryptase and proteases along with TNF-α triggers a cascade of events that leads to mucosal inflammation and tight junction breakdown (Overman et al., 2012). Overman et al. (2012) showed that TNF-α secreted from CRF-activated mast cells changes tight junction structure by initiating pathways that lead to phosphorylation and activation of MLCK. Furthermore, recent information reported by Hu et al. (2013) shows the early weaning stress reduces gastrointestinal integrity by altering expression of tight junction proteins. In addition to weaning-associated changes in mast cell density Pie et al. (2004) showed that during the first 2 d post-weaning there is a spike in most inflammatory cytokines that corresponds to transient gut inflammation and is linked with excessive post-weaning chloride ion secretion and diarrhea. Hu et al. (2013) showed that early-weaning stress induced TNF-α-dependent MAPK signaling pathways that lead to activation of transcription factors associated with induction of inflammatory mediators (ERK, P38, and JNK) and was associated with increased gastrointestinal

permeability. Furthermore, Spreeuwenberg et al. (2001) showed poor post-weaning feed intake induced inappropriate gastrointestinal inflammation and resulted in an increase in the density of mucosal cytotoxic T cells. In addition to poor feed consumption induced by weaning stress, post-weaning dehydration is of concern. Poor water intake can occur during the post-weaning period due to stress-induced lethargy or lack of water during transportation. A dearth of information exists for the effects of poor water consumption following weaning on swine physiology although a recent report by Horn et al. (2014) showed that an acute post-weaning water deprivation event resulted in a spike in serum CRF and affects nursery pig gastrointestinal function by reducing ileal villus height and altering mucosal tight junction gene expression. Furthermore, Horn et al. (2014) showed that a 24-h post-weaning feed and water deprivation event impacted growth performance throughout the nursery period.

Nursery pig management strategies exist to minimize the impact of psychological and environmental stressors post-weaning. Such strategies include limiting pen stocking density, stimulating pigs to move at least two times daily for the first wk post-weaning, and increasing pig weaning age (PIC, 2013). With the exception of increasing weaning age, there is little empirical evidence in modern scientific literature that links the aforementioned management strategies to improved performance or health. On the contrary, nutritional management of nursery pigs to minimize post-weaning stress effects has been well studied and reviewed. Supplementation of dairy products as a highly digestible source of carbohydrates and AA has been shown to improve nursery pig performance, gut health, and alter gut microbial ecology as reviewed by Thacker (1999). Furthermore, the benefits of spray-dried plasma (SDP)

supplementation to nursery pigs was reviewed by Lalles (2007). Briefly, recent studies show supplementation of SDP increases feed intake, stimulates growth factors such as IGF-1, enhances intestinal morphology, and reduces *E.coli*-associated morbidity (Van Dijk et al., 2001; Touchette et al., 2002). The health-promoting benefits associated with SDP are assumed to be related to immunoglobin and AA content (Lalles, 2007). Supplementation of crystalline AA Glu, Gln, Gly, Ala, Arg, and Cys have also been shown to improve nursery pig performance through enhancement of gastrointestinal function. Glutamine and glutamate serve as a metabolic fuel for enterocytes and dietary supplementation has been shown to mitigate post-weaning villous atrophy as reviewed by Lalles (2007). Furthermore, Gly and Ala have been shown to enhance porcine gastrointestinal secretory factors (Ewtushick et al., 2000) and dietary supplementation of Arg has been shown to mitigate post-weaning villus atrophy by serving as a precursor to polyamines (Harte et al., 2003). Prebiotic supplements have been shown to elicit changes in intestinal microflora and mucosal immunity although the "positive" gut changes have not been consistently connected to reduced morbidity or improved performance (Lalles, 2007). However, probiotic supplements have been well documented to alter microbial ecology, immune dynamics, and morbidity as reviewed by Lalles (2007). For instance, several *Lactobacillus* strains have been shown to reduce *E.coli* pathogenesis in-vivo (Van Nevel et al., 2003 and 2005) and supplementation with live yeast *saccharomyces cerevisiae* has been shown to reduced intestinal inflammation and post-weaning diarrhea (Baum et al., 2002; Taras et al., 2006). Supplementation of botanical compounds, such as carvacol and thymol have been linked to modulation of intestinal microflora and reduced *E.coli* pathogenesis (Manzanilla et al., 2004). To date,

a wide degree of variability in botanical product efficacy has been reported and will be further discussed in a later section.

Pigs are particularly sensitive to heat stress due to lack of sweat glands, modern housing facilities, and hot weather endemic to the Midwestern and Southeastern United states where pigs are typically raised. Heat stress has been well documented to depress feed intake in order to reduce metabolic heat production. Recent evidence shows that heat stress also has detrimental effects on pig digestive physiology (Kerr et al., 2003; Pearce et al, 2013a,b; Pearce et al., 2014). Pearce et al. (2014) showed that an acute heat stress (37˚C) in growing pigs results in an immediate decrease in feed intake that corresponds to decreases in ghrelin, cholecystokinin, gastric inhibitory peptide. This data suggests that perception of heat stress induced behavioral and metabolic changes by impacting gastrointestinal neuropeptides. During periods of heat stress redistribution of blood flow occurs to allow more efficient heat dissipation, which in turn reduces nutrient and oxygen delivery to intestinal tissues (Lambert, 2009). Subsequently, intestinal tissues become hypoxic and ATP stores are depleted which results in intestinal integrity being compromised (Lambert, 2009). In heat-stressed rats Hall et al. (2001) reported that hypoxic conditions in the gut resulted in ATP depletion and cellular necrosis which resulted in a decrease in TEER, an increase in serum LPS, and tight junction opening. Furthermore, the increase in serum LPS due to reduced gastrointestinal integrity induced systemic and local inflammation. In growing pigs Pearce et al. (2013a,b and 2014) showed that a heat stressor can reduce TEER within 2 h of initiation, which results in up to a 45% increase in serum LPS. Furthermore, Pearce et al. (2013a) showed that heat stress induced changes in metabolism and

intestinal nutrient utilization by increasing glucose transport, but decreasing sucrase and maltase activity. Pearce et al. (2013a) hypothesized that the heat stress-induced increase in glucose transport was related to increased passive glucose transport as discussed by Kellet and Brot-Laroche (2005) and the decrease in brush border enzymes was related to intestinal sloughing. Several studies in pigs report short-term damage to intestinal morphology due to heat stress which is likely related to hypoxia-induced tissue necrosis (Pearce et al., 2013a,b; Pearce et al., 2014). Furthermore, Pearce et al. (2013 a,b) showed that heat stress-induced changes in gastrointestinal permeability were related to changes in tight junction gene and protein expression. In-vitro research by Yang et al. (2007) show that loss of epithelial barrier function was due to hypoxia-induced up-regulation of HIF-1α which ultimately leads to activation of MLCK, rearrangement of actin filaments, and changes in tight junction expression. It should be noted that tolerance of heat stress on tight junction dynamics has been observed and is hypothesized as a protective mechanism (Dokladny et al., 2006). In rats and mice long-term exposure to heat leads to up-regulation of the tight junction protein OC (Ruell et al., 2004). In-vitro research also shows that heat shock proteins 70 and 72 bind to OC proteins to help mitigate heat-stress induced degradation, whereas heat shock factor 1 binds to the OC promoter to increase gene expression (Dokladny et al., 2006). Considerable research exists on the impact of short- and long-term heat stress on pig growth performance. Pearce et al. (2013b) showed that in 46-kg pigs exposed to a constant, 24-h heat stressor (35.5˚C) there was a 1.6˚C increase in body temperature and a 5 and 53% reduction in BW and FI, respectively. Kerr et al. (2005) reported a 23 and 14% reduction in gain and ADFI in pigs exposed to a 36-d heat stressor (33˚C), whereas

Hyun et al. (1998) reported a 10% reduction in ADG and ADFI during a 4-wk heat stressor (34˚C).

Management strategies to mitigate heat stress in growing pigs and sows have been extensively studied and shown beneficial. Such strategies include use of temperature-activated ventilation fans, evaporative cooling systems, and direct water application (Renadeau et al., 2010). Modern air-movement and water misting systems have been shown to increase finisher pig gain during a natural and cylic (above 29˚C) heat stressor by 25 and 13 %, respectively (McGlone et al., 1988). Furthermore, modification of dietary nutrient content has been shown to be an effective strategy to mitigate heat stress in finishing pigs (Renadeau et al., 2010). Supplementation of dietary fat to increase dietary energy concentration has been shown to improve performance in heat-stressed finishing, but not growing pigs as reviewed by Renadeau et al. (2010). Additionally, reduction of dietary CP and supplementation of crystalline AA has been shown to increase lean-growth efficiency in heat-stressed finishing pigs (Kerr et al., 2003). It has been hypothesized that lower dietary CP results in less heat production associated with disposal of excess AA and therefore lowers total heat production. Supplementation of dietary antioxidants and AA such has been shown to mitigate heat stress in rodent and poultry models although limiting information exists for swine (Renadeau et al., 2010). In rats, Hall et al. (2001) showed that supplementation with vitamins A, C, and E mitigated heat-associated oxidative stress. Furthermore, supplementation with glutamine prior to a heat stress event has been shown to improve intestinal integrity, survival rate, and production of protective heat-shock proteins in rats (Ruell et al., 2004).

1.8. General review: phytochemical feed additives in swine and poultry production

Phytochemical additives are plant-derived products that have bioactive function (Windisch et al., 2008). Recently, concerns with inclusion of growth promoting antibiotics in livestock diets, a more developed understanding of the relationship between gastrointestinal health and growth efficiency, and ability to more precisely extract and analyze plant compounds has led to a spike in interest around phytochemical feed additives (Windisch et al., 2008). Phytochemical feed additives include herbs, spices, essential oils, oleoresins, and purified plant compounds (Windisch et al., 2008). Due to lack of industry standardization and difficulty in measuring bioactive components the modes of action of phytochemical feed additives are poorly understood. Furthermore, in some cases the bioactive compound concentration in phytochemical feed additives can be influenced by plant growing conditions and extraction methods which contribute to difficulty in determining product efficacy. Nonetheless, phytochemical compounds have been well documented to contain antioxidant, antimicrobial, and immune and gut modulatory properties using in-vitro and rodent models (Windisch et al., 2008; Diaz-Sanchez et al, 2015).

Plant-derived compounds have been well documented to modulate intestinal microflora which results in reduced morbidity and improved growth performance (Windisch et al., 2008; Diaz-Sanchez et al., 2015). Furthermore, the improvement in growth performance due to supplementation of phytochemicals has been shown in poultry to be related to a decreased incidence of sub-clinical enteropathogen infections (Brennan et al., 2003). The mode of action of plant-derived compounds on microbial

ecology is usually related to disruption of bacterial membranes, reduced pathogen virulence capacity, or modulation of microflora resulting in a higher concentration of "beneficial" microbes in the gut lumen (Diaz-Sanchez et al., 2015). Phenolic plant-derived compounds such as carvacol (oregano), thymol (thyme), eugenol (clove), cinnamaldehyde (cinnamon), and allicin (garlic) have all been documented to be antimicrobial by causing disruption of bacterial membranes (Kollanoor-Johny et al., 2010, 2012). In most cases, plant-derived essential oils are more inhibitory to gram-positive microflora because the compounds generally do not penetrate the outer membrane of gram-negative bacteria (Diaz-Sanchez et al., 2015). In broilers, dietary supplementation of essential oils has been shown to reduce intestinal *E.coli* and C. *Perfrigens* (Jamroz et al., 2006). Furthermore, Cross et al. (2007) and De Martino et al. (2009) reported essential oil mixtures reduce coliforms in broiler chicken excreta. In addition to direct antimicrobial effects, it may be possible that the modes of action of plant-derived compounds are related to a change in pathogen virulence or microbial modulation. Interestingly, in broilers fed diets supplemented with eugenol, a decrease in *Salmonella* motility was observed and gene expression analysis showed the eugenol supplementation decreased motility and adherence genes motA, flhC, hila, hilD, and invF (Kollanoor-Johny et al., 2012). In broilers, yerba mate and grape seed extract have been attributed to an increase in intestinal *Lactobacillus* and *Pediococcus* which resulted in improved resistance to enteric pathogens (Viveros et al., 2011; Gonzalez-Gil et al., 2014). Swine and poultry literature shows that supplementation of plant-derived compounds can increase intestinal *Lactobacillus* and *Bifidobacteria* populations leading to a decrease in intestinal pH and enhanced nutrient utilization (Jang et al. 2004). The

aforementioned phytochemical-induced microbial modulation increases the capacity for nutrient utilization which has been demonstrated by improved apparent nutrient retention or digestibility in pigs (Cho et al., 2006), broilers (Hernandez et al., 2004) and turkeys (Seskeviciene et al., 2005).

Antioxidant actions of phytochemicals in feed and in-vivo have been reported (Cuppett and Hall, 1998; Windisch et al., 2008; Diaz-Sanchez et al., 2015). Phenolic terpene phytochemicals such as rosmarinic acid, thymol, and carvacol have been shown to protect in-feed lipids from oxidation in swine and poultry feeds although efficacy of the aforementioned phytochemicals relative to standard feed antioxidants such as α-tocopherols is unknown (Cuppett and Hall, 1998). Limited information exists on the ability of phytochemicals to influence oxidative balance in swine and poultry. Garlic-derived compounds have been well documented to have radical scavenging properties and stimulate antioxidant enzymes systems as reviewed by Amagase et al. (2001) and Amagase (2006). A recent study in nursery pigs shows reduced oxidative stress in the intestinal mucosa of pigs following weaning due to dietary supplementation of a product containing tea polyphenols (Zhu et al., 2012). Furthermore, some research shows dietary supplementation of phytochemicals can reduce meat lipid oxidation and improve meat quality although a reduction in lipid oxidation is usually associated with phytochemical-specific off flavors in cooked meat (Diaz-Sanchez et al., 2015).

Evidence from in-vitro studies shows that phytochemicals can influence cellular immune dynamics although there is a dearth of information exploring the consequences of phytochemicals on monogastric species immune function (Windisch et al., 2008; Diaz-Sanchez et al., 2015). Kyo et al. (2001) show that garlic compounds have immune

stimulatory properties by using in-vitro and rodent models. Furthermore, Zhao et al. (2011) showed that phytochemicals can inhibit activation of pattern recognition receptors which may explain why some phytochemicals reduce intestinal inflammation. Studies by Youn et al. (2006 and 2008) showed that both curcumin and cinnamaldehyde inhibit LPS-induced TLR4 dimerization which prevents activation of the NF-κB inflammatory pathway. Furthermore, curcumin, helenalin, and other plant-derived compounds inhibit NOD2 activation of NF-κB and Youn et al. (2005) showed resveratrol and ECGC can inhibit MYD88 TRIF downstream of TLR-3 and 4 to mitigate NF-κB activation. Taken altogether, these studies suggests that phytochemicals may improve animal performance through immune modulatory pathways although there is limiting supporting evidence in monogastric species. In broilers, Lee et al. (2003) demonstrated that turmeric oleoresins reduced *C. perfringens* infection by stimulating immune function and Kim et al. (2013) showed that supplementation of garlic compounds improved resistance to Eimeria by enhancing innate and adaptive immune dynamics. In pigs, supplementation of Echinacea was shown to enhance immune stimulation following vaccination (Maass et al., 2005). Additionally in nursery pigs, Liu et al. (2013) showed that dietary supplementation of capsicum oleoresin, garlic compounds, and turmeric oleoresin mitigated an *E.coli* infection by influencing mucosal innate immune function and reducing intestinal inflammation. Liu et al. (2014) subsequently conducted microarray analysis of intestinal mucosal due to supplementation of the aforementioned phytochemicals and found all supplements influenced markers of mucosal immune function .

Although the growth promoting mode of action of phytochemicals is complex and not fully understood, it is likely that the gut is the link between phytochemical mode of action and growth performance improvements (Windisch et al., 2008; Diaz-Sanchez et al., 2015). Using a rat model, Rao et al. (2003) showed that various spices could increase rat pancreatic lipase activity and Kreydiyyeh et al. (2003) reported improved glucose absorption due to supplementation with anise. In broilers, Lee et al. (2003) reported supplementation of essential oils increased trypsin and amylase activity whereas Jamroz et al. (2006) reported changes in mucin dynamics with essential oil supplementation. Furthermore, Manzilla et al. (2004) reported supplementation of a combination of essential oils and capsicum reduced gastric emptying in swine. Supplementation of saponins (Yucca) to swine and poultry has been shown to reduce intestinal ammonia concentrations and urease activity which is related to improved intestinal health (Diaz-Sanchez et al., 2015). The aforementioned improvements in enzyme activity and gut secretions are likely related microbial modulation and production of VFA as discussed previously. As shown in a review conducted by Windisch et al. (2008) the impact of phytochemicals on intestinal morphology is inconclusive.

Recent evidence from human and rodent nutritional studies suggests that phytochemicals may improve health through hormesis. Mattson et al. (2008) defined hormesis as an adaptive stress response to a low concentration of a compound, recognized as toxic. Mattson (2008) discussed that some phytochemicals exert beneficial stress adaptation mechanisms by impacting heat shock proteins, antioxidant systems, and growth and neurotropic factors, which leads to activation of cytoprotective

and detoxification enzymes. As discussed by Son et al. (2008) ferulic acid (tomatoes) modifies oxidative stress and protective mechanisms in cells and green-tea bioactive components increase hemeoxygenase-1 which confers hydrogen peroxide resistance. Furthermore, phenethyl isothiocyanate (root crops) has been well documented to induce protective mechanisms by hormetic action. To date, most data shows that hormetic actions of phytochemicals converge at the antioxidant response element (ARE) transcription factor pathway (Mattson, 2008; Son et al., 2008). Evidence from in-vitro and rodent studies suggest that the health-improving mechanisms of phytochemicals may be related to hormetic actions although scant evidence exists in swine and poultry.

Phytochemical feed additives have been suggested to stimulate feed intake in swine although there is a dearth literature to support this claim (Windisch et al., 2008). On the contrary, reports in swine show that supplementation of products containing oregano, thyme, and garlic can decrease feed palatability in a dose-dependent fashion (Holden, 1998; Windisch et al., 2008). Improvements in growth performance and efficiency due to phytochemical supplementation have traditionally been attributed to microbial modulation (Roth and Kirchgessner, 1998) although recent evidence suggests phytochemical impacts on immune dynamics, antioxidant status, and cellular health may also be contributing factors (Diaz-Sanchez et al., 2015). Reviews by Rodehutscord and Kluth (2002) and Windisch et al. (2008) show the impact of phytochemicals on swine performance are highly variable and general conclusions on efficacy cannot be made. On the other hand, the majority of studies evaluating the impact of phytochemicals on broiler chicken growth performance show an improvement in feed

efficiency, but not gain or FI, as shown in meta-analyses conducted by Windisch et al. (2008) and Diaz-Sanchez et al. (2015).

1.9. Specific review: garlic as a phytochemical supplement

Supplementation of raw garlic, crude garlic preparations, aged garlic, and specific garlic phytochemical compounds has been shown to impact human and animal metabolism and health (Amagase et al., 2001; Amagase, 2006). Historically, garlic became of interest in the early 1940's when Cavallito and Bailey (1944) published a manuscript showing that garlic-derived allicin was highly antimicrobial. Due to the concurrent development of penicillin and the soon-followed discovery that some garlic compounds are highly unstable, garlic was discarded as a viable antimicrobial option (Amagase, 2006). Recently, the use of garlic compounds to promote human and animal health has become of more interest due to more accurate extraction and purification techniques and a push from public health officials to minimize antibiotic use, resulting in well over 1,000 scientific publications on garlic in the preceeding 20 years (Amagase et al., 2001).

The chemistry of garlic is shown in Figure 1-2. Little bioactive activity exists in the whole garlic bulb due to compound compartmentalization and the bioactive AA precursors mainly include γ-glutamyl Cys (0.90%) and alliin (1.8 %) (Amagase et al., 2001; Amagase 2006). Following a cutting or crushing event the enzyme allinase reacts with alliin forming the highly antimicrobial yet unstable compound allicin (Amagase et al., 2001). Allicin is spontaneously and rapidly broken down to more stable oil-soluble

bioactive components, including diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), ajoenes, and vinyldithiins (Amagase et al., 2001). The aforementioned oil-soluble compounds are commonly referred to as stench compounds because of their characteristic odor associated with garlic. At the same time, the water soluble S-allyl Cys (SAC) variants are formed through degradation of γ-glutamyl Cys by γ-glutamyl transpeptidase. Other water soluble compounds are also formed and include glycosides, lectins, and vitamins, although SAC predominates (Amagase et al., 2001). There is little information on the bioavailability of garlic compounds in livestock species although much work has been down in rodents and humans (Amagase et al., 2001; Amagase, 2006). Amagase (2006) reported that 60 to 70% of protected alliin can be absorbed by rodents and is mainly metabolized to downstream oil-soluble bioactive components in the liver. Human and rodent studies show that minimal allicin is absorbed and it is assumed that allicin consumed from fresh garlic cannot pass through the intestinal mucus layer (Amagase, 2006). Other oil-soluble garlic compounds, such as DADS have been shown to be rapidly oxidized following consumption. Pushpendran et al. (1980) showed that liver DADS was highest in the liver cytosol 90 min following consumption and was never detected in the urine, which suggest rapid metabolism in the body. The aforementioned evidence has led to the hypothesis that oil-soluble garlic compounds have little bioactivity post-absorption. Studies in rats show that the water-soluble compound SAC is 98% available, broken down to N-acetyl SAC in the liver and kidneys, and is excreted in the urine (Nagae et al., 1994). Consequently, N-acetyl SAC can be used as a biomarker of fresh garlic or garlic supplement consumption (Amagase et al., 2001). Garlic supplements that

currently exists include garlic essential oils (high in oil-soluble compounds), garlic oil macerate (high in alliin and oil-soluble compounds), garlic powder (little bioactivity), and aged-garlic products (high in water-soluble compounds) (Amagase et al., 2001).

Early research showed garlic-derived allicin was bactericidal for gram positive and gram negative bacteria, antifungal, and antiviral (Anki and Mirelman, 1999). Allicin has been clearly shown to inhibit the activity of thiol-containing enzymes such as phosphotransacetyl-CoA synthetase, acetate kinase, alchohol dehydrogenase, thioredoxin reductase, and RNA polymerase, which ultimately leads to cellular death (Focke et al., 1990). Although the actions of allicin can impact both mammalian cells and bacteria, the impact on bacteria is more severe due to a lack of endogenous glutathione, which is critical for reactivation of thiolated enzymes (Anki and Mirelman, 1999). Furthmore, Davis et al. (1994) showed that a garlic extract containing 34% allicin, 44% thiosulfinates, and 20% vinyldithiins was antifungal and prevent mycotoxin production although the mode of action is not understood. Additionally, Anki and Mirelman (2001) showed that allicin is highly anti-parasitic and can inhibit growth of Entamoeba histolytica, Giardia lamblia, and Leishmania major, and other human parasites. Because allicin is highly unstable, damaging to enterocytes, and may be inactivated at the mucus layer, it generally is not considered a viable antimicrobial agent for livestock. In broilers, Shanmugavelu et al. (2004) showed that supplementation of garlic meal resulted in increased cecal fermentation activity and Cross et al. (2011) and Olukosi and Dono (2014) showed changes in cecal volatile fatty acid production and a decrease in intestinal pH, respectively. Ramiah et al. (2014) showed supplementation of 0.50% garlic product to broilers reduced intestinal *E.coli* and increased *lactobacillus*,

whereas Robyn et al. (2013) showed that a garlic oil high in DADS was able to mitigate *C.jejuni*. To the author's knowledge there are currently no studies exploring the implications of garlic compounds on intestinal ecology in swine.

Garlic compounds have been shown to influence oxidative status directly by scavenging reactive oxygen species (ROS) or preventing lipid peroxidation or indirectly by influencing antioxidant enzyme systems (Borek, 2001). Using endothelial cell models the antioxidant functions of oil- and water-soluble garlic compounds have been well documented. Wei and Lau (1998) and Yamasaki et al. (1997) showed that aged-garlic extract and SAC could mitigate hydrogen-peroxide induced increases in ROS. Likewise, Horie et al. (1989 and 1992) showed lipid-soluble garlic compounds could mitigate oxidant intermediates. Further in-vitro evidence showed that garlic compounds can mitigate oxidative stress by influencing antioxidant enzyme mechanisms (Borek, 2001). Wei and Lau (1998) and Geng et al. (1997) showed that aged-garlic extract and SAC improved cellular oxidative balance by increasing superoxide dismutase, catalase, and glutathione activity. Furthermore, Kohda et al. (2013) showed that aged-garlic extract enhances hemeoxygenase-1 and NAD(P)H quinone oxidoreductase 1, two enzymes involved in maintaining cellular oxidative status. Limiting in-vivo information exists to support the aforementioned in-vitro results. Pedrazza-Chaverri et al. (2001) showed the dietary supplementation of garlic to rats reduced hepatic and renal hydrogen peroxide generation which corresponded to a decrease in catalase gene expression. Pedrazza-Chaverri et al. (2001) hypothesized that the gene expression changes were related to an anti-peroxide effect of the garlic compounds. In pigs, mucosal gene profile analysis conducted by Liu et al. (2014) showed that garlic product

supplementation resulted in up-regulation of approximately 10 genes related to oxidation reduction pathways, suggesting the garlic compounds influence intestinal oxidative balance. It should be noted that many of the aforementioned garlic effects on oxidative balance suggests that garlic compounds may have hormetic action, although to date there are no studies linking garlic-induced changes in oxidant status to changes in stress adaptation pathways.

Recent evidence from in-vitro, rodent, human, and livestock studies show garlic compounds alter immune dynamics. Kyo et al. (1997) showed in a foundational study that aged-garlic extract had an anti-allergic effect in rats by inhibiting histamine release from basophils and later Kyo et al. (2001) showed a similar extract mitigated IgE-mediated skin reactions in mice. Furthermore, Kyo et al. (2001) showed that aged-garlic extract is immune stimulatory by increasing proliferation of rat splenocytes and lymphocytes, augmenting concavalin A, and enhancing natural killer (NK) cell activity. Interestingly, in the same study Kyo et al. (2001) showed that aged-garlic extract mitigated stress-induced changes of immune function in mice. Likewise, Alma et al. (2014) recently reported an increase in urinary IL-12 in human subjects consuming a garlic powder product. Because IL-12 is critical for T cell differentiation into T_{H1}-type cells and NK cell activity this data supports previous rodent studies (Kyo et al. 1997, 2001). Clemmens et al. (2010) isolated two proteins from raw garlic (QR-1 and QR-2) that were shown to be immune modulatory using an in-vitro model, although to date there is no evidence that shows the aforementioned proteins are present in downstream bioactive garlic products. In isolated leghorn lymphocytes, Hanieh et al. (2012) showed that a garlic product augmented concavalin A stimulation in an IL-12 dependent manner

similar to reports by Kyo et al. (2001). Furthermore, Jafari et al. (2012) reported that a garlic product altered innate immune function of broilers by impacting complement proteins and Kim et al. (2013) showed that an isolated garlic compound, propyl thiosulphinate, enhanced adaptive immunity and performance of broilers infected with *E. acervulina*. Interestingly, Kim et al. (2013) also showed that supplementation of propyl thiosulphinate decreased gastrointestinal inflammation by influencing gene-expression pathways related to TLR-3 and TLR-5 signaling. In pigs, Tatara et al. (2008) showed that supplementation of an aged-garlic product in the milk replacer of young pigs impacted lysozyme and ceruplasmin activity, whereas Yan et al. (2012) showed that long-term supplementation of a garlic meal to grow-finish pigs influenced serum IgG and lymphocyte concentration. These results suggest that garlic products can influence both innate and adaptive porcine immune function. Furthermore, Liu et al. (2013) showed that garlic product supplementation to *E.coli*-infected nursery pigs reduced intestinal inflammatory cytokines and ileal neutrophil concentration, suggesting garlic compounds can exert an anti-inflammatory effect. This theory is supported by previous in-vitro work conducted by Lang et al. (2004) in which they showed oil-soluble garlic compounds inhibited spontaneous and induced release of inflammatory cytokines in Caco-2 cells. Liu et al. (2014) attempted to shed light on the mechanism of action of garlic compounds on mucosal immune function in nursery pigs by conducting a gene expression profile study. In that study Liu et al. (2014) showed that garlic compounds up-regulate complement and coagulation immune cascades and down-regulate phagocytic mechanisms although the specific mode of action could not be determined. Garlic compounds have been shown to up- and down-regulate immune

functions using in-vitro and in-vivo research models but due to the complexity and variability of results clear conclusions on the specific mode of action cannot be made.

Previous research shows garlic compounds have implications on lipid metabolism and hepatic detoxification mechanisms (Amagase, 2006). In a classical study by Hu et al. (1999) it was shown that supplementation of garlic-derived diallyl sulfide reduced mortality and LDH release (a marker of liver damage) in rats exposed to toxic levels of acetaminophen. Hu et al. (1999) showed that diallyl sulfide reduced acetaminophen toxicity by impacting hepatic P450 enzyme activity. These results suggest that garlic compounds may impact drug and mycotoxin hepatic detoxification mechanisms. Much interest has been given to garlic supplementation to reduce serum cholesterol in humans and a recent meta-analysis by Reid et al. (2013) showed that the majority of human clinical studies show long-term garlic supplementation results in a change in the serum lipid profile. Specifically, Reid et al. (2013) found that long-term garlic product supplementation resulted in an 8% decrease in low-density lipoproteins that corresponded to a 38% decrease in coronary complications. Rodent studies show supplementation of garlic products decrease hepatic lipogenic and cholesterolgenic enzymes such as fatty acid synthase, malic enzyme, and glucose-6-dehydrogenase as reviewed by Reid et al. (2013). In broilers, Qureshi et al. (1983) showed various garlic fractions decrease hepatic biosynthesis of fatty acids and cholesterol by decreasing hepatic HMG-CoA-reductase, cholesterol 7α hydroxylase, and fatty acid synthase enzymes. Furthermore, Chowdhury et al. (2002) showed that 6 wk supplementation of a garlic paste to laying hens resulted in a decrease in serum and yolk cholesterol.

Previous research in swine and poultry show that garlic-derived products can impact gastrointestinal health, nutrient utilization, and growth performance (Tatara et al., 2008;Yan et al., 2012; Liu et al., 2013). Although the mode of action of garlic compounds has not been fully elucidated it is likely that health and performance improvements are related to the previously discussed effects of garlic compounds on immune status, microbial ecology, antioxidant status, and metabolism. A clear distinction should be made between the impact of fresh garlic and downstream bioactive components on gastrointestinal function. In a review conducted by Amagase et al. (2001) it was clearly shown in rats that allicin oxidizes gastrointestinal tissue and results in mucosal tissue damage, especially in the stomach. On the other hand, the impact of downstream garlic bioactive components is generally associated with improvements in gastrointestinal health (Tatara et al., 2008; Liu et al., 2013; Badr and Al-Mulhim, 2014). Badr and Al-Mulhim (2014) showed that supplementation of up to 200 mg of aged garlic to rats for 30 d resulted in mitigation of indomethacin-induced gastric lesions, reduced TNF-α, and restoration of cellular antioxidant systems which suggests that garlic compounds may have gut protective functions. Furthermore, Tatara et al. (2008) showed that supplementation of oil- and water-soluble garlic compounds in milk replacer for 8 d improves villus height throughout the small intestine and Liu et al. (2013) showed that supplementation of a garlic product reduced *E.coli*-induced scours and resulted in improved ileal villus height. Improvements in gastrointestinal health have been linked to improved gut secretions, nutrient retention, and growth performance (Pluske et al., 1997). Olukosi and Dono (2014) showed an improvement in ileal digestible energy, and apparent total tract digestiblity of energy and N with

garlic meal supplementation to broilers. The aforementioned improvements corresponded with changes in intestinal pH but did not translate into an improvement in growth performance. Furthermore, Kim et al. (2013) and Ramiah et al. (2014) reported that oil-soluble garlic components increased broiler gain which was related to garlic compound-induced changes in intestinal function. Similarly, in young pigs Tatara et al. (2008) and Huang et al. (2011) reported growth performance improvements that were related to improved morphology or reduced diarrhea, respectively. In grow-finish pigs, Yan et al. (2012) showed that long-term supplementation of a garlic product linearly improved DM and N digestibility that corresponded to improvements in cumulative gain and feed efficiency. It would be remiss not point out that several broiler and pig studies have shown garlic-derived products or compounds influence markers of gut health, metabolism, or immune status but do not improve growth performance (Holden, 1998; Chen et al., 2008; Liu et al., 2014, Olukosi and Dono 2014). Discrepancies in product efficacy are likely related to variation in bioactive compounds, route of administration, duration of supplementation, and animal health.

The literature shows that supplementation of garlic-derived products and compounds show promise as a growth and health promoter in swine and poultry. Further research investigating the impact of garlic compounds on intestinal ecology and markers of immune status, and gut health is warranted before practical application. Furthermore, careful attention should be given to quantification and reporting of bioactive garlic components in all published literature, which will allow for clear interpretation of results and more accurate trial to trial comparisons.

1.10. Conclusions

Review of the literature shows that digestive physiology of young pigs is impacted by weaning-dependent and weaning-independent mechanisms that influence intestinal architecture and digestive secretions. Concurrent with the weaning period, pigs do not have a fully-formed mucosal immune system and are prone to over activation of innate immune mechanisms. Post-weaning changes in digestive physiology and inappropriate mucosal immune activation cause gastrointestinal dysfunction and are directly related to short- and long-term disease susceptibility and depression of growth performance. Furthermore, environmental, nutritional, or psychological stressors at weaning can exacerbate gastrointestinal dysfunction. Extensive information from the literature shows gastrointestinal dysfunction can be alleviated by dietary supplementation of various nutrients or feed additives. In-vitro and rodent studies show that garlic-derived bioactive compounds may be a viable strategy to mitigate stress-induced gastrointestinal dysfunction although scant supporting evidence exists in monogastric species. Hence, a series of studies were designed to characterize the effects of an acute post-weaning feed and water deprivation event and to determine if oral supplementation of garlic-derived DADS + DATS to nursery pigs enhances intestinal function and growth performance.

1.11. References

Alma, E., A. Eken, H. Ercil, K. Yelsel, and N. Daglioglu. 2014. The effect of garlic powder on human urinary cytokine excretion. Urology J. 11(1): 1309-1315.

Amagase, H., B.L. Petesch, H. Matsuura, S. Kasuga, and Y. Itakura. 2001. Intake of garlic and its bioactive components. J. Nutr. 131: 955S–962S.

Amagase, H. 2006. Clarifying the real bioactive cconstituents of garlic. J. Nutr. 136: 716S-725S.

Ankri, S., and D. Mirelman. 1999. Antimicrobial properties of allicin from garlic. Microb. Infect. 2: 125−129.

Argenzio, R.A. 1993. Secretory functions of the gastrointestinal tract. In: M.J. Swenson and W.O. Reece, editors, Duke's physiology of domestic animals. Cornstock Publishing associates, Ithaca, New York, USA. p. 349.

Badr, G.M., and J.A. Al-Mulhim. 2014. The protective effect of aged garlic extract on nonsteroidal anti-inflammatory drug-induced gastric inflammations in male albino rats. Evid. Based Complement. Alternat. Med. 2014: 759642.

Bailey, M., K. Haverson, C. Inman, C. Harris, P. Jones, G. Corfield, B. Miller, and C. Stokes. 2005. The development of the mucosal immune system pre- and post-weaning: balancing regulatory and effector function. Proc. Nutr. Soc. 64: 451–457.

Baum, B., E.M. Liebler-Tenorio, M.L. Enss, J.F. Pohlenz, and G. Breves. 2002. Saccharomyces L. and *Bacillus cereus* var. Toyoi influence the morphology and the mucins of the intestine of pigs. Zeitschrift fu¨r Gastroenterologie 40: 277-284.

Borek, C. 2001. Antioxidant health effects of aged garlic extract. J. Nutr. 131: 1010S-1015S.

Brennan, J., J. Skinner, D. A. Barnum, and J. Wilson. 2003. The efficacy of bacitracin methylene disalicylate when fed in combination with narasin in the management of necrotic enteritis in broiler chickens. Poult. Sci. 82:360–363.

Brooks, P.H., C.A. Moran, J.D. Beal, V. Demeckova, and A. Campbell. 2001. Liquid feeding for the young piglet. In: M.A. Varley and J. Wiseman, editors, The weaner pig: nutrition and management, CAB International, Wallingford, UK, p. 153 -178.

Brosnahan, A.J., and D. R. Brown. 2012. Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. Vet Microbiol. 156(3-4): 229–237.

Burkey, T.E., K.A. Skjolaas, and J. E. Minton. 2009. Porcine mucosal immunity of the gastrointestinal tract. J. Anim. Sci. 87: 1493-1501.

Cai, X., X. Chen, X. Wang, C. Xu, Q. Guo, L. Zhu, S. Zhu, and J. Xu. 2013. Pre-protective effect of lipoic acid on injury induced by H2O2 in IPEC-J2 cells. Mol. Cell Biochem. 378:73-81.

Cameron, H., and M. Perdue. 2005. Stress impairs murine intestina barrier function: Improvement by glucagon-like peptide-2. J. Pharmacol. Exp. Ther. 314:214–220.

Cavallito, C.J. and J.H. Bailey. 1944. Allicin, the antibacterial principle of Allium sativum: isolation, physical properties and antibacterial action. J. Am. Chem. Soc. 66:1950-1951.

Chen, Y.J., I.H. Kim, J.H. Cho, J.S. Yoo, Q. Wang, Y. Wang, and Y. Haung. 2008. Evaluation of dietary L-carnitine or garlic powder on growth performance, dry matter and nitrogen digestibilities, blood profiles and meat quality in finishing pigs. Anim. Feed Sci. Tech. 141(1): 141-152.

Clemmens, F., S.N. Pramod, and Y.P. Venkatesh. 2010. Identity of the immunomodulatory proteins from garlic (Allium sativum) with the major garlic lectins or agglutinins. Int. Immunopharmacol.10: 316–324.

Cho, J. H., Y. J. Chen, B. J. Min, H. J. Kim, O. S. Kwon, K. S. Shon, I. H. Kim, S. Kim, and A. Asamer. 2006. Effects of essential oils supplementation on growth performance, IgG concentration and fecal noxious gas concentration of weaned pigs. Asian-australas. J. Anim. Sci. 19:80–85.

Chowdhury, S.R., and S. D. Chowdhury, T. K. Smith. 2002. Effects of dietary garlic on cholesterol metabolism in laying hens. Poul. Sci. 81:1856–1862.

Cranwell, P.D., 1995. Development of the neonatal gut and enzyme systems. In: M.A. Varley, editor, The neonatal pig: development and survival. CAB International, Wallingford. Oxon, UK, pp. 99-154.

Corring, T. 1980. Endogenous secretions of the pig. In: A.G. Low and I.G. Partridge, editors, Current concepts of digestion and absorption in pigs. National institute for research in dairying, Reading, UK. p. 136.

Cross, D. E., R. M. McDevitt, K. Hillman, and T. Acamovic. 2007. The effect of herbs and their associated essential oils on performance, dietary digestibility and gut microflora in chickens from 7 to 28 days of age. Brit. Poult. Sci. 48:496–506.

Cross, D.E. , R.M. Mcdevitt, and T. Acamovic. 2011. Herbs, thyme essential oil and condensed tannin extracts as dietary supplements for broilers, and their effects on performance, digestibility, volatile fatty acids and organoleptic properties. Br. Poult. Sci. 52: 227-337.

Cunningham, K.E., and J.R. Turner. 2012. Myosin light chain kinase: pulling the strings of epithelial tight junction function. Ann. N.Y. Acad. Sci. 1258(1): 34-42.

Cuppett, S. L., and C. A. Hall. 1998. Antioxidant activity of Labiatae. Adv. Food Nutr. Res. 42:245–271.

Davin, R. 2013. Importance of intestinal cells for gut health-IPEC studies. Intern. Pig Topics. 28(2): 8-9.

Davis, L.E., J. Shen, and R.E. Royer. 1994. In vitro synergism of concentrated Allium sativum extract and amphotericin B against *Cryptococcus neoformans*. Planta Med. 60: 546–549.

De Martino, L., V. de Feo, and F. Nazzaro. 2009. Chemical composition and in vitro antimicrobial and mutagenic activities of seven lamiaceae essential oils. Molecules. 14:4213–4230.

Diaz-Sanchez, S., D. D'Souza, D. Biswas, and I. Hanning. 2015. Botanical alternatives to antibiotics for use in organic poultry production. Poult. Sci. 94: 1419-1430.

Diesing, A., C. Nossol, P. Panther, N. Walk, A. Post, J. Kluess, P. Kreutzmann, S. Dänicke, H. Rothkötter, and S. Kahlert. 2011. Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. Toxicology Letters. 200: 8-18.

Dokladny, K., P. L. Moseley, and T. Y. Ma. 2006. Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. Am. J. Physiol. Gastrointest. Liver Physiol. 290:G204-G212.

Dunsford, B.R., D.A. Knabe, and W.E. Haensly. 1989. Effect of dietary soybean meal on the microscopic anatomy of the small intestine in the early-weaned pig. J. Anim. Sci. 67, 1855-1863.

Ewtushick, A.L., R.F.P. Bertolo, and R.O. Ball. 2000. Intestinal development of early-weaned piglets receiving diets supplemented with selected amino acids or polyamines. Can. J. Anim. Sci. 80: 653-662.

Focke, M., A. Feld, K. Lichtenthaler. 1990. Allicin, a naturally occurring antibiotic from garlic, specifically inhibits acetyl-CoA synthetase. FEBS Lett. 261: 106-108.

Foltman, B.K., G. Harlow, P. Hoen, and P.K. Nielson. 1995. Comparitive investigations of porcine proteases and their zymogens. In: K. Takahashi, editor, Proceedings of the 5th international conference on aspartic proteases. Plenum Press, New York, USA. p. 41.

Geens M., and T.A. Niewold. 2010. Preliminary Characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enterotoxigenic *Escherichia coli, Escherichia coli,* and *E. coli* lipopolysaccharide. Comp. Funct. Genomics. 2010:e469583.

Geng, Z., Y. Rong, B.H.S. Lau. 1997. S-Allyl cysteine inhibits activation of nuclear factor kappa B in human T cells. Free Radic. Biol. Med. 23: 345–350.

Gonzalez-Gil, F., S. Dıaz-Sanchez, S. Pendleton, A. Andino, N. Zhang, C. Yard, N. Crilly, F. Harte, and I. Hanning. 2014. Yerba Mate enhances probiotic bacteria growth in vitro but as feed additive does not reduce *S. Enteritidis* colonization in vivo. Poult. Sci. 93:434-440.

Gonzalez-Mariscal, L., A. Betanzos, P. Nava, and B.E. Jaramillo. 2003. Tight junction proteins. Prog. Biophys. Mol. Biol. 81:1-44.

Hall, G.A., and T.F. Byrne. 1989. Effects of age and diet on small intestinal structure and function in gnotobiotic piglets. Res. Vet. Sci. 47, 387-392.

Hall, D. M., G. R. Buettner, L. W. Oberley, L. Xu, R. D. Matthes, and C. V. Gisolfi. 2001. Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia. Am. J. Physiol. Heart Circ. Physiol. 280:H509–H521.

Hampson, D.J. 1986. Alterations in piglet small intestinal structure at weaning. Res. Vet. Sci. 40, 32-40.

Hanieh, H., K. Narabara, Y. Tanaka, Z. Gu, A. Abe, and Y. Kondo. 2012. Immunomodulatory effects of Alliums and Ipomoea batata extracts on lymphocytes and macrophages functions in White Leghorn chickens. Anim. Sci. J. 83: 68-76.

Harte, R.D., A.K. Shoveller, R.F.P. Bertolo, and R.O. Ball. 2003. Cystine supplementation adversely affects small intestinal morphology. In: R.O. Ball, editor, Proceedings of the 9th International Symposium on Digestive Physiology in Pigs. University of Alberta Publisher, Banff, CA. p. 213-215.

Herdt, T. 1992. Digestion and absorption: the nonfermentive process. In: J.G. Cunningham, editor, Textbook of veterinary physiology. Saunders, Philidelphia, PA, USA. p. 286.

Hernandez, F., J. Madrid, V. Garcia, J. Orengo, and M. D. Megias. 2004. Influence of two plant extracts on broilers performance, digestibility, and digestive organ size. Poult. Sci. 83:169–174.

Holden, P.J., J. McKean, and E. Franzenburg. 1998. Botanicals for pigs-garlic. Iowa State University, Swine Research Report.

Horie, T., T. Murayama, T. Mishima, I. Fumiyaki, Y. Minamide, and T. Fuwa, S. Awaxu.1989. Protection of liver microsomal membranes from lipid peroxidation by garlic extract. Planta Med. 55: 506–508.

Horie, T., S. Awazu, Y. Itakura, and T. Fuwa.1992. Identified diallyl polysulfides from an aged garlic extract which protects the membranes from lipid peroxidation. Planta Med. 58: 468–469.

Horn, N., F. Ruch, G. Miller, K. M. Ajuwon, and O. Adeola. 2014. Impact of acute water and feed deprivation events on growth performance, intestinal characteristics, and serum stress markers in weaned pigs. J. Anim. Sci. 92: 4407-4416.

Huang, R.H., X.S. Qiu, F.X., Shi, C.L. Hughes, Z.F. Lu, and W.Y. Zhu. 2011. Effects of dietary allicin on health and growth performance of weanling piglets and reduction in attractiveness of faeces to flies. Animal. 5: 304–311.

Hu, J.J., J.S. Yoo, M. Lin, E.J. Wang, and C.S. Yang. 1996. Protective effects of diallyl sulfide on acetaminophen-induced toxicities. Food Chem. Toxicol. 34:963–969.

Hu, C. H., K. Xiao, Z. S. Luan, and S. Jong. 2013. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. J. Anim. Sci. 91:1094–1101.

Hyun, Y., M. Ellis, G. Riskowski, and R.W. Johnson. 1998. Growth performance of pigs subjected to multiple concurrent environmental stressors. J. Anim. Sci. 76: 721-727.

Ivanov, A., C. A. Parkos, and A. Nusrat. 2010. Cytoskeletal regulation of epithelial barrier function during inflammation. Am. J. Path. 177(2): e102.2353.

Jafari, R. A., M. Ghorbanpoor, and M. Makkei. 2012. Effect of garlic supplementation on complement activity in broiler chickens. Comp. Clin. Pathol. 21: 183-185.

Jamroz, D., T. Wertelecki, M. Houszka, and C. Kamel. 2006. Influence of diet type on the inclusion of plant origin active substances on morphological and histochemical characteristics of the stomach and jejunum walls in chicken. J. Anim. Physiol. Anim. Nutr. (Berl.) 90:255–268.

Jang, I. S., Y. H. Ko, H. Y. Yang, J. S. Ha, J. Y. Kim, S. Y. Kang, D. H. Yoo, D. S. Nam, D. H. Kim, and C. Y. Lee. 2004. Influence of essential oil components on growth performance and the functional activity of the pancreas and small intestine in broiler chickens. Asian-australas. J. Anim. Sci. 17:394–400.

Kellett, G.L. and E. Brot-Laroche. 2005. Apical GLUT2: A major pathway of intestinal sugar absorption. Diabetes. 54: 3056-3062.

Kerr, B.J., J. T. Yen, J. A. Nienaber, and R. A. Easter. 2003. Influences of dietary protein level, amino acid supplementation and environmental temperature on performance, body composition, organ weights and total heat production of growing pigs. J. Anim. Sci. 81: 1998-2007.

Kieta, A.V., and J.D. Soderholm. 2010. The intestinal barrier and its regulation by neuroimmune Factors. Neurogastroenterol. Motil. 22: 718–733.

Kim, D., H.S. Lillehoj, S.H. Lee, E. P. Lillehoj, and D. Bravo. 2013. Improved resistance to Eimeria acervulina infection in chickens due to dietary supplementation with garlic metabolites. Br. J. Nutr. 109: 76-88.

Klobasa, F., E. Werhahn, and J.E. Butler. 1987. Composition of sow milk during lactation. J. Anim. Sci. 64: 1458.

Kohda, K., H. Goda, K. Itoh, K. Samejima, and T. Fukuuchi. 2013. Aged garlic extract reduces ROS production and cell death induced by 6-hydroxydopamine through activation of the Nrf2-ARE pathway in SH-SY5Y cell. Pharmacology Pharm. 4: 31-40.

Kollanoor -Johny, A., M. J. Darre, A. M. Donoghue, D. J. Donoghue, and K. Venkitanarayanan. 2010. Antibacterial effect of trans-cinnamaldehyde, eugenol, thymol and carvacrol against *Salmonella Enteritidis* and *Campylobacter jejuni* in vitro. J. Appl. Poult. Res. 19:237–244.

Kollanoor-Johny, A., T. Mattson, S. A. Baskaran, M. A. Amalaradjou, S. Babapoor, B. March, S. Valipe, M. Darre, T. Hoagland, D. Schreiber, M. I. Khan, A. Donoghue, D. Donoghue, and K. Venkitanarayanana. 2012. Reduction of *Salmonella enterica* serovar Enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds trans-cinnamaldehyde and eugenol. Appl. Env. Microbiol. 78:2981–2987.

Kreydiyyeh, S. I., J. Usta, K. Knio, S. Markossian, and S. Dagher. 2003. Aniseed oil increases glucose absorption and reduces urine output in the rat. Life Sci. 74:663–673.

Kyo, E., N. Uda, M. Kakimoto, K. Yokoyama, M. Ushijima, I. Sumioka, S. Kasuga, Y. Itakura. 1997. Anti-allergic effects of aged garlic extract. Phytomedicine. 4: 335–340.

Kyo, E., N. Uda, S. Kasuga, and Y. Itakura. 2001. Immunomodulatory effects of aged garlic extract. J. Nutr. 131:1075S–1079S.

Lallès, J. P., G. Boudry, C. Favier, N. Le Floc'h, I. Luron, L. Montagne, I. P. Oswald, S. Pié, C. Piel, and B. Seve. 2004. Gut function and dysfunction in young pigs: Physiology. Anim. Res. 53:301-316.

Lalles, J.P., P. Bosi, H. Smidt, and C. R. Stokes. 2007. Nutritional management of gut health in pigs around weaning. Proc. Nutr. Soc. 66: 260-268.

Lambert, G. P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J. Anim. Sci. 87(E suppl.):E101–E108.

Lammers, P.J., D.R. Stender, and M.S. Honeyman. 2007. Environmental needs of the pig. IPIC: NPP210.

Lang, A., M. Lahava, E. Sakhninia, I. Barshack, H.H. Fiddera, B. Avidana, E. Bardana, R. Hershkovizc, S. Bar-Meira, and Y. Chowersa. 2004. Allicin inhibits spontaneous and TNF-α induced secretion of proinflammatory cytokines and chemokines from intestinal epithelial cells. Clin. Nutr. 23: 1199–1208.

Lee, K. W., H. Everts, H. J. Kappert, M. Frehner, R. Losa, and A. C. Beynen. 2003. Effects of dietary essential oil components on growth performance, digestive enzymes and lipid metabolism in female broiler chickens. Br. Poult. Sci. 44:450–457.

Liu, Y., N.Y. Fatheree, N. Mangalat, and J.M. Rhoads. 2010. Human-derived probiotic *Lactobacillus reuteri* strains differentially reduce intestinal inflammation. Am. J. Physiol. Gastrointest. Liver Physiol. 299:G1087-G1096.

Liu, Y., M. Song, T.M. Che, A.S. Almeida, J.J. Lee, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2013. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs. J. Anim. Sci. 91:5294-5306.

Liu, Y., M. Song, D. Che, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2014. Effects of capsicum oleoresin, garlic botanical, and turmeric oleoresin on gene expression profile of ileal mucosa in weaned pigs. J. Anim. Sci. 92:3426-3440.

Lodemann, U., R. Einspanier, F. Scharfen, H. Martens, and A. Bondzio. 2013. Effects of zinc on epithelial barrier properties and viability in a human and a porcine intestinal cell culture model. Toxicolology in Vitro. 27: 834–843.

Low, A.G., and T. Zebrowska. 1989. Digestion in pigs. In: T. Zebrowska, editor, Protein metabolism in farm animals. Oxford University Press, Oxford, UK. p. 53.

Maass, N., J. Bauer, B. R. Paulicks, B. M. Bohmer, and D. A. Roth- Maier. 2005. Efficiency of Echinacea purpurea on performance and immune status in pigs. J. Anim. Physiol. Anim. Nutr. (Berl.) 89:244–252.

Makkink, C.A., G.P. Negulescu, Q. Guixin, and M.W.A. Verstegen. 1994. Effect of dietary protein source on feed intake, growth, pancreatic enzyme activities and jejunal morphology in newly-weaned pigs. Br. J. Nutr. 72, 353-368.

Manzanilla, E. G., J. F. Perez, M. Martin, C. Kamel, F. Baucells, and J. Gasa. 2004. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. J. Anim. Sci. 82:3210–3218.

Mattson M.P., and A. Cheng. 2006. Neurohormetic phytochemicals: Low-dose toxins that induce adaptive neuronal stress responses. Trends Neurosci. 29:632–639.

Mattson, M.P. 2008. Hormesis defined. Ageing Res. Rev. 7 (1): 1-7.

Mayer, E.A. 2000. The neurobiology of stress and gastrointestinal disease. Gut. 47: 861-869.

Manzanilla, E.G., J.F. Perez, M. Martin, C. Kamel, F. Baucells, and J. Gasa. 2004. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. J. Anim. Sci. 82: 3210-3218.

Miller, B.G., C.T. Whittemore, C.R. Stokes, and E. Telemo. 1994. The effect of delayed weaning on the development of oral tolerance to soya-bean protein in pigs. Br. J. Nutr. 71: 615-625.

McLamb, B.L., A.J. Gibson, E.L.Overman, C. Stahl, and A.J. Moeser. 2013. Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic *E. coli* challenge and exacerbates intestinal injury and clinical disease. PLOS ONE. 8(4): e59838.

McGlone J.J., W.F. Stansbury, and L.F. Tribble. 1987. Effects of heat and social stressors and within-pen weight variation on young pig performance and agonistic behavior. J. Anim. Sci. 65: 456-462.

Mcpherson, A.J., K.D. McCoy, F.E. Johansen, and P. Brandtzaeg. 2008. The immune geography of IgA induction and function. Mucosal Immun. 1: 11-22.

Moeser, A.J., C.V. Klok, K.A. Ryan, J.G. Wooten, and D. Little. 2007. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am. J. Physiol. Gastrointest. Liver Physiol. 292: G173–G181.

Moughan, P.J., M.J. Birtles, P.D. Cranwell, W.C. Smith, and M.Pedraza. 1992. The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. World Rev. Nutr. Diet. 67: 40-113.

Murphy, K. 2012. The mucosal immune system. In: Janeway's Immunobiology (8th edition). Garland Science, New York, USA. p. 465-508.

Nagae, S., M. Ushijima, S. Hatono, J. Imai, S. Kasuga, H. Matsuura, Y. Itakura, and Y. Higashi. 1994. Pharmacokinetics of the garlic compound S-allyl cysteine. Planta Med. 60:214-217.

Nusrat, A, J. R. Turner, and J. L. Madara. 2000. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am. J. Physiol. Gastrointest. Liver Physiol. 279:G851-G857.

Overman, E.L., J.E. Rivier, and A.J. Moeser. 2012. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-a. PLOS ONE. 7(6): e39935.

Olukosi, O.A., and N. D. Dono. 2014. Modification of digesta pH and intestinal morphology with the use of benzoic acid or phytobiotics and the effects on broiler chicken growth performance and energy and nutrient utilization. J. Anim. Sci. 92:3945–3953.

Pearce, S.C., V. Mani, T. E. Weber, R. P. Rhoads, J. F. Patience, L. H. Baumgard, and N. K. Gabler. 2013a. Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs. J. Anim. Sci. 91: 5183-5193.

Pearce, S.C., V. Mani, R. L. Boddicker, J. S. Johnson, T. E. Weber, J. W. Ross, R. P. Rhoads, L. H. Baumgard, and N. K. Gabler. 2013b. Heat stress reduces intestinal barrier integrity and favors intestinal glucose transport in growing pigs. PLOS ONE. 8(8): e70215.

Pearce, S.C., M. V. Sanz-Fernandez, J. H. Hollis, L. H. Baumgard, and N. K. Gabler. 2014. Short-term exposure to heat stress attenuates appetite and intestinal integrity in growing pigs. J. Anim. Sci. 92: 5444-5454.

Pedraza-Chaverrí, J., M. Granados-Silvestre,O. N. Medina-Campos, P. D. Maldonado, I. M. Olivares-Corichi, and M. E. Ibarra-Rubio. 2001. Post-transcriptional control of catalase expression in garlic-treated rats. Mol. Cell. Biochem. 216: 9-19.

Pestova, M.I., R.E. Clift, J.R. Vickers, M.A. Franklin, and A.G. Mathew. 2000. Effect of weaning and dietary galactose supplementation on digesta glycoproteins in pigs. J. Sci. Food Agric. 80: 1918-1924.

PIC. 2013. Wean-to-Finish Manual. PIC, Hendersonville, TN, USA.

Pie, S., J.P. Lalles, F. Blazy, J. Laffitte, B. Seve, and I.P. Oswald. 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. J. Nutr. 134: 641-647.

Pluske, J. R., D. J. Hampson, and I. H. William. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: A review. Livest. Prod. Sci. 51:215-236.

Pushpendran, C.K., T.P.A. Devasagayam, G.J. Chintalwar, A. Banerji, and J. Eapen. 1980. The metabolic fate of [-35S]-diallyl disulfide in mice. Experientia. 36:1000-1001.

Qureshi, A. A., N. Abuirmeileh, Z. Z. Din, C. E. Elson, and W. C. Burger. 1983. Inhibition of cholesterol and fatty acid biosynthesis in liver enzymes and chicken hepatocytes by polar fractions of garlic. Lipids 18:343–348.

Ramiah, S. K., I. Zulkifli, N. Asyikin, A. Rahim, M. Ebrahimi, and G. Y. Meng. 2014. Effects of two herbal extracts an virginiamycin supplementation on growth performance, intestinal microflora population and fatty acid composition in broiler chickens. Austr. Asian J. Anim. Sci. 27: 375-382.

Rao, R. R., K. Platel, and K. Srinivasan. 2003. In vitro influence of spices and spice-active principles on digestive enzymes of rat pancreas and small intestine. Nahrung 47:408–412.

Ried, K., C. Toben, and P. Fakler. 2013. Effect of garlic on serum lipids: an updated meta-analysis. Nutr. Rev. 71(5):282–299.

Renaudeau, D., C. Anais, L. Tel, and J. L. Gourdine. 2010. Effect of temperature on thermal acclimation in growing pigs estimated using a nonlinear function. J. Anim. Sci. 88:3715-3722.

Robyn, J., G. Rasschaert, D. Hermans, F. Pasmans, and M. Heyndrickx. 2013. Is allicin able to reduce *Campylobacter jejuni* colonization in broilers when added to drinking water? Poult. Sci. 92:1408-1418.

Rodehutscord, M., and H. Kluth. 2002. Tierfu¨ tterung ohne antibiotisch wirkende Leistungsfo¨rderer. Zuchtungskunde 74:445–452.

Roth, F. X., and M. Kirchgessner. 1998. Organic acids as feed additives for young pigs: Nutritional and gastrointestinal effects. J. Anim. Feed Sci. 8:25–33.

Ruell, P., K. Hoffman, C. Chow, and M. Thompson. 2004. Effect of temperature and duration of hyperthermia on HSP72 induction in rat tissues. Mol. Cell. Biochem. 267:187-194.

Sambuy, Y., I. Angelis, G. Ranaldi, M.L. Scarino, A. Stammati, and F. Zucco. 2005. The Caco-2 as a model of the intestinal barrier and cell-culture factors on Caco-2 cell functional characteristics. Cell Biol. Toxicol. 21: 1-26.

Sangild, T., M. Silver, A.L.Fowden, A. Turvey, and B. Foltmann. 1994. Adrenocortical stimulation of stomach development in the prenatal pig. Biol. Neonate. 65(6): 378-389.

Schneedberger, E.E., and R.D Lynch. 2004. The tight junction: a multifunctional complex. Am. J. Physiol. Cell Physiol. 268: C1212-C1228.

Seskeviciene, J., J. Jankowski, and K. Kozlowski. 2005. Effect of probiotic preparation and phytogeneous feed additive on digestibility of nutrients, metabolizability of gross energy and content of metabolizable energy of a practical feed ration for fattening turkeys. Arch. Geflugelkd. 69:107–109.

Shanmugavelu, S., J.D. Brooker, T. Acamovic, and A.J. Cowieson. 2004. Spring meeting of the WPSA UK branch papers. Brit. Poult. Sci. 45 (Suppl. 1): S9-S10.

Shepherd, E.J., P.A. Helliwell, O.J. Mace, E.L. Morgan, N. Patel, and G.L. Kellett. 2004. Stress and glucocorticoid inhibit apical GLUT2-trafficking and intestinal glucose absorption in rat small intestine. J. Physiol. 561(1): 281-290.

Smith, F., J.E. Clark, B.L. Overman, C.C. Tozel, J.H. Huang, J.E. Rivier, A.T. Blikslager, and A.J. Moeser. 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 298(3):G352-G363.

Son, T.G., S. Camandola, and M.P. Mattson. 2008. Hormetic dietary phytochemicals. Nueromol. Med. 10(4): 236-246.

Spreeuwenberg, M. A. M., J. M. A. J. Verdonk, H. R. Gaskins, and M.W. A. Verstegen. 2001. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. J. Nutr. 131:1520-1527.

Taras, D., W. Vahjen, M. Macha, and O. Simon. 2006. Performance, diarrhea incidence, and occurrence of *Escherichia coli* virulence genes during long-term administration of a probiotic *Enterococcus faecium* strain to sows and piglets. J. Anim. Sci. 84: 608-617.

Tatara, M.R., E. Śliwa, K. Dudek, A. Gawron, T. Piersiak, P. Dobrowolski , J. Mosiewicz, , A. K. Siwicki, and T. Studziński T. 2008. Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared on artificial sow. Ann. Agric. Environ. Med. 15: 63–69.

Thacker, P.A. 1999. Nutritional requirements of early weaned pigs: a review. Pig News Information 20, 13N-24N.

Touchette, K.J., J.A. Carroll, G.L. Allee, R.L. Matteri, C.J. Dyer, L.A. Beausang, and M.E. Zannelli. 2002. Effect of spray-dried plasma and lipopolysaccharide exposure on weaned pigs: Effects on the immune axis of weaned pigs. J. Anim. Sci. 80: 494-501.

Van Dijk, A.J., H. Everts, N.J.A. Nabuurs, R.J.C.F. Margry, and A.C. Beynen. 2001. Growth performance of weaned pigs fed spray dried animal plasma: a review. Livestock Prod. Sci. 68: 263-274.

Van Nevel, C.J., J.A. Decuypere, N. Dierick, and K. Molly. 2003. The influence of Lentinus edodes (Shiitake mushroom) preparations on bacteriological and morphological aspects of the small intestine in piglets. Archives fu¨r Tierernahrung 57: 399-412.

Van Nevel, C.J., J.A. Decuypere, N.A. Dierick, and K. Molly. 2005. Incorporation of galactomannans in the diet of newly weaned piglets: effect on bacteriological and some morphological characteristics of the small intestine. Arch. Anim. Nutr. 59: 123-138.

Van der Meulen J., S.J. Koopmans, R.A. Dekker, and A. Hoogendoorn. 2003. Weaning at 4 or 7 weeks: consequences for intestinal function and growth. In: R.O. Ball, editor, Proceedings of the 9th International Symposium on Digestive Physiology in Pigs, Banff, AB, Canada. p. 140–142.

Viveros, A., S. Chamorro, M. Pizarro, I. Arija, C. Centeno, and A. Brenes. 2011. Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks. Poult Sci. 9:566–578.

Wei, Z., and B.H., Lau. 1998. Garlic inhibits free radical generation and augments antioxidant enzyme activity in vascular endothelial cells. Nutr. Res. 18:61–70.

Wijtten, P. J., J. van der Meulen, and M. W. Verstegen. 2011. Intestinal barrier function and absorption in pigs after weaning: A review. Br. J. Nutr. 105(7):967–981.

Windisch, W., K. Schedle, C. Plitzner, and A. Kroismayr. 2008. Use of phytogenic products as feed additives for swine and poultry. J. Anim. Sci. 86: E140-E148.

Yan, L., Q.W. Meng, and I.H. Kim. 2012. Effects of fermented garlic powder supplementation on growth performance, nutrient digestibility, blood characteristics, and meat quality in grow-finish pigs. Anim. Sci. J. 83: 411-417.

Yang, P.C., S.H. He, and P.Y. Zheng. 2007. Investigation into the signal transduction pathway via which heat stress impairs intestinal epithelial barrier function. Gastroentero. 22: 1883-1831.

Yamasaki, T., and B.H. Lau. 1997. Garlic compounds protect vascular endothelial cells from oxidant injury. Folia Pharmacol. Jpn.110(Suppl.1):138–141.

Yeh, Y. and S. Yeh. 1994. Garlic reduces plasma lipids by inhibiting hepatic cholesterol and triacylglycerol synthesis. Lipids 29: 189–193.

Yen, J. 2001. Anatomy of the digestive system and nutritional physiology. In: A.J. Lewis and L.L. Southern, editors, Swine Nutrition. CRC Press, New York, USA. p. 19-30.

Youn, H.S., S.I. Saitoh, K. Miyake, and D.H. Hwang. 2006. Inhibition of homodimerization of Toll-like receptor 4 by curcumin. Biochem Pharmacol. 72:62–69.

Youn, H.S., J.Y. Lee, K.A. Fitzgerald, H.A. Young, S. Akira, and D.H. Hwang. 2005. Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: Molecular targets are TBK1 and RIP1 in TRIF complex. J Immunol.175:3339-3346.

Youn, H.S., J.K. Lee, and Y.J. Choi. 2008. Cinnamaldehyde suppresses toll-like receptor 4 activation mediated through the inhibition of receptor oligomerization. Biochem. Pharmacol.75:494–502.

Zanello, G., M. Berri, J. Dupont, P. Sizaret, and R. D'Inca. 2011. Saccharomyces cerevisiae modulates immune gene expressions and inhibits ETEC mediated ERK1/2 and p38 signaling pathways in intestinal epithelial cells. PLoS ONE 6(4): e18573.

Zhao, L., J.Y. Lee, and D.H. Hwang. 2011. Inhibition of pattern recognition receptor-mediated inflammation by bioactive phytochemicals. Nutr. Rev. 69(6): 310-320.

Zhu, L.H., K. L. Zhao, X. L. Chen, and J. X. Xu. 2012. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. J. Anim. Sci. 90: 2581-2589.

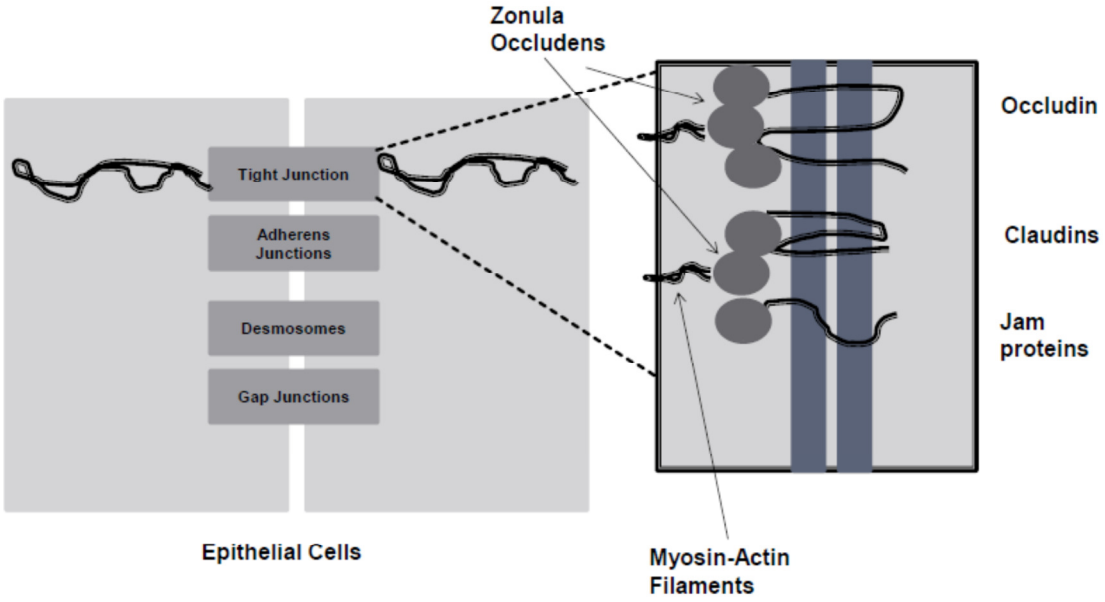

Figure 1-1. The tight junction complex. The gut barrier includes protein complexes such as tight junctions, adherens junctions, desmosomes, and gap junctions that span epithelial cells. The tight junction complex consists of 3 major families of proteins that include: Occludins (gate-keeper proteins), Claudins (selective pore proteins), and zonula occludens (control proteins). Membrane spanning proteins are anchored to intracellular myosin-actin filaments through zonula occludens.

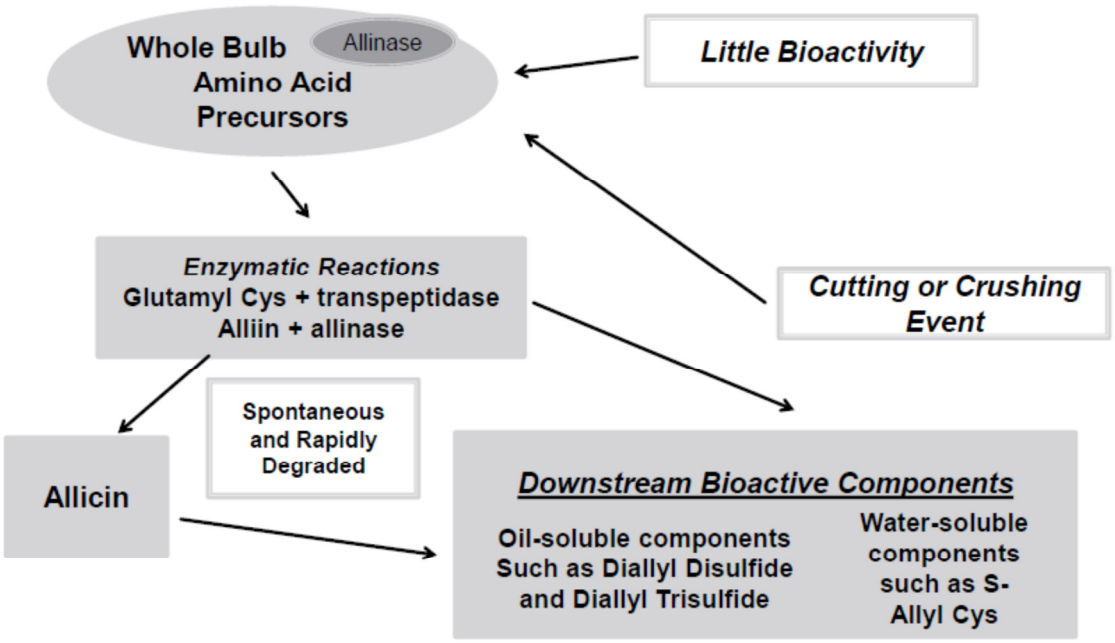

Figure 1-2. Garlic chemistry and bioactivity.

CHAPTER 2. IMPACT OF ACUTE WATER AND FEED DEPRIVATION EVENTS ON GROWTH PERFORMANCE, INTESTINAL CHARACTERISTICS AND SERUM STRESS MARKERS IN WEANED PIGS

2.1. Abstract

The impact of acute stressors (24-h feed or water deprivation) on growth performance, intestinal characteristics and serum stress markers in weaned pigs was evaluated. Pigs (6.21 ± 0.29 kg) were allotted in a randomized complete block design to four treatments on the basis of body weight at the time of weaning. There were 8 mixed-sex pigs in each of 12 pens per treatment. Treatments were arranged as a 2 × 2 factorial and consisted of a feed or water stressor that included a 0- or 24-h deprivation period post-weaning and pigs were subsequently allowed access to feed and water. Growth performance was measured 1, 7, 14, and 28 d post-weaning. Serum and intestinal samples were taken 1 and 7 d post-weaning. Serum was analyzed for cortisol and corticotrophin releasing factor and villus height, crypt depth, and mast cell density were measured in the jejunum and the ileum. Expression of mucin (MUC2), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1) genes were measured on d 1 and 7 post-weaning in the jejunum and ileum by real-time PCR. There was a decrease ($P < 0.05$) in ADG with the water stressor 1-d post weaning although subsequently there were improvements ($P <$

0.05) in ADG and feed efficiency. Furthermore, the water stressor reduced ADFI during the last 14 d of the trial and cumulatively ($P < 0.05$). Seven d post-weaning there was an increase ($P < 0.05$) in jejunal villous height to depth ratio due to the feed stressor and a decrease ($P < 0.05$) in the ileal villous height to depth ratio due to the water stressor. There was an increase ($P < 0.05$) in serum cortisol levels due to the water stressor both 1 and 7 d post-weaning. Furthermore, there was an increase in serum corticotrophin releasing factor 1 but not 7 d post-weaning due to the water stressor ($P < 0.05$). The feed stressor reduced ($P < 0.05$) TNF-α gene expression and the water stressor reduced ($P < 0.05$) OC gene expression in the jejunum 1 d post weaning. In the ileum, there was a reduction in CL-1 and ZO-1 gene expression ($P < 0.05$) due to the water stressor 7 d post weaning. The results from the current investigation showed that a 24-h feed and water deprivation at the time of weaning has negative impacts on growth performance, intestinal characteristics and serum stress responses immediately following the stress event and throughout the nursery period.

2.2. Introduction

Weaning is a stressful event in pigs and is associated with psychological, environmental, and dietary stressors (Lalles et al., 2004). Brooks et al. (2001) suggested that most pigs consume minimal feed during the first 24-h post-weaning and approximately 10% of pigs do not consume any feed during the first 48-h post-weaning. Weaning stress-induced reduction in feed intake is marked by villous atrophy and crypt hyperplasia that contribute to short- and long-term negative impacts on growth performance, nutrient absorption, gut secretions, scours, and disease susceptibility in weaned pigs (Pluske et al., 1997; Lalles et al., 2004; Wijtten et al., 2011). Previous research shows a link between water flow rate and feed efficiency suggesting a relationship between water intake and growth efficiency in nursery pigs (Neinaber and Hahn, 1984) that may be related to gastrointestinal dysfunction. A dearth of information exists on the impact of limited water intake at weaning, either during transport or from stress-induced lethargy, on nursery pig performance and gastrointestinal (GI) health. Studies have shown a link between weaned pig stress, serum cortisol and CRF, and gut dysfunction (Moeser et al., 2007; Smith et al., 2010; Overman et al., 2012). Previous research shows a link between stress and inflammatory cytokine level in early-weaned pigs (Hu et al., 2013), which can impact gastrointestinal permeability and function by altering tight junction proteins (Lambert et al., 2009; Hu et al., 2013).

Several studies show a link between nursery pig stress and feed consumption which relates to gastrointestinal function and furthermore previous literature suggests a link between water consumption and feed intake in weaned pigs (Pluske et al., 1997;

Patience, 2012). Therefore in the current experiment, our objective was to investigate the impact of an acute (24-h) feed, water, or feed plus water deprivation events in pigs immediately post-weaning and we hypothesized that the acute stressors would affect growth performance, gut morphology, serum stress markers, and intestinal cytokine and tight junctions during the nursery period. To achieve our objectives we measured growth performance, intestinal morphology, intestinal cytokine and tight junction gene expression, and serum cortisol and CRF.

2.3. Materials and methods

All animal procedures were approved by the JBS United Animal Care and Use Committee.

Animals and treatments

A total of 384 PIC 337 × C29 weaned, mixed-sex pigs (initial BW = 6.21 ± 0.29 kg, 19 d old) were used in a randomized complete block design with BW as the blocking factor. The pigs were housed in pens (1.21 × 1.11 m) equipped with one cup drinker and one feed trough. Room temperature was 34.4°C at weaning and reduced by 0.5°C each subsequent day of the trial. There were 4 treatments arranged as a 2 × 2 factorial that consisted of 1) control (no feed or water deprivation), 2) 24-h feed deprivation event, 3) 24-h water deprivation event, and 4) 24-h feed plus water deprivation event. There were 12 replicate pens per treatment with 8 mixed-sex pigs per pen. Deprivation events were administered immediately following weaning and following the deprivation events, pigs were returned to normal management procedures.

Following the stressor, pigs were allowed free access to feed and water and were fed a mash-based feeding program that met or exceeded NRC requirements (NRC, 2012) and consisted of starter, transition and late nursery diets for 7, 7, and 14 d post-weaning, respectively (Table 2-1). Pigs were vaccinated for circovirus and mycoplasma on d 0 and 14 post-weaning (Circumvent, Merck /Animal Health). Pigs and feeders were weighed on 1, 7, 14, and 28 d post-weaning to calculate ADG, ADFI, and G:F for four growth phases which included: phase 1 (0 to 1 d post weanng), phase 2 (2 to 7 d post weaning), phase 3 (8 to 14 d post weaning), and phase 4 (15 to 28 d post weaning). On 1 and 7 d post-weaning, 1 pig per pen was randomly selected, blood was collected via venipuncture into serum collection tubes (CORVAC serum separator tube, Tyco Healthcare Group LP, Mansfield, MA, USA), and subsequently euthanized via CO_2 asphyxiation. Immediately following euthanasia, 10-cm segments of the jejunum and ileum were excised for histological and gene expression measurements.

Serum stress markers

Blood was collected at approximately the same time each day to minimize the effect of diurnal rhythms. Following blood collection serum was separated via centrifugation (20 min, 10,000 × g at 4°C) and stored at -80°C until analysis. Serum levels of CRF and cortisol were determined using commercial ELISA kits (CRF: Phoenix Pharmaceuticals, Belmont, CA; cortisol: R&D systems, Minneapolis, MN).

Histological measurements

Excised intestinal segments were flushed in ice-cold 10% phosphate-buffered saline, stapled to a cardboard background, and fixed in 10% buffered formalin for 4 d. Samples were subsequently dehydrated with ethanol, cleared with Sub-X®

(Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). Segments were sliced (5 µm) and stained with hematoxylin and eosin by the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth were measured and villus height to crypt depth ratio was calculated. Means from at least 4 villi per segment were analyzed for differences. Villus length is defined as the length from the villus tip to the valley between villi and crypt depth is defined as the length between the crypt opening and base.

Mast cell counts

Frozen sections (4 µm) from the ileum were prepared and fixed in Carnoy's fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 10 min. Sections were then stained with 0.50% toluidine blue in 0.50 N HCl at room temperature for 30 min. Sections were viewed at the 20X objective and counted using a grid. There were 6 slides per treatment group for each of 1 and 7 d post-weaning with 4 viewing fields selected per slide. Cell density is expressed as ileal mucosal mast cells per mm^2 tissue. Due to sample availability only ileal mast cell density was determined.

RNA isolation, cDNA Synthesis, and real-time PCR

Immediately after removal, intestinal segments were cut in half, exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were immediately placed in 5 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C until RNA isolation. RNA was extracted according to the Trizol protocol (Invitrogen, Grand Island, NY). Reverse transcription was carried out using the MMLV reverse transcription system of Promega and real-time PCR was performed on

a Bio-Rad iCyler using a reaction mix that consisted of 0.50 µg cDNA, 0.075 nmol of each forward and reverse primers, SYBR Green Master (Roche, Basel, Switzerland), and nuclease-free water (Ambion, Austin, TX) to reach a reaction volume of 20 µl per well. Following a 5-min incubation period at 95ºC, reactions were cycled 50 times using the following protocol: 10s at 95ºC, 20 s at 55ºC, and 72ºC for 30 s. Gene expression of mucin (MUC2), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1) were measured using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping control. Primers used are presented in Table 2-2.

Statistical analysis

Data was analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) with the fixed effects of feed and water stressor and the random effect of block. The following statistical model was used in the analysis: $Y_{ijk} = \mu + W_i + F_j + B_k + (WF)_{ij} + \varepsilon_{ijk}$ where Y is the response criterion; μ is the overall mean; W_i is the effect of i^{th} water stressor (i = 1, 2); F_j is the effect of j^{th} feed stressor (j = 1, 2); B_k is the effect of k^{th} block (k = 1, 2, 3, ……., 12); $(WF)_{ij}$ is the interaction effect between i^{th} water stressor and j^{th} feed stressor; and ε_{ijk} is the error term. Simple effect means were separated using the PDIFF option in SAS where there was an interaction of water and feed stressors. Significant difference was defined as $P \leq 0.05$ and $0.05 \leq P \leq 0.10$ was considered a tendency. Model assumptions for normality and equal variances were validated by the Shapiro-Wilks test (Proc Univariate) and Brown and Forsythe test (Proc GLM), respectively.

2.4. Results

Growth performance (Table 2-3) was recorded on 1, 7, 14, and 28 d post-weaning that correspond to phases 1, 2, 3, and 4, respectively. On 1 d post-weaning, immediately following the stressors, there was a reduction in final body weight (FBW) and ADG ($P < 0.01$) due to the water stressor. During the second growth phase, there was no effect of the feed stressor or feed × water interaction although there was an improvement in ADG and G:F in pigs due to the water stressor ($P < 0.01$). There were no performance differences during phase 3. During phase 4 of the experiment, there were performance differences due to the previous stressor that coincided with an increase in barn temperature due to unusually warm weather. There was an effect of the water stressor on phase 4 ADFI and cumulative ADFI ($P = 0.02$). Furthermore, there was a tendency for the water stressor to reduce phase 4 BW and ADG ($P < 0.10$) and cumulative ADG ($P = 0.06$).

Intestinal histology measurements are reported in Table 2-4. In the jejunum, the average villous heights were 271 and 312 µm and average crypt depths were 96 and 134 µm on 1 and 7 d post-weaning, respectively. There were no differences in jejunum villous height immediately following the stressors, however, there was a tendency ($P = 0.08$) for an increase in villous height due to the feed stressor 7 d post-weaning. For jejunal crypt depth, there were no differences immediately following the stressors, although there was a tendency ($P = 0.06$) for the water stressor and an effect of the feed stressor ($P = 0.03$) to reduce crypt depth 7 d post-weaning. There was a significant impact of the feed stressor to increase the villous height to depth ratio ($P = 0.01$) 7 d

post-weaning. In the ileum the average villous height was 353 and 364 µm and average crypt depth was 87 and 90 µm on 1 and 7 d post-weaning, respectively. There were no differences in ileal villous height immediately following the stressor, however, there was a tendency ($P = 0.07$) for the feed stressor to reduce height and a feed × water interaction ($P = 0.03$) 7 d post-weaning. There was a tendency for an increase ($P = 0.06$) in crypt depth immediately following the feed stressor (1 d post weaning), although no differences in crypt depth were observed 7 d post weaning. Villous height to depth ratio immediately following the stress events was not affected by treatment, however, there was a decrease ($P = 0.03$) in the height to depth ratio due to the water stressor 7 d post-weaning.

Serum cortisol and CRF data are reported in Table 2-4. The average serum cortisol concentrations were 28.15 and 32.95 ng/mL on 1 and 7 d post-weaning, respectively, and the average serum CRF levels were 0.39 and 0.28 ng/mL on d 1 and 7 post-weaning, respectively. There was an increase ($P < 0.05$) in serum cortisol due to the water stressor on both 1 and 7 d post-weaning. Furthermore, there was an increase ($P = 0.02$) in serum CRF due to the water stressor 1 d but not 7 d post-weaning.

The average mast cell densities, determined only in ileal mucosal tissues on 1 and 7 d post-weaning, were 244.9 and 206.4 cells/mm^2 , respectively (Figure 2-1). Immediately following the stress event there was no effect of the feed, water, or a feed × water stressor interaction on mast cell density. There was a tendency ($P = 0.10$) for mast cell density to be higher due to the feed stressor on 7 d post-weaning, although there was no effect of the water stressor or a feed × water stressor interaction.

Gene expression data are reported in Tables 2-5 and 2-6. Immediately following the feed stressor in the jejunum, there was a decrease in TNF-α gene expression (P = 0.01) and tendency for a decrease in IL-6 gene expression (P = 0.07). Furthermore, there was an effect of the water stressor to increase expression of MUC2 (P = 0.05) and to decrease expression of OC (P = 0.02). Conversely, in the ileum immediately following the stress event, there was an effect of the water stressor to increase OC gene expression (P = 0.03). Seven days post weaning there was no impact of stressors on jejunal gene expression although there was a reduction in ileal CL-1 and ZO-1 expression (P = 0.01) due to the water stressor.

2.5. Discussion

Literature suggests a link among weaned-pig gut function, feed intake and gut fill, and weaning stressors (Pluske et al., 1997; Spreeuwenberg et at. 2001; Lalles et al., 2004). According to Brooks et al. (2001), approximately 50 and 10% of pigs do not consume feed within 24 and 48 h post-weaning, respectively. Limited research exists investigating the impact of dehydration stress on nursery pig performance and gut function although dehydration is a concern in nursery pigs due to stress-induced lethargy and lack of access to water during post-weaning transit.

In the current experiment our aim was to induce an acute stress event without causing moderate or severe mortality and morbidity. Overall pig performance in the current trial was good with mortality below 1% (2 pigs) and fall-off pigs (visual morbidity) at 6% (22 pigs). Stressors were administered for 24 h post-weaning, which

was determined at the time of separation from the sow (0800 h) through the following morning. The feed stressor had no impact on immediate ADG although there was an impact of water deprivation on ADG with the water and feed plus water-deprived pigs losing 80 and 82% more weight relative to non-stressed pigs, respectively. The minimal feed intake and gain observed in the current trial during the first day post-weaning is consistent with the "transient growth check" period as reported in the literature (Pluske et al., 1997; Lalles et al., 2004). During the second phase of the trial (1 to 7 d post-weaning) there was no impact of the previous feed stressor on performance, although there was compensatory gain and a subsequent improvement in feed efficiency due to the water stressor. Indications from the daily experimental notes were that water-stressed pigs had increased water intake for up to 36 h following the 24-h water stressor. Cumulatively, pigs deprived of water and feed plus water tended to be lighter and had increased ADFI, which can mainly be accounted for by performance differences during phase 4 of the experiment.

Changes in gut morphology at weaning, largely due to limited feed intake, have been reported (Pluske et al., 1997). Post-weaning villous atrophy may be attributed to epithelial cell loss or reduced cell renewal; the latter is generally associated with deeper crypts (Pluske et al., 1997). In the current study, there were no consistent effects of treatment on gut histology immediately following the stressors. It is likely that the stressors were not severe enough to cause an immediate effect on histological measurements. Conversely, histological measurements on 7 d post-weaning in the jejunum showed the feed stressor increased villous height to depth ratio, suggesting a compensatory response in the 6 d following. In the ileum 7 d post-weaning the water

stressor reduced villous height to depth ratio, which is largely attributed to reduced villous height. The feed × water interaction at 7 d post-weaning for villous height suggests that water stressor attenuated the tendency for an increase in villous height in feed-stressed pigs. The tendency for an increase in ileal villous height 7 d post-weaning is not clear because it is not supported by changes in feed intake patterns although histological changes may be due to compensatory gain observed in water-stressed pigs. These data suggest that acute feed and water stressors impact GI morphology in a site-dependent manner. Relative to information from the literature in nursery pigs, the overall means for villous height in the proximal and distal segments of the intestine were slightly lower although the mean crypt depth values were similar to those reported in literature (Pluske et al., 1997; Moeser et al., 2007; Yang et al., 2012). The reduced villous heights in the current study relative to other published studies are likely due to differences in analytical methodology. Generally, villous height decreases distally in the gut, but this was not the case in the current study. It is possible that the aforementioned villi characteristics vary in newly-weaned pigs due to rapid changes in gut morphology and similar results have been reported by Zijlstra et al. (1996). Physiological stressors at weaning are generally linked to reduced voluntary feed intake (Pluske et al., 1997). In the current study however, the water stressor affected gut morphology despite the fact that the stressor did not affect feed intake during the time of intestinal sampling. Moeser et al. (2007) reported no changes in gut morphology in pigs exposed to an early weaning stress, although Albinsson and Anderson (1990) reported a decrease in crypt-cell proliferation in weaned pigs suffering from post-weaning stressors up to 5 wk post-weaning.

Previous research in pigs and rodents show a link between weaning stressors and changes in central and peripheral stress mediators (Aisa et al., 2008; Smith et al., 2010). In the current study, serum stress markers cortisol and CRF were measured immediately and 6 d following the stress events (1 and 7 d post weaning) as indicators of stress markers that impact gut permeability. There was no impact of the feed stressor or feed × water stressor interaction on serum cortisol and CRF. Immediately following the water stressor there was a 60% increase in both serum cortisol and CRF. Furthermore, the water stressor increased serum cortisol, but not CRF, 7 d post-weaning. The cortisol and CRF concentrations observed in the current study are similar as reported by Wu et al. (2000) and Moeser et al. (2007) for CRF and Jiang et al. (2000) for cortisol. Previous research shows that a surge in cortisol and CRF is common at weaning (Worsae and Schmidt, 1980; Moeser et al., 2007) and activation of CRF and glucocorticoid receptors results in increased gut permeability and secretions (Moeser et al., 2007; Teitelbaum et al., 2008; Smith et al., 2010). Furthermore, research by Wu et al. (2000) suggested that a post-weaning cortisol surge supports greater arginine synthesis through increased citrulline production, which supports various metabolic and immune processes in nursery pigs. Moeser et al. (2007) showed that the relationship between CRF and gut permeability was more strongly correlated compared to cortisol, which suggests that CRF is a better indicator of the impact of a stressor on gut permeability. Mast cells are part of the innate immune system, and upon activation release various inflammatory mediators. Inflammatory mediators including histamine, cytokines, and proteases have been shown to influence intestinal permeability and further recruit immune cells (Metcalfe et al., 1997). Smith et al. (2010) and Overman et al. (2012) reported that

CRF binds to receptors on mast cells causing activation and degranulation, which influences porcine intestinal permeability. In the present study, an increase in CRF was observed immediately following the water stressor only and serum cortisol was elevated due to the water stressor in pigs at both sampling time points indicating that acute water deprivation affects stress mediators immediately following the event and subsequently for at least 6 d. The reason for a lack of increase in serum CRF due to the water stressor 7 d post-weaning is not fully known although it is known that a negative feedback loop exists in which elevated cortisol levels suppress central CRF release (Smith et al., 2010).

Previous research shows a link between elevated levels of stress mediators and increased mast cell activation and hypertrophy (Santos et al., 2001; Smith et al., 2010). In the current experiment, there was no effect of treatment on mast cell density in the ileum immediately following the stressor, however, there was a tendency for increased mast cell density due to the feed stressor 7 d post-weaning. Smith et al. (2010) showed an increase in mucosal mast cell density, several days post stress event, due to an early weaning stressor that correlated with elevated CRF and cortisol. Furthermore, Santos et al. (2001) observed mucosal mast cell hyperplasia in chronically-stressed rats. The lack of significant differences in the current experiment may be due to the acute nature of the stressors and lack of a long-term spike in serum CRF. More specifically, the serum data from the current experiment, along with low morbidity and mortality, suggests the events induced stress during the time of deprivation, were quickly alleviated following deprivation, and did not induce chronic stress. The tendency for an increase mast cell density in the ileum due to the feed stressor 7 d post-weaning suggests the feed stressor

impacts mast cell hyperplasia differently than the acute water stressor although further investigation is needed.

Tight junctions form a selective paracellular barrier and are influenced by GI cytokines and enzymes (Li et al., 2012). A link has been established between intestinal tight junction gene expression and intestinal permeability (Hu et al., 2013). Hu et al. (2013) showed that in early weaned pigs, ZO-1, OC, and CL-1 gene expression decreased following weaning and coincided with negative changes in intestinal permeability. In the current study there was a decrease in jejunal but increase in ileal OC gene expression immediately following the water stressor. The discrepancy between jejunal and ileal OC gene expression following the water stressor is not clear and warrants further investigation. Furthermore, there was a decrease in ileal CL-1 and ZO-1 gene expression 7-d post-weaning due to the water stressor. Previous research in environmentally-stressed nursery pigs shows a link between CRF, mast cell activation, and gut permeability (Moeser et al., 2007; Smith et al., 2010; Overman et al., 2012). Additionally, there is clearly a link between inflammatory cytokines such as TNF-α and IL-6 and tight junction protein structure (Ma et al., 2005). Previous research showed that IFN-γ reduced ZO-1 gene expression (Blikslager et al., 2007) and recently, a link has been established between increased TNF-α and IL-6 gene expression and decreased ZO-1 and claudin-1 gene expression in weaned pigs (Hu et al., 2013). In the current study there was an impact of the feed stressor and a feed × water interaction for reduced TNF-α gene expression immediately following the stressor. Likewise, there was a tendency for a decrease in IL-6 gene expression due to the feed stressor immediately following the event. The decrease in the aforementioned cytokines may be due to the

lack of partially-digested feed ingredients in the gut of feed-deprived pigs, such as soy proteins, that have been shown to elicit an immune response (Friesen et al., 1993; Engle, 1994). The reason there was no increase in inflammatory cytokine expression in the current study may be due to the acute nature of the stressors or intestinal sampling time. Mucins are an integral part of the gastrointestinal mucus layer and changes in mucin gene expression could result in changes in nutrient digestibility, mucus viscosity, and mucus barrier function (Horn et al., 2008). In the current study there was an increase in MUC2 gene expression due to the water stressor in the jejunum immediately following the stressor. To the author's knowledge there are no other reports linking water deprivation to changes in mucin gene expression. Increased MUC2 gene expression could be a stress coping or protective mechanism resulting in a thicker mucus layer, although further research is needed. In the current study there was a link between the water stressor and decreased tight junction gene expression although there was not an increase in cytokine gene expression or mast cell density. This could be attributed to the acuteness of the stressor when compared to other published literature (Smith et al., 2010; Hu et al., 2013) and further research investigating time-dependent changes in gene expression following a stressor are needed.

An acute water but not feed stressor has short- and long-term impacts on growth performance. There was an increase in serum stress mediators cortisol and CRF following the water stressor indicating that water deprivation is a more potent psychological stressor when compared with feed deprivation. Furthermore, the water and feed stressors had implications on gut morphology that suggest site-specific changes. The water stressor affected tight junction gene expression following the

stressor although there was no increase in cytokine gene expression. These data suggest that an acute water deprivation event has an immediate and subsequent impact on nursery pig performance, serum stress markers, and gut morphology independent of a reduction in feed intake. Therefore, nutritional and management strategies should be adopted to minimize acute dehydration in nursery pigs at weaning.

2.6. Acknowledgments

The authors would like to express their appreciation to JBS United for their financial and technical support of this trial. Further appreciation is given to the employees of Burton Russell Farm at JBS United. The authors would also like to express appreciation to Aly Gautier, Dr. Meliza Ward, and Hang Lu, Purdue University Department of Animal Sciences, for their assistance with laboratory analyses and Tracy Wiegand, Purdue University Veterinary School, for her assistance with histological measurements.

2.7. References

Aisa B., R. Tordera, B. Lasheras, J. Del Rio , and M. J. Ramirez. 2008. Effects of maternal separation on hypothalamic-pituitary-adrenal responses, cognition and vulnerability to stress in adult female rats. Neuroscience 154: 1218–1226.

Albinsson, A.R.-M. and G.K.A. Andersson. 1990. Subclinical characteristics of the wasting pig syndrome. Res. Vet. Sci. 49, 71-76.

Blikslager, A.T., A.J. Moeser, J.L. Gookin, S.L. Jones, and J. Odle. 2007. Restoration of barrier function in injured intestinal mucosa. Physiol. Rev. 87: 545-564.

Brooks, P.H., C.A. Moran, J.D. Beal, V. Demeckova, and A. Campbell. 2001. Liquid feeding for the young piglet, in: Varley M.A., Wiseman J. (Eds.), The weaner pig: nutrition and management, CAB International, Wallingford, UK, pp. 153 -178.

Feldman G.J., J. M. Mullin, and M. P. Ryan. 2005. Occludin: structure, function and regulation. Adv. Drug Del. Rev. 57, 883-917.

Hall, G.A. and T.F. Byrne. 1989. Effects of age and diet on small intestinal structure and function in gnotobiotic piglets. Res.Vet. Sci. 47, 387-392.

Hu, C.H., K. Xiao, Z.S. Luan, and S. Jong. 2013. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. J. Anim. Sci. 91, 1093-1101.

Horn, N.L., S.S. Donkin, T.A. Applegate, and O. Adeola. 2008. Intestinal mucin dynamics: response of broiler chicks and White Pekin ducklings to dietary threonine. Poult. Sci. 88(9): 1906-1914.

Hampson, D.J. 1986. Alterations in piglet small intestinal structure at weaning. Res. Vet. Sci. 40, 32-40.

Lalles, J.P., G. Boudry, C. Favier, N. LE floc'h, I. Luron, L. Montagne, I.P. Oswald, S. Pie, C. Piel, and B. Seve. 2004. Gut function and dysfunction in young pigs: physiology. Anim. Res. 53: 301–316.

Lambert, G.P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J. Anim. Sci. 87 (E suppl.): E101-E108.

Li, X., S. Akhart, and M.A. Choudry. 2012. Alteration in intestine tight junction protein phosphorylation and apoptosis is association with increase in IL-18 levels following alcohol intoxication and burn injury. Biochem. Biophys. Acta. 1822: 186-203.

Ma, T., M.A. Biovin, D. Ye, A. Pedram, and H.M. Said. 2005. Mechanism of TNF-α modulation of Caco-2 intestinal epithelial tight junction barrier: Role of myosin light-chain kinase protein expression. Am. J. Physiol. 288: G422-G430.

Metcalfe, D.D., D. Baram, and Y.A. Mekori. 1997. Mast cells. Physiol. Rev. 77: 1033–1079.

Moeser, A.J., C.V. Klok, K.A. Ryan, J.G. Wooten, and D. Little. 2007. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am. J. Physiol. Gastrointest. Liver Physiol. 292: G173–G181.

NRC, 2012. Nutrient requirements of Swine. 11th rev ed. Natl. Acad. Press, Washington, D.C.

Neinaber, J.A. and G.L. Hahn. 1984. Effects of Water Flow Restriction and Environmental Factors on Performance of Nursery-Age Pigs. Publications from USDA-ARS / UNL Faculty. Paper 466.

Overman, E.L., J.E. Rivier, and A.J. Moeser. 2012. CRF Induces Intestinal Epithelial Barrier Injury via the Release of Mast Cell Proteases and TNF-a. PLoS ONE 7(6) e39935.

Patience, J.F. The importance of water in pork production. 2012. Anim. Front. 2(2):28-35.

Pluske, J.R., D.J. Hampson, and I.H. William. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review, Livest. Prod. Sci. 51, 215–236.

Santos, J., P.C. Yang, J.D. Soderholm, M. Benjamin, and M.H. Perdue. 2001. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. Gut. 49: 630-636.

Smith, F., J.E. Clark, B.L. Overman, C.C. Tozel, J.H. Huang, J.E. Rivier, A.T. Blikslager, and A.J. Moeser. 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 298(3):G352-G363.

Spreeuwenberg, M.A.M., J.M.A.J. Verdonk, H.R. Gaskins, and M.W.A. Verstegen. 2001. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. J. Nutr. 131: 1520-1527.

Teitelbaum, A.A., M.G. Gareau, J. Jury, P.C. Yang, and M.H. Perdue. 2008. Chronic peripheral administration of corticotropin-releasing factor causes colonic barrier dysfunction similar to psychological stress. Am. J. Physiol. Gastrointest. Liver Physiol. 295: G452–G459.

Wijtten, P.J., J. van der Meulen, and M.W. Verstegen. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. Br. J. Nutr.105 (7):967-81.

Worsae, H., and M. Schmidt. 1980. Plasma cortisol and behavior in early weaned piglets. Acta. Vet. Scand. 21(4): 640-657.

Wu, G., C. J. Meininger, K. Kelly, M. Watford, and S. M. Morris, Jr. 2000. A Cortisol surge mediates the enhanced expression of pig intestinal pyrroline-5-carboxylate synthase during weaning. J. Nutr. 130: 1914–1919.

Yang, C.M., P. R. Ferket, Q. H. Hong, J. Zhou, G. T. Cao, L. Zhou, and A. G. Chen. 2012. Effect of chito-oligosaccharide on growth performance, intestinal barrier function, intestinal morphology and cecal microflora in weaned pigs. J. Anim. Sci. 90:2671-2676.

Zijlstra, R.T., K.Y. Whang, R.A. Easter, and J. Odle. 1996. Effect of feeding a milk replacer to early-weaned pigs on growth, body composition, and small intestinal morphology, compared with suckled littermates. J. Anim. Sci. 74 (12): 2948-2959.

Table 2-1. Ingredient composition of diets

Item	Phase 1	Phase 2	Phase 3
Ingredients, g/kg			
Corn	450.5	522.8	690.1
Soybean meal	225.0	275.0	275.0
Whey permeate	200.0	100.0	-
Plasma	60.0	20.0	-
Fish meal	38.0	63.0	-
Monocalcium phosphate[1]	9.0	6.0	9.0
Limestone[2]	7.5	5.0	10.0
Salt	2.5	3.5	6.5
Vitamin premix[3]	1.0	1.0	1.0
Mineral premix[4]	0.8	0.8	0.8
Selenium premix[5]	0.5	0.5	0.5
L-Lys HCL	2.8	1.4	4.3
DL-Met	1.7	1.0	1.5
L-Thr	0.7	-	1.3
Total	1,000	1,000	1,000
Calculated nutrients and energy, as-fed basis			
ME, kcal/kg	3317.6	3297.8	3260.6
CP, g/kg	225.0	234.0	186.0
Ca, g/kg	8.6	8.1	5.9
P, g/kg	8.0	7.4	5.3
Nonphytate P, g/kg	6.4	5.5	3.3
Ca:P	1.08	1.09	1.12
SID Lys, g/kg	13.9	13.0	12.1

[1] Contained 17% Ca and 21.1 % P.

[2] Contained 38% Ca.

[3] Vitamin premix per kilogram of diet: vitamin A, 5,090 IU; vitamin D3 1,270 IU ; vitamin E 22 IU, vitamin K activity 7.2 mg; menadione, 2,412 µg; vitamin B12, 22.4 µg; riboflavin 8.11 mg; d-pantothenic acid 61.20; and niacin 106.59 mg.

[4] Mineral premix supplied per kilogram of diet: Cu (as copper chloride), 18 mg; I (as ethylenediamine dihydroiodide) 0.95 mg; Fe (as iron carbonate) 334 mg; Mn (as manganese oxide) 60 mg; and zinc (as zinc oxide) 150 mg.

[5] Supplied as 300 µg of Se per kilogram of diet.

Table 2-2. Primers used for RT-PCR[1]

Gene	Primer (5`-3`)
GAPDH (forward)	GGGCATGAACCATGAGAAGT
GAPDH (reverse)	TGTGGTCATGAGTCCTTCCA
MUC2 (forward)	CAACGGCCTCTCCTTCTCTGT
MUC2 (reverse)	GCCACACTGGCCCTTTGT
TNF-α (forward)	ATGGGCCCCCAGAAGGAAGAG
TNF-α (reverse)	GATGGCAGAGAGGAGGTTGAC
IL-6 (forward)	TCTGGGTTCAATCAGGAGACCTGC
IL-6 (reverse)	TGCACGGCCTCGACATTTCCC
CL-1 (forward)	TACTTTCCTGCTCCTGTC
CL-1 (reverse)	AAGGCGTTAATGTCAATC
OC (forward)	ATCAACAAAGGCAACTCT
OC (reverse)	GCAGCAGCCATGTACTCT
ZO-1 (forward)	GAGTTTGATAGTGGCGTT
ZO-1 (reverse)	GTGGGAGGATGCTGTTGT

[1] GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MUC2 = mucin 2; TNF-α = tumor necrosis alpha; IL-6 = interleukin 6; CL-1 = claudin 1; OC = occludin; ZO-1 = zonula occuden 1.

Table 2-3. Growth performance response of pigs to feed, water, or feed + water stressor [1,2,3]

Treatment	1	2	3	4		*P*-value		
Description[3]	Control	Feed stressor	Water stressor	Feed + water stressor	SEM[4]	Feed stressor	Water stressor	Feed × water stressor
IBW, kg	6.21	6.22	6.20	6.23	0.29	0.67	0.78	0.67
Phase 1								
FBW, kg	6.17	6.22	5.92	5.93	0.27	0.22	<0.01	0.74
ADG, kg	-0.05	-0.01	-0.25	-0.28	0.03	0.68	<0.01	0.29
ADFI, kg	0.01	0.00	0.02	0.00	0.00	<0.01	0.91	0.91
G:F, kg:kg	-5.00	0.00	-12.50	0.00	0.04	<0.01	0.07	0.07
Phase 2								
FBW, kg	6.97	7.02	6.95	6.86	0.29	0.89	0.21	0.34
ADG, kg	0.13	0.13	0.17	0.15	0.01	0.26	<0.01	0.11
ADFI, kg	0.15	0.15	0.15	0.14	0.01	0.22	0.49	0.49
G:F, kg:kg	0.82	0.84	1.13	1.11	0.09	0.85	<0.01	0.60
Phase 3								
FBW, kg	9.89	9.84	9.83	9.77	0.53	0.76	0.69	0.92
ADG, kg	0.39	0.38	0.38	0.38	0.04	0.63	0.90	0.31
ADFI, kg	0.33	0.31	0.30	0.30	0.01	0.12	0.12	0.67
G:F, kg:kg	1.23	1.23	1.24	1.27	0.15	0.88	0.39	0.61
Phase 4								
FBW, kg	14.52	14.21	14.05	13.95	0.43	0.39	0.09	0.46
ADG, kg	0.33	0.31	0.30	0.30	0.04	0.41	0.09	0.27
ADFI, kg	0.58	0.56	0.54	0.54	0.02	0.25	0.02	0.38
G:F, kg:kg	0.56	0.55	0.56	0.54	0.06	0.80	0.65	0.87
Cumulative								
ADG, kg	0.29	0.27	0.27	0.26	0.01	0.12	0.06	0.81
ADFI, kg	0.39	0.37	0.37	0.36	0.01	0.18	0.02	0.77
G:F, kg:kg	0.73	0.74	0.74	0.74	0.01	0.58	0.42	0.53

[1] There were 4 treatments with 10 to 12 replicate pens per treatment. Pens were mixed sex with 8 pigs per pen at the start of the trial.
[2] Phase 1 lasted 1 d, phase 2 lasted 6 d, phase 3 lasted 7 d, and phase 4 lasted 14 d.
[3] IBW = initial bodyweight, FBW=final bodyweight.
[4] SEM = standard error of the mean with the highest SEM among treatment means reported.

Table 2-4. Intestinal histology and serum cortisol and corticotrophin releasing factor responses of pigs to feed, water, or feed + water stressor [1]

Treatment	1	2	3	4		*P*-value		
Description	Control	Feed stressor	Water stressor	Feed + water stressor	SEM[2]	Feed stressor	Water stressor	Feed × water stressor
				Jejunum				
Villus height, µm								
1 d	271.60	284.27	258.15	269.17	33.30	0.70	0.66	0.97
7 d	258.81	338.64	325.98	336.49	27.07	0.08	0.20	0.17
Crypt depth, µm								
1 d	100.72	96.24	92.68	92.71	13.92	0.85	0.58	0.83
7 d	136.50	109.29	161.53	130.83	11.71	0.03	0.06	0.85
				Ileum				
Villus height, µm								
1 d	355.24	354.94	306.56	394.24	52.10	0.33	0.91	0.34
7 d	327.45[a]	453.50[c]	340.18[ab]	336.47[ab]	33.58	0.07	0.12	0.03
Crypt depth, µm								
1 d	74.70	104.29	78.47	91.29	10.84	0.06	0.66	0.43
7 d	87.46	80.50	99.43	93.37	11.05	0.57	0.20	0.97
				Serum				
Cortisol, ng/ml								
1 d	13.64	27.40	37.25	34.29	5.76	0.43	0.02	0.34
7 d	16.31	26.00	36.94	52.53	9.50	0.14	0.05	0.82
CRF[3], ng/ml								
1 d	0.21	0.27	0.55	0.53	0.13	0.85	0.02	0.77
7 d	0.26	0.24	0.35	0.25	0.13	0.65	0.68	0.73

[1] Intestinal samples for histology and blood samples were taken from 1 randomly selected pig per pen on 1 or 7 d post weaning. Four villi and crypts were identified for each sample and the corresponding mean of those samples were used for analysis. There were 5 to 8 replicates for histological and serum samples.
[2] SEM = standard error of the mean with the highest SEM among treatment means reported.
[3] CRF = corticotrophin releasing factor.

Table 2-5. Jejunal gene expression responses of pigs to feed, water, or feed + water stressor [1]

Treatment	1	2	3	4		*P*-value		
Description	Control	Feed stressor	Water stressor	Feed + water stressor	SEM[2]	Feed stressor	Water stressor	Feed × water stressor
MUC 2 (arbitrary units)								
1d[3]	3.76	4.09	3.82	5.91	2.11	0.58	0.05	0.97
7 d	4.75	4.44	0.84	0.58	1.77	0.88	0.05	0.95
TNF-α (arbitrary units)								
1 d	4.05[a]	0.45[b]	1.10[b]	1.31[b]	0.73	0.01	0.07	< 0.01
7 d	2.44	1.18	1.04	1.04	0.52	0.12	0.06	0.12
IL-6 (arbitrary units)								
1 d[3]	2.58	0.44	5.73	2.70	2.09	0.07	0.26	0.73
7 d	13.08	8.56	10.01	6.23	5.66	0.94	0.23	0.39
CL-1 (arbitrary units)								
1 d	1.35	0.89	1.35	2.24	1.06	0.81	0.46	0.46
7 d	0.67	0.14	0.41	0.49	0.15	0.13	0.70	0.07
OC (arbitrary units)								
1 d	0.93	1.41	0.56	0.54	0.24	0.34	0.02	0.29
7 d	1.45	0.78	1.14	1.15	0.54	0.49	0.95	0.48
ZO-1 (arbitrary units)								
1 d	1.30	1.81	1.26	1.07	0.32	0.60	0.21	0.27
7 d	2.87	1.99	1.99	2.27	0.97	0.71	0.72	0.48

[1] Mucosa samples were taken from 1 randomly selected pig per pen for 4 to 7 replicates on 1 or 7 d post weaning. The housekeeping gene used was GAPDH.
[2] SEM = standard error of the mean with the highest SEM among treatment means reported.
[3] *P*-value from transformed data using a log transformation.

Table 2-6. Ileal gene expression responses of pigs to feed, water, or feed + water stressor [1]

Treatment	1	2	3	4		*P*-value		
Description	Control	Feed stressor	Water stressor	Feed + water stressor	SEM[2]	Feed stressor	Water stressor	Feed × water stressor
MUC 2 (arbitrary units)								
1 d[3]	1.09	5.10	3.83	8.18	4.01	0.15	0.97	0.47
7 d[3]	4.40	8.05	6.61	0.92	3.41	0.69	0.25	0.50
TNF-α (arbitrary units)								
1 d	0.76	0.97	0.77	0.29	0.28	0.58	0.16	0.20
7 d[3]	2.13	6.32	2.14	1.03	2.01	0.91	0.18	0.22
IL-6 (arbitrary units)								
1 d[3]	0.65	1.46	0.41	0.41	0.33	0.33	0.11	0.36
7 d[3]	3.09	7.23	1.91	3.56	3.26	0.55	0.64	0.67
CL-1 (arbitrary units)								
1 d	1.46	1.72	1.76	3.54	0.79	0.16	0.15	0.28
7 d[3]	4.80	3.46	1.19	0.84	0.99	0.95	0.01	0.38
OC (arbitrary units)								
1 d	0.54	0.16	0.84	1.13	0.30	0.89	0.03	0.34
7 d[3]	5.26	2.21	2.86	1.71	1.53	0.41	0.36	0.88
ZO-1 (arbitrary units)								
1 d	0.53	0.50	0.56	0.70	0.22	0.75	0.55	0.64
7 d[3]	5.70	3.60	2.87	0.96	1.14	0.32	0.01	0.88

[1] Mucosa samples were taken from 1 randomly selected pig per pen for 4 to 8 replicates on 1 or 7 d post weaning. The housekeeping gene used was GAPDH.
[2] SEM = standard error of the mean with the highest SEM among treatment means reported.
[3] *P*-value from transformed data using a log transformation.

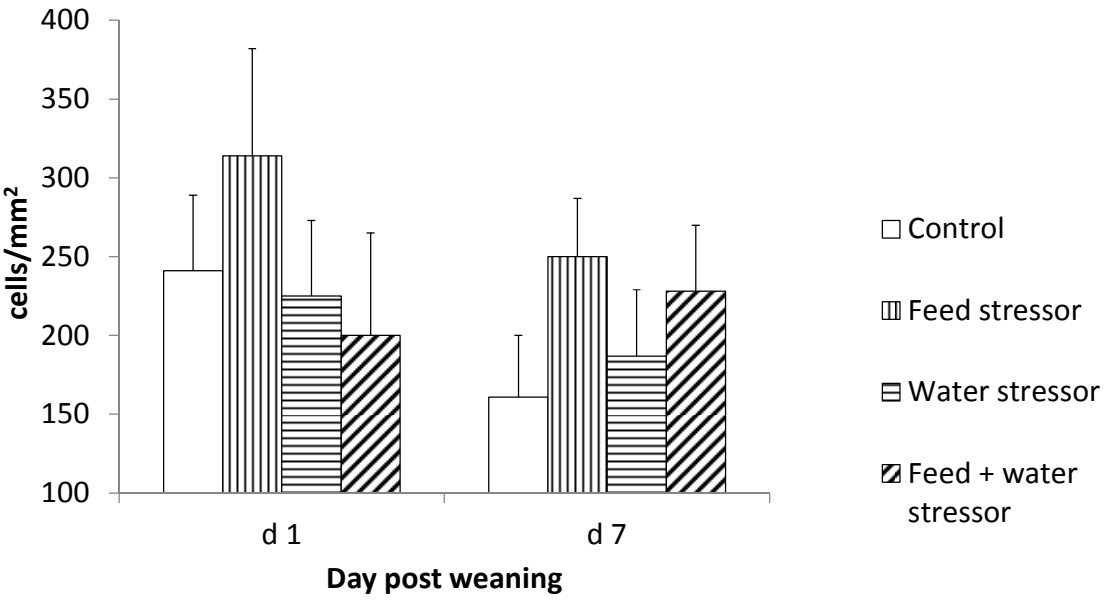

Figure 2-1. Effect of acute weaning stressors on ileal mast cell density. Mast cell counts were performed for 6 pigs per treatment and are expressed as the number of toluidine-blue positive cells per mm^2 tissue. On 1 d post-weaning $P = 0.58$, 0.18, and 0.30 for the effects of the feed stressor, water stressor, and feed × water interaction, respectively. On 7 d post-weaning $P = 0.10$, 0.95, and 0.53 for effects of the feed stressor, water stressor, and feed x water interaction, respectively.

CHAPTER 3. IMPACT OF ACUTE FEED AND WATER DEPRIVATION AT WEANING AND SUBSEQUENT HEAT STRESS EVENT ON GROWTH PERFORMANCE, SERUM STRESS MARKERS AND ILEAL MUCOSA CHARACTERISTICS IN NURSERY PIGS

3.1. Abstract

The current experiment was conducted to investigate the impact of a feed and water deprivation event at weaning and subsequent heat stress event on growth performance, serum stress markers, and ileal characteristics in nursery pigs. Mixed-sex pigs were allotted on the basis of IBW (7.0 kg ± 0.89) in a RCBD with treatments in a split-plot arrangement and consisting of the whole-plot factor of with or without a 24-h feed + water deprivation event at weaning and the sub-plot factor of with or without a cyclic 3-d heat stress event starting 27 d post-weaning. Growth performance was measured throughout the experiment and on 1, 27, and 30 d post-weaning one pig from each pen was selected, blood was collected for measurement of serum cortisol, corticotrophin releasing factor (CRF), and endotoxins, and a segment of the mid ileum was excised for morphological measurements. Furthermore, an ileal mucosal scraping was taken and gene expression of mucin (MUC2), tumor necrosis factor alpha (TNF-α), glucose transporter 2 (GLUT2), claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1) was measured by RT PCR. Feed + water deprivation reduced ADG ($P < 0.01$) 1 d post-weaning and body weights ($P < 0.05$) up to 27 d post-weaning. At 30 d

post-weaning, there was both a reduction due to heat stress and interaction between the stress events on ADG and ADFI ($P < 0.05$). There was an increase ($P < 0.05$) in serum CRF and endotoxins and a tendency for an increase ($P = 0.09$) in serum cortisol due to the deprivation event 1 d post-weaning. There was a reduction in villous height to crypt depth ratio ($P = 0.05$) due to the deprivation event 1 d post-weaning and an interaction between the stress events ($P = 0.05$) for crypt depth on d 30 post-weaning. Gene expression of GLUT 2 tended to decrease ($P = 0.09$), CL-1 tended to increase ($P = 0.10$), and OC decreased ($P = 0.05$) due to the deprivation event 1 d post-weaning. Expression of the OC gene decreased ($P < 0.05$) due to the deprivation event 27 d post-weaning and OC and ZO-1 gene expression tended to decrease ($P = 0.07$) due to the heat stress event 30 d post-weaning. Results from the current study show a link between feed and water deprivation at weaning and subsequent heat stress response with implications on growth, serum stress markers, and ileal characteristics.

3.2. Introduction

During the nursery phase, pigs are exposed to weaning, environmental temperature, and dietary stressors that are linked to reduced performance and health and gastrointestinal (GI) dysfunction (Pluske et al., 1997; Lalles et al., 2004; Smith et al. 2010). Previous research shows a connection between feed and water deprivation, markers of stress, and GI dysfunction in weaned pigs (Pluske et al., 1997; Hu et al., 2013; Horn et al., 2014). Brooks et al. (2001) showed that approximately 50 and 10 % of pigs do not consume feed for the first 24- and 48-h post-weaning. Low post-weaning

feed intake has been shown to cause villus atrophy and crypt hyperplasia that corresponds to short- and long-term negative impacts on growth, gut function, and disease susceptibility (Pluske et al., 1997; Lalles et al., 2004; Wijtten et al., 2011). Water intake can be limited in weaned pigs due to transport time or weaning-associated lethargy although limited information exists on the effect of dehydration on nursery pig GI function. Our lab recently showed that a 24-h post-weaning water deprivation event resulted in reduced growth performance, increased serum stress markers, and changes in markers of mucosal integrity (Horn et al., 2014). Heat stress in pigs is associated with a reduction in performance, changes in metabolism, and reduced gut integrity (Pearce et al., 2013a,b). Briefly, heat stress reduces blood flow to GI tissues for body temperature regulation, which causes a hypoxic environment in the gut and reduces mucosal integrity (Yan et al., 2006; Lambert, 2009; Yu et al., 2010l; Pearce et al. 2013a). In a production setting, pigs are commonly exposed to multiple concurrent or subsequent stressors, therefore it is necessary to investigate stressor interactions (Hyun et al., 1998). In grow-finish pigs Morrow-Tesch et al. (1994) and Hyun et al. (1998) showed that concurrent heat and social stressors impacted growth performance additively although little information exists on the impact of subsequent stressors on growth performance during the nursery phase.

Several nursery pig studies show a link between individual stressors and gut function (Pluske et al., 1997; Pearce et al., 2013a; Horn et al., 2014), however a dearth of information exists exploring interactions between stressors during the nursery phase. Therefore, our objective was to investigate the impact of a 24-h feed and water deprivation event at weaning followed by a cyclic, 3-d heat stress event starting at 27 d

post-weaning. We hypothesized that the stress events would affect growth performance, gut morphology, serum stress markers, and intestinal cytokine and tight junctions. To achieve our objectives we measured growth performance, serum cortisol, CRF, and endotoxins, ileal morphology, and ileal mucosa cytokine and tight junction gene expression.

3.3. Materials and methods

All animal procedures were approved by the JBS United Animal Care and Use Committee.

Animals and treatments

A total of 260 PIC 337 × C29 weaned, mixed-sex pigs (initial BW = 7.0 ± 0.89 kg, 19 d old) were used in a randomized complete block design with BW as the blocking factor. The pigs were housed in pens (1.21 × 1.11 m) equipped with one fixed-nipple drinker and one space dry-box feeder (4.87 × 1.52 × 0.30 m). Room temperature was 34.4°C at weaning and reduced by 0.5°C each subsequent day of the trial until the heat stress event. Treatments were in a 2 × 2 split-plot arrangement with the whole-plot factors consisting of with or without a 24-h feed + water deprivation event at weaning and the sub-plot factor of with or without a cyclic 3-d heat stress event starting 27 d post-weaning. There were 22 whole-plot replicate pens per treatment and 11 sub-plot replicate pens per treatment with 5 to 6 mixed-sex pigs per pen. The deprivation event was similar as described by Horn et al. (2014). Briefly, the deprivation event consisted of withholding of feed and water for a period of 24-h

following weaning. Following the stressor, pigs were allowed free access to feed and water and were fed a mash-based feeding program that met or exceeded NRC requirements (NRC, 2012) and consisted of starter, transition and late nursery diets for 7, 7, and 21 d post weaning, respectively (Table 3-1). The heat stress event consisted of a cyclic, 8-h increase in room temperature to 35˚C (61% relative humidity) starting from 27 to 29 d post-weaning. Evening and overnight room temperatures were allowed to drop down to 29˚C during the 3-d heat stress period. Room temperature was maintained at 23˚C (55% relative humidity) for pigs not exposed to the heat stress event. Rectal temperatures were recorded from the same 2 pigs per pen 24-h before the heat stress event, daily during the heat stress event midpoint, and 24-h following the heat stress event. Pigs and feeders were weighed on 1, 7, 14, 21, 27, 30, and 35 d post-weaning to calculate ADG, ADFI, and G:F for the respective growth phases. On 1, 27, and 30 d post-weaning, 1 pig per pen was randomly selected, blood was collected via venipuncture into serum collection tubes (CORVAC serum separator tube, Tyco Healthcare Group LP, Mansfield, MA, USA), and subsequently euthanized via CO_2 asphyxiation. Immediately following euthanasia, 10-cm segments of the ileum were excised for histological and gene expression measurements.

Serum stress markers

Blood was collected at approximately the same time each day to minimize the effect of diurnal rhythms. Following blood collection serum was separated via centrifugation (20 min, 10,000 × g at 4°C) and stored at -80°C until analysis. Serum levels of CRF and cortisol were determined using commercial ELISA kits (CRF: Phoenix Pharmaceuticals, Belmont, CA; cortisol: R&D systems, Minneapolis, MN).

Serum endotoxin concentrations were measured by chromogenic assay using the Pierce LAL Chromogenic Endotoxin Kit (Fisher Scientific, Pittsburgh, PA). Briefly, the serum samples were heat shocked for 15 min at 70˚C and diluted 100 fold in pyrogen-free water. Serum endotoxins catalyze cleavage of p-nitroaniline (PNA) from Ac-Ile-Glu-Ala-Arg-PNA, which forms a colormetric substrate that is measured at 410 nm. Therefore, the amount of PNA detected by colormetric assay is proportional to the amount of endoxotin present in the sample, which was interpolated from a standard curve.

Histological measurements

Excised intestinal segments were flushed in ice-cold 10% phosphate-buffered saline, stapled to a cardboard background, and fixed in 10% buffered formalin for approximately 30 d. Samples were subsequently dehydrated with ethanol, cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). Segments were sliced (5 µm) and stained with hematoxylin and eosin by the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth were measured and villus height to crypt depth ratio was calculated. Means from at least 4 villi per segment were analyzed for differences. Villus length is defined as the length from the villus tip to the valley between villi and crypt depth is defined as the length between the crypt opening and base.

RNA isolation, cDNA Synthesis, and real-time PCR

Immediately after removal, intestinal segments were cut in half, exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were

immediately placed in 5 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C until RNA isolation. Extraction of RNA was according to the Trizol protocol (Invitrogen, Grand Island, NY). Reverse transcription was carried out using the MMLV reverse transcription system of Promega and real-time PCR was performed on a Bio-Rad iCyler using a reaction mix that consisted of 0.50 µg cDNA, 0.075 nmol of each forward and reverse primers, SYBR Green Master (Roche, Basel, Switzerland), and nuclease-free water (Ambion, Austin, TX) to reach a reaction volume of 20 µl per well. Following a 5-min incubation period at 95ºC, reactions were cycled 50 times using the following protocol: 10s at 95ºC, 20 s at 55ºC, and 72ºC for 30 s. Gene expression of mucin (MUC2), glucose transporter 2 (GLUT2), tumor necrosis factor alpha (TNF-α), claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1) were measured using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping control. Primers used are presented in Table 3-2.

Statistical analysis

Data was analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) appropriate for a split-plot design with the whole-plot factor of feed + water deprivation event and sub-plot factor of heat stress event. Simple effect means were separated using the PDIFF option in SAS where there was an interaction of whole- and sub-plot factors. Significant difference was defined as $P \leq 0.05$ and $0.05 \leq P \leq 0.10$ was considered a tendency.

3.4. Results

There were no treatment-specific instances of mortality or morbidity throughout the trial. Furthermore, there was no treatment effect ($P < 0.10$) on daily scour scores. Rectal temperatures were recorded 24-h before and after, and daily during the heat stress event from two pigs per pen (data not shown). There was no effect of treatment on rectal temperatures 24-h before or after the heat stress event. There was, on average, a 0.35 ˚C daily increase ($P < 0.05$) in rectal temperature at the midpoint of the heat stressor. Growth performance data are reported in Tables 3-3 and 3-4. All pigs lost weight from 0 to 1 d post-weaning, although pigs exposed to the deprivation event were 0.23 kg lighter ($P = 0.01$) compared with non-stressed pigs. From 1 to 7 d post-weaning, there was an 8.7% increase in ADG, but 9.1% decrease in ADFI ($P < 0.05$) relative to non-stressed pigs due to the deprivation event. Bodyweights of feed + water-deprived pigs continued to be lighter (by 0.60 kg) compared with non-stressed pigs ($P = 0.03$) on 27 d post-weaning. Immediately following the heat stress event (30 d post-weaning) there was an effect of the sub-plot heat stress event and a stress event interaction ($P < 0.05$) for ADG and ADFI and a tendency ($P = 0.10$) for FBW. Relative to pigs exposed to no stress event, there was a 0.92, 0.65, and 1.92 kg decrease in FBW at 30 d post-weaning for the heat stress only, feed + water deprivation only, and feed + water stress and heat stress, respectively. There was no impact ($P > 0.10$) of stress factors the week following the heat stress event (30 to 35 d post-weaning). Cumulatively, there was a tendency for a feed + water deprivation and heat stress event

interaction ($P = 0.10$) for FBW and ADG and a heat stress event and stress event interaction ($P = 0.03$) for ADFI.

Ileal morphology data are reported in Tables 3-3 and 3-4. The average villus and crypt depth were 284 and 97 μm 1 d post-weaning, respectively. Villous height was decreased ($P = 0.03$) by 27% but crypt depth was not impacted ($P > 0.10$) 1 d post-weaning with feed+water deprivation. The average villus height and crypt depth were 354 and 220 μm and 323 and 180 μm on 27 and 30 d post-weaning, respectively. There was no impact ($P > 0.10$) of treatment on ieal morphology 27 d post-weaning although there was an interaction effect ($P = 0.05$) 30 d post-weaning. The interaction effect shows that pigs exposed to no stress events had the deepest crypts.

Serum marker data are reported in Tables 3-5 and 3-6. Immediately following the deprivation event (1 d post-weaning) there was a tendency ($P = 0.09$) for a 29 % increase in serum cortisol and an 8.1 and 94 % increase ($P < 0.05$) in serum CRF and endotoxins, respectively. Furthermore, on 27 d post-weaning there was a tendency ($P = 0.07$) for a 37% increase in serum endotoxins and on 30 d post-weaning, there was a tendency ($P = 0.08$) for an increase in serum CRF due to the deprivation event.

Ileal mucosa gene expression analyses are presented in Tables 3-7 and 3-8. Expression of MUC2, GLUT2, TNF-α, CL-1, OC, and ZO-1 genes were measured with GAPDH as the housekeeping gene. There was no impact ($P > 0.10$) of treatment on GADPH gene expression. On 1 d post-weaning there was a tendency for a decrease ($P = 0.09$) in GLUT2 gene expression, a tendency for an increase ($P = 0.10$) in CL-1 gene expression, and a decrease ($P = 0.05$) in OC gene expression due to the deprivation

event. Furthermore, there was a decrease ($P < 0.01$) in OC gene expression due to the deprivation event on 27 d post-weaning. On 30 d post-weaning, there was no impact of the deprivation event on gene expression although there was a tendency for a decrease ($P = 0.07$) in OC and ZO-1 gene expression due to the heat stress event.

3.5. Discussion

A relationship exists between stress events during the nursery period, short- and long-term performance, and gastrointestinal function (Pluske et al., 1997; Lalles et al., 2004; Smith et al., 2010). Within the first 24-h post-weaning it has been estimated that approximately 50 % of pigs do not consume feed (Brooks et al., 2001), which is marked by poor pig performance, increased disease susceptibility, and atrophy of intestinal morphology (Pluske et al., 1997). Furthermore, dehydration is common during the post-weaning period and in an experimental setting has been shown to be associated with an increase in markers of stress and reduced gut integrity (Horn et al., 2014). The impact of heat stress on feed intake and metabolism in growing pigs has been well documented (Collin et al., 2001; Baumgard and Rhoads, 2011), however recent literature shows heat stress also results in hypoxia-associated changes in GI integrity and cellular absorptive properties (Yu et al., 2010; Pearce et al., 2013a; Pearce et al., 2014). Previous research shows stressors are additive (Hyun et al., 1998) although there is a dearth of information exploring the relationship between a heat stressor that is subsequent to a post-weaning deprivation stressor in nursery pigs. Because stress events commonly occur concurrent or subsequent to each other it is critical to

understand stressor interactions. Furthermore, an understanding of the impact of common nursery pig stressors on performance and GI function will allow for development of management and nutritional strategies to help mitigate the stress-induced negative effects.

For the current experiment there was no mortality and fall-off pigs (visual morbidity) was below 5%. Immediately following weaning all pigs lost weight although the pigs exposed to the deprivation event had 4% lower BW compared to control pigs. During the first 1 to 3 d following weaning it is common for nursery pigs to lose weight and this period has been referred to as the "transient growth check" period as reported by Pluske et al. (1997) and Lalles et al. (2004). During the period following the deprivation event (2 to 7 d post-weaning) pigs exposed to the deprivation event had improved gains although their BW remained 0.13 kg lighter compared to control pigs. The improvement in gain during the immediate period following the stressor is related to water gorging and is similar to a previous experiment reported by our lab (Horn et al., 2014). However, at 27 d post-weaning the pigs exposed to the 24-h post-weaning deprivation event remained 0.60 kg lighter compared to control pigs. Following the 3-d, cyclic heat stress event there was a 16 and 20% reduction in ADG and ADFI, respectively, which resulted in a 1.1 kg lighter pig. Furthermore, an interaction existed that shows pigs exposed to both the deprivation and heat stress events had lower ADFI compared to other treatment groups. The interaction carried over cumulatively and shows the pigs exposed to both stress events have the lowest overall gain and ADFI. It has been well documented that pigs are susceptible to heat stress due to lack of sweat glands which corresponds to increased basal metabolic rate

and urination, and decreased feed intake (D'Allaire et al., 1996). Increased water losses along with heat-induced increases in appetite-suppressing neuropeptides such as ghrelin are largely responsible for attenuated pig performance (Pearce et al., 2014). There is limited information on the impact a cyclic heat stress event in nursery pigs, therefore comparing results from the current trial to other published studies is limited. Pearce et al. (2013b) showed that in 46-kg pigs exposed to a constant, 24-h heat stress (35.5˚C) there was a 1.6˚C increase in body temperature and a 5 and 53% reduction in BW and FI, respectively. Kerr et al. (2005) reported a 23 and 14% reduction in gain and ADFI in pigs exposed to a 36-d heat stressor (33˚C), whereas Hyun et al. (1998) reported approximately a 10% reduction in ADG and ADFI during a 4-wk heat stressor (34˚C). A dearth of information exists on the interactive effects of subsequent stressors in nursery pigs although Hyun et al. (1998) reported that a stressor interaction existed in pigs concurrently exposed to heat and social stress.

Poor feed intake during the immediate post-weaning period results in limited energy for the rapidly developing gut which has been shown to reduce intestinal villus height and GI function (Pluske et al., 1997). For the current experiment, there was a 27% reduction in ileal villus height immediately following the deprivation event although there was recovery in deprivation-induced morphological effects by 27 d post-weaning. Previous studies reported little impact of nursery pig stress events on intestinal morphology (Moeser et al., 2007) and it is not clear why there was such a drastic effect of the deprivation event for the current study. Reduced villus height indicates reduced potential for nutrient utilization during the first week post-weaning along with increased susceptibility to enteric pathogens (Lalles et al., 2004) and further research is warranted

to better understand the practical implications of these results. There was no impact of the heat stress event on ileal morphology following the 3-d event although there was a stressor interaction that shows pigs not exposed to any stressor had the deepest crypts. During heat stress, blood flow to GI tissues is limited, which limits oxygen and nutrient delivery and results in limited gut tissue growth or atrophy (Yan et al., 2006; Pearce et al. 2013a). Pearce et al. (2013a and 2014) showed that when growing pigs were exposed to a constant heat stressor there was a reduction in villus height and an increase in crypt depth within 24-h following initiation of the stressor. Conversely, Yu et al. (2010) reported in pigs that morphological changes were not shown until 3 d into a cyclic heat stressor. For the current experiment it is likely that there was no impact of the heat stressor on ileal morphology due to its cyclic and limited nature. The stressor interaction on crypt depth indicates increased cellular proliferation and further work measuring molecular markers of proliferation and differentiation are needed to better understand these results.

Previous studies have shown that serum CRF and cortisol are markers of nursery pig stress and are linked to stress-induced changes in growth and gastrointestinal function (Moeser et al. 2007; Smith et al., 2010; Horn et al., 2014). For the current study there were 28 and 8% increases in serum cortisol and CRF immediately following the post-weaning deprivation event. The data reported in the current study are consistent with reports by Smith et al. (2010) and Horn et al. (2014). Stress-induced changes in central and peripheral CRF release have been shown to increase intestinal mast cell activation and recruitment and can be a contributing factor to inappropriate intestinal secretion of TNF-α (Overman et al., 2012). Changes in mucosal immunity can

alter enterocyte tight junction protein structure and subsequently increase intestinal permeability (Lambert, 2009). Glucocorticoids are a critical part of the stress-adaptation response and drive stress-induced metabolic changes. There was no impact of the heat stress event or a stress event interaction for the current study. Yu et al. (2010) reported an increase in serum cortisol each day following a cyclic heat stressor using a mini pig model. Compared to Yu et al. (2010), pigs in the current study were exposed to a lower degree of heat stress (35˚C vs. 40˚C) for a longer duration (8 h vs. 5 h) which could explain the lack of change in serum cortisol levels. Under normal conditions, bacterial cell wall components, such as endotoxins, should be very low in the serum due to properly functioning physical barriers. An increase in serum endotoxin level can lead to endotoxemia and activation of immune and hepatic detoxification mechanisms (Hall et al., 2001). Clinically, endotoxemia can manifest as increased body temperature, reduced feed intake, lethargy, and in severe cases septic shock and death. Furthermore, it has been well documented that changes in serum endotoxin level are related to reduced intestinal integrity in rodents, chickens, pigs, and humans (Bouchama et al., 1991; Hall et al., 2001; Cronje et al., 2007; Pearce et al., 2013a,b). For the current study, there was a 16-fold increase in serum endotoxin levels due to water deprivation event 1 d post-weaning. Furthermore, there was a 1.6-fold increase in serum endotoxins due to the deprivation event 27 d post-weaning. The increase in serum endotoxins at 27 d post-weaning is not clear since it does not correlate with any changes in intestinal integrity. It may be possible that the aforementioned changes are related to a stressor-induced microbial population shift rather than changes in intestinal integrity and warrants further investigation from a microbiology perspective. Previous research

shows an increase in serum endotoxin levels in growing pigs exposed to a constant heat stress event are linked to increased intestinal permeability (Pearce et al., 2013a; Pearce et al., 2014). For the current study there was no impact of the heat stress event or a stress event interaction on serum endotoxin levels. The discrepancy between the current study and previous reports is likely due to the duration and intensity of the heat stress event.

Tight junction protein complexes form a selective pore between epithelial cells and can be influenced by stress, cytokines, and proteolytic enzymes (Li et al., 2012). Hu et al. (2013) demonstrated in pigs that changes in tight junction gene expression were related to weaning stress-induced changes in gut permeability. Changes in tight junction gene and protein expression are driven by myosin light chain kinase (MLCK) actions on cytoskeletal proteins (Lambert, 2009). Serum stress mediators, cytokines, and hypoxia have been shown to be inducers of MLCK and are related to loss of intestinal integrity (Overman et al., 2012; Pearce et al., 2013a). For the current study there was a decrease in gene expression of the tight junction protein OC immediately following the deprivation stress event that was maintained up to 27 d post-weaning. These results are consistent with those reported by our lab in a previous experiment using a deprivation stressor (Horn et al., 2014) and by those reported by Hu et al. (2013) when pigs were exposed to general weaning stress. Previous research shows a link between inflammatory cytokines and changes in tight junction gene expression (Ma et al., 2005) although similar results were not observed for the current trial. The discrepancy between the current experiment and the literature may be due to sampling time and stress event intensity. As previously mentioned, during heat stress changes in

blood flow lead to a hypoxic environment in the gut resulting in limited delivery of energy and nutrients to enterocytes (Yan et al., 2006). Resulting consequences include changes in tight junction structure and increased intestinal permeability. For the current study we showed that at the end of the cyclic, 3-d heat stress event there was a decrease in expression of tight junction genes OC and ZO-1. Pearce et al. (2013a) showed a decrease in ZO-1 gene expression and an increase in OC gene expression at the end of a constant, 3-d heat stressor in growing pigs. Furthermore, it has been reported that tight junction gene expression patterns change throughout a heat stress event (Pearce et al., 2013a). The discrepancy between OC gene expression results for the current study and previous studies are likely related to sample timing. Mucins are a major component of the mucus layer and serve an integral role in GI function and protection (Atuma et al., 2001; Horn et al., 2008). For the current experiment there was no impact of the deprivation stressor on MUC2 gene expression, which is consistent with results reported by Horn et al. (2014). Limited information exists on the impact of heat stress on mucin dynamics in pigs. Pearce et al. (2014) reported an increase in MUC2 gene expression during a heat stressor and the authors suggested the aforementioned increase was a protective mechanism. Further work is needed to understand gastrointestinal secretions over the time course of a heat stressor. During psychological stress, glucocorticoids have been shown to decrease GLUT2 activity and abundance (Shepherd et al., 2004). Consistently, we observed a 2.7-fold decrease in GLUT2 gene expression immediately following the deprivation event. Shepherd et al. (2004) suggested the decrease in GLUT2 is a mechanism to prevent intestinal counteraction of the lytic actions of cortisol during stress. Conversely, during a heat stress event an increase in intestinal

glucose transport is believed to be a protective mechanism to minimize cellular damage (Kellet and Brot-Laroche, 2005). For the current study, there was no change in GLUT 2 gene expression due to the heat stress event which is consistent with results reported by Pearce et al. (2013b). It is possible that increases in heat-stress associated glucose uptake are mainly mediated by SGLT-1 due to the ability of the transporter to cotransport water and Na along with glucose (Wright and Loo, 2006). There were no stressor interactions observed for ileal mucosa gene expression markers which is likely related to the limited and cyclic nature of the heat stress event and timing of sampling for the current study.

A 24-h feed and water deprivation event at weaning has short- and long-term implications on growth performance and results in a 0.60-kg lighter pig up to 27 d post-weaning. During the heat stress event, weight gain and feed intake were reduced and a feed + water deprivation by heat stressor interaction existed that showed pigs exposed to both stress events subsequently had the poorest performance. Furthermore, the deprivation event increased serum stress markers, negatively affected ileal morphology, and altered ileal mucosa genes related to nutrient absorption and cellular integrity. The heat stress event did not impact serum stress markers or ileal morphology, although tight junction gene expression was altered. These results show that nursery pig performance is impacted by subsequent stress events and therefore special focus should be given to developing nutritional and management strategies that help mitigate stress during the nursery phase.

3.6. Acknowledgements

The authors would like to express their appreciation to JBS United, Inc. and Biomatrix International for their financial and technical support of this trial. Further appreciation is given to the employees of Burton Russell Farm at JBS United.

3.7. References

Atuma, C., V. Strugala, A. Allen, and L. Holm. 2001. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am. J. Physiol. 280: G922-929.

Baumgard, L.H., and R.P. Rhoads. 2011. Effects of environment on metabolism. In: Collier, R.J., editor. Environmental Physiology: John Wiley and Sons, Inc.

Bouchama, A., R. Parhar, A. el-Yazigi, K. Sheth, and S. al-Sedairy. 1991. Endotoxemia and release of tumor necrosis factor and interleukin 1 alpha in acute heatstroke. J. Appl. Physiol. 70:2640–2644.

Brooks, P.H., C.A. Moran, J.D. Beal, V. Demeckova, and A. Campbell. 2001. Liquid feeding for the young piglet, in: Varley M.A., Wiseman J. (Eds.), The weaner pig: nutrition and management, CAB International, Wallingford, UK, pp. 153 -178.

Collin, A., J. van Milgen, and J. Le Dividich. 2001. Modeling the effect of high, constant temperature on food intake in young growing pigs. J. Anim. Sci. 72: 519–529.

Cronje, P. B. 2007. Gut health, osmoregulation and resilience to heat stress in poultry. Aust. Poult. Sci. Symp. 19: 9–13.

D'Allaire, S., R. Drolet, and D. Brodeur. 1996. Sow mortality associated with high ambient temperatures. Can. Vet. J. 37: 237–239.

Hall, D. M., G. R. Buettner, L. W. Oberley, L. Xu, R. D. Matthes, and C. V. Gisolfi. 2001. Mechanisms of circulatory and intestinal barrier dysfunction during whole body hyperthermia. Am. J. Physiol. Heart Circ. Physiol. 280:H509–H521.

Horn, N., F. Ruch, G. Miller, K. M. Ajuwon, and O. Adeola. 2014. Impact of acute water and feed deprivation events on growth performance, intestinal characteristics, and serum stress markers in weaned pigs. J. Anim. Sci. 92: 4407-4416.

Hu, C.H., K. Xiao, Z.S. Luan, and S. Jong. 2013. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. J. Anim. Sci. 91: 1093-1101.

Hyun, Y., M. Ellis, G. Riskowski, and R.W. Johnson. 1998. Growth performance of pigs subjected to multiple concurrent environmental stressors. J. Anim. Sci. 76: 721-727.

Kellett, G.L. and E. Brot-Laroche. 2005. Apical GLUT2: A Major Pathway of Intestinal Sugar Absorption. Diabetes. 54: 3056-3062.

Kerr, B.J., J. T. Yen, J. A. Nienaber, and R. A. Easter. 2003. Influences of dietary protein level, amino acid supplementation and environmental temperature on performance, body composition, organ weights and total heat production of growing pigs. J. Anim. Sci. 81: 1998-2007.

Lalles, J.P., G. Boudry, C. Favier, N. LE floc'h, I. Luron, L. Montagne, I.P. Oswald, S. Pie, C. Piel, and B. Seve. 2004. Gut function and dysfunction in young pigs: physiology. Anim. Res. 53:301–316.

Lambert, G.P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J. Anim. Sci. 87 (E suppl.): E101-E108.

Li, X., S. Akhart, and M.A. Choudry. 2012. Alteration in intestine tight junction protein phosphorylation and apoptosis is association with increase in IL-18 levels following alcohol intoxication and burn injury. Biochem. Biophys. Acta. 1822: 186-203.

Ma, T., M.A. Biovin, D. Ye, A. Pedram, and H.M. Said. 2005. Mechanism of TNF-α modulation of Caco-2 intestinal epithelial tight junction barrier: Role of myosin light-chain kinase protein expression. Am. J. Physiol. 288: G422-G430.

Moeser, A.J., C.V. Klok, K.A. Ryan, J.G. Wooten, and D. Little. 2007. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am. J. Physiol. Gastrointest. Liver Physiol. 292: G173–G181.

Morrow-Tesch, J. L., J. J. McGlone, and J. L. Salak-Johnson. 1994. Heat and social effects pig immune parameters. J. Anim. Sci. 72: 2599-2609.

NRC, 2012. Nutrient requirements of Swine. 11th rev ed. Natl. Acad. Press, Washington, D.C.

Overman, E.L., J.E. Rivier, and A.J. Moeser. 2012. CRF Induces Intestinal Epithelial Barrier Injury via the Release of Mast Cell Proteases and TNF-a. PLOS ONE. 7(6): e39935.

Pearce, S.C., V. Mani, T. E. Weber, R. P. Rhoads, J. F. Patience, L. H. Baumgard, and N. K. Gabler. 2013a. Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs. J. Anim. Sci. 91: 5183-5193.

Pearce, S.C., V. Mani, R. L. Boddicker, J. S. Johnson, T. E. Weber, J. W. Ross, R. P. Rhoads, L. H. Baumgard, and N. K. Gabler. 2013b. Heat stress reduces intestinal barrier integrity and favors intestinal glucose transport in growing pigs. PLOS ONE. 8(8): e70215.

Pearce, S.C., M. V. Sanz-Fernandez, J. H. Hollis, L. H. Baumgard, and N. K. Gabler. 2014. Short-term exposure to heat stress attenuates appetite and intestinal integrity in growing pigs. J. Anim. Sci. 92: 5444-5454.

Pluske, J.R., D.J. Hampson, and I.H. William. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review, Livest. Prod. Sci. 51: 215–236.

Shepherd, E.J., P.A. Helliwell, O.J. Mace, E.L. Morgan, N. Patel, and G.L. Kellett. 2004. Stress and glucocorticoid inhibit apical GLUT2-trafficking and intestinal glucose absorption in rat small intestine. J. Physiol. 561(1): 281-290.

Smith, F., J.E. Clark, B.L. Overman, C.C. Tozel, J.H. Huang, J.E. Rivier, A.T. Blikslager, and A.J. Moeser. 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 298(3):G352-G363.

Wijtten, P.J., J. van der Meulen, and M.W. Verstegen. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. Br. J. Nutr.105(7): 967-81.

Wright, E.M., and D.D. Loo. 2000. Coupling between Na+, sugar, and water transport across the intestine. Ann. N.Y. Acad. Sci. 915: 54–66.

Yan, Y., Y. Zhao, H. Wang, and M. Fan. 2006. Pathophysiological factors underlying heatstroke. Med. Hypotheses 67: 609–617.

Yang, P.C., S.H. He, and P.Y. Zheng. 2007. Investigation into the signal transduction pathway via which heat stress impairs intestinal epithelial barrier function. Gastroentero. 22: 1883-1831.

Yu, J., P. Yin, F. Liu, G. Cheng, K. Guo, A. Lu, X. Zhu, W. Luan, J. Xu. 2010. Effect of heat stress on the porcine small intestine: A morphological and gene expression study. Comp. Biochem. Physiol. A. 156: 119-128.

Table 3-1. Ingredient composition of diets

Item	Phase 1	Phase 2	Phase 3
Ingredients, g/kg			
Corn	450.5	522.8	690.1
Soybean meal	225.0	275.0	275.0
Whey permeate	200.0	100.0	-
Plasma	60.0	20.0	-
Fish meal	38.0	63.0	-
Monocalcium phosphate[1]	9.0	6.0	9.0
Limestone[2]	7.5	5.0	10.0
Salt	2.5	3.5	6.5
Vitamin premix[3]	1.0	1.0	1.0
Mineral premix[4]	0.8	0.8	0.8
Selenium premix[5]	0.5	0.5	0.5
L-Lys HCL	2.8	1.4	4.3
DL-Met	1.7	1.0	1.5
L-Thr	0.7	-	1.3
Total	1,000	1,000	1,000
Calculated nutrients and energy, as-fed basis			
ME, kcal/kg	3317.6	3297.8	3260.6
CP, g/kg	225.0	234.0	186.0
Ca, g/kg	8.6	8.1	5.9
P, g/kg	8.0	7.4	5.3
Nonphytate P, g/kg	6.4	5.5	3.3
Ca:P	1.08	1.09	1.12
SID Lys, g/kg	13.9	13.0	12.1

[1] Contained 17% Ca and 21.1 % P.

[2] Contained 38% Ca.

[3] Vitamin premix per kilogram of diet: vitamin A, 5,090 IU; vitamin D3 1,270 IU ; vitamin E 22 IU, vitamin K activity 7.2 mg; menadione, 2,412 µg; vitamin B12, 22.4 µg; riboflavin 8.11 mg; d-pantothenic acid 61.20; and niacin 106.59 mg.

[4] Mineral premix supplied per kilogram of diet: Cu (as copper chloride), 18 mg; I (as ethylenediamine dihydroiodide) 0.95 mg; Fe (as iron carbonate) 334 mg; Mn (as manganese oxide) 60 mg; and zinc (as zinc oxide) 150 mg.

[5] Supplied as 300 µg of Se per kilogram of diet.

Table 3-2. Primers used for RT-PCR

Gene[1]	Primer (5`-3`)
GAPDH (forward)	GGGCATGAACCATGAGAAGT
GAPDH (reverse)	TGTGGTCATGAGTCCTTCCA
MUC2 (forward)	CAACGGCCTCTCCTTCTCTGT
MUC2 (reverse)	GCCACACTGGCCCTTTGT
GLUT2 (forward)	CTGCTTGGTCTATCTGCTGTG
GLUT2 (reverse)	TTGATGCTTCTTCCCTTTCTTT
TNF-α (forward)	ATGGGCCCCCAGAAGGAAGAG
TNF-α (reverse)	GATGGCAGAGAGGAGGTTGAC
CL-1 (forward)	TACTTTCCTGCTCCTGTC
CL-1 (reverse)	AAGGCGTTAATGTCAATC
OC (forward)	ATCAACAAAGGCAACTCT
OC (reverse)	GCAGCAGCCATGTACTCT
ZO-1 (forward)	GAGTTTGATAGTGGCGTT
ZO-1 (reverse)	GTGGGAGGATGCTGTTGT

[1]GAPDH= glyceraldehyde-3-phosphate dehydrogenase, MUC2= mucin 2, GLUT2= glucose transporter 2, TNF-α= tumor necrosis factor α, CL-1= claudin 1, OC= occludin, and ZO-1= zonula occludens 1.

Table 3-3. Growth performance and ileal morphology responses of pigs exposed to a 24-h post-weaning feed and water deprivation event

	Treatment			
	No feed and water deprivation	Feed and water deprivation	SEM[1]	*P*-value
Growth performance[2]				
IBW, kg	6.99	6.95	0.19	0.32
0 to 1 d post-weaning				
FBW, kg	6.84	6.58	0.18	< 0.01
ADG, kg	-0.16	-0.39	0.01	< 0.01
ADFI, kg	0.04	0.00	< 0.01	< 0.01
G:F, kg:kg	-4.00	0.00	< 0.01	< 0.01
1 to 7 d post-weaning				
FBW, kg	8.14	8.01	0.19	0.01
ADG, kg	0.21	0.23	0.01	0.01
ADFI, kg	0.22	0.20	0.01	0.02
G:F, kg:kg	0.96	1.20	< 0.01	< 0.01
7 to 14 d post-weaning				
FBW, kg	10.26	10.02	0.23	0.07
ADG, kg	0.31	0.30	0.01	0.53
ADFI, kg	0.35	0.34	0.01	0.38
G:F, kg:kg	0.89	0.89	0.01	0.97
14 to 21 d post-weaning				
FBW, kg	13.61	13.13	0.27	0.01
ADG, kg	0.50	0.45	0.02	0.04
ADFI, kg	0.67	0.62	0.01	0.01
G:F, kg:kg	0.73	0.73	0.01	0.77
21 to 27 d post-weaning				
FBW, kg	17.30	16.71	0.31	0.03
ADG, kg	0.62	0.59	0.02	0.08
ADFI, kg	0.91	0.87	0.02	0.15
G:F, kg:kg	0.68	0.67	0.01	0.33
Ileal morphology[3]				
1 d post-weaning				
Villous height, µm	328	241	31.67	0.03
Crypt depth, µm	93	101	6.80	0.38
VH:CD[4]	3.57	2.44	0.30	0.05
27 d post-weaning				
Villous height, µm	386	352	51.76	0.57
Crypt depth, µm	218	221	29.20	0.94
VH:CD[4]	1.80	1.75	0.30	0.92

[1] SEM= standard error of the mean.

[2] There were 20 to 22 replicates per treatment with 5 to 6 pigs per pen at the start of the trial.

[3] There were 12 to 16 replicates per treatment.

[4] villous height to crypt depth ratio.

Table 3-4. Growth performance and ileal histology responses of pigs exposed to a heat stress event subsequent to a post-weaning feed and water deprivation event[1]

	Treatment				SEM[2]			*P*-value		
Whole-plot factor	No feed or water deprivation		Feed and water deprivation		Whole-plot	Sub-plot	Interaction	Whole-plot	Sub-plot	Interaction
Sub-plot factor	Heat stress	No heat stress	Heat stress	No heat stress						
Growth performance[3]										
27 to 30 d post-weaning										
FBW, kg	18.65	19.57	17.65	18.92	0.38	0.47	0.54	0.12	0.10	0.08
ADG, kg	0.56[a]	0.65[ab]	0.56[a]	0.69[b]	0.03	0.03	0.03	0.69	0.01	0.03
ADFI, kg	0.81[a]	0.98[b]	0.77[a]	0.99[b]	0.03	0.03	0.03	0.57	< 0.01	< 0.01
G:F, kg:kg	0.68	0.65	0.74	0.68	0.02	0.02	0.03	0.08	0.17	0.14
30 to 35 d post-weaning										
FBW, kg	21.54	22.77	21.08	22.32	0.41	0.52	0.56	0.38	0.16	0.11
ADG, kg	0.60	0.59	0.61	0.62	0.01	0.01	0.02	0.23	0.91	0.48
ADFI, kg	0.95	0.95	0.97	1.02	0.02	0.02	0.03	0.14	0.45	0.32
G:F, kg:kg	0.64	0.62	0.63	0.61	0.01	0.01	0.02	0.73	0.34	0.78
0 to 35 d post-weaning										
Gain, kg	15.27	15.53	14.10	15.36	0.32	0.36	0.44	0.14	0.16	0.10
ADG, kg	0.44	0.44	0.40	0.40	0.01	0.01	0.01	0.14	0.16	0.10
ADFI, kg	0.58[ac]	0.61[c]	0.56[b]	0.59[ab]	0.01	< 0.01	0.01	0.15	0.03	0.03
G:F, kg:kg	0.75	0.72	0.72	0.74	0.01	0.02	0.02	0.54	0.81	0.45
Ileal morphology[4]										
30 d post-weaning										
Villous height, µm	318	329	309	336	18.10	17.22	27.33	0.93	0.43	0.87
Crypt depth, µm	156[a]	215[b]	179[a]	171[a]	11.58	10.79	15.35	0.44	0.14	0.05
VH:CD [5]	2.11	1.59	1.76	1.97	0.13	0.13	0.19	0.88	0.51	0.19

[1]Difference in superscript denotes difference between simple means.

[2]SEM = standard error of the mean.

[3]There were 9 to 11 replicates per treatment with 4 to 6 pigs per pen.

[4]There were 6 to 8 replicates per treatment.

[5] Villous height to crypt depth ra

Table 3-5. Serum measurements of pigs exposed to a 24-h post-weaning feed and water deprivation event[1]

	Treatment			
	No feed and water deprivation	Feed and water deprivation	SEM[2]	*P*-value
1 d post-weaning				
Cortisol, ng/mL	40.53	57.46	6.32	0.09
CRF, ng/mL[3]	1.57	1.71	0.05	0.03
Endotoxins, EU/mL[4]	0.83	13.03	3.15	0.02
27 d post-weaning				
Cortisol, ng/mL	45.84	45.17	5.80	0.94
CRF, ng/mL[3]	2.04	2.04	0.02	0.77
Endotoxins, EU/mL[4]	37.04	58.68	8.81	0.07

[1] There were 9 to 12 replicates per treatment.

[2] SEM= standard error of the mean.

[3] CRF = corticotrophin releasing factor.

[4] EU/mL = endotoxin units per mL.

Table 3-6. Serum measurements of pigs exposed to a heat stress event subsequent to a post-weaning feed and water deprivation event [1]

	Treatment				SEM[2]			*P*-value		
Whole-plot factor	No feed or water deprivation		Feed and water deprivation		Whole-plot	Sub-plot	Interaction	Whole-plot	Sub-plot	Interaction
Sub-plot factor	Heat stress	No heat stress	Heat stress	No heat stress						
30 d post-weaning										
Cortisol, ng/mL	33.49	33.50	37.79	27.67	3.78	4.19	5.39	0.87	0.54	0.63
CRF, ng/mL[3]	1.64	1.66	1.68	1.74	0.02	0.02	0.03	0.08	0.26	0.51
Endotoxin, EU/mL[4]	7.04	14.53	13.56	15.51	3.49	3.32	5.47	0.38	0.25	0.53

[1]There were 4 to 8 replicates per treatment with 4 to 6 pigs per pen.
[2]SEM = standard error of the mean.
[3] CRF= corticotrophin releasing factor.
[4] EU/mL = endotoxin units per mL.

Table 3-7. Ileal mucosa gene expression of pigs exposed to a 24-h post-weaning feed and water deprivation event[1,2,3]

	Treatment			
	No feed and water deprivation	Feed and water deprivation	SEM[4]	*P*-value
1 d post-weaning				
MUC2[5]	0.33	1.79	0.70	0.24
GLUT2[5]	3.20	1.18	0.95	0.09
TNF-α[5]	1.70	0.39	1.18	0.26
CL-1	1.56	1.92	0.82	0.10
OC[5]	3.06	0.72	0.71	0.05
ZO-1[5]	1.67	1.54	0.82	0.95
27 d post- weaning				
MUC2[5]	2.80	2.15	1.81	0.14
GLUT2	1.71	0.99	0.48	0.33
TNF-α	3.04	0.84	0.55	0.22
CL-1[5]	1.14	1.81	0.91	0.86
OC[5]	1.62	0.87	0.49	< 0.01
ZO-1	2.02	1.04	0.59	0.30

[1] There were 4 to 8 replicates per treatment.

[2] MUC2 = mucin; GLUT2 = glucose transporter 2; TNF-α = tumor necrosis factor alpha; CL-1 = claudin 1; OC = occludin; ZO-1 = zonula occludens 1.

[3]Gene expression relative to housekeeping gene GAPDH.

[4] SEM= standard error of the mean.

[5] Data was transformed to meet model assumptions.

Table 3-8. Ileal mucosa gene expression of pigs exposed to a heat stress event subsequent to a post-weaning feed and water deprivation event[1,2,3]

	Treatment				SEM[4]			*P*-value		
Whole-plot factor	No feed or water deprivation		Feed and water deprivation		Whole-plot	Sub-plot	Inter-action	Whole-plot	Sub-plot	Interaction
Sub-plot factor	Heat stress	No heat stress	Heat stress	No heat stress						
30 d post-weaning										
MUC2	1.89	2.08	1.12	2.04	0.41	0.37	0.59	0.46	0.37	0.62
GLUT2[5]	1.04	1.43	1.14	1.24	0.21	0.23	0.33	0.63	0.22	0.50
TNF-α[5]	1.47	1.44	1.18	1.49	0.33	0.32	0.49	0.73	0.45	0.86
CL-1	1.94	3.16	3.39	2.78	0.71	0.76	1.09	0.66	0.78	0.79
OC	0.85	1.19	0.88	1.19	0.13	0.12	0.18	0.88	0.07	0.34
ZO-1[5]	1.02	1.39	0.87	1.29	0.18	0.23	0.29	0.84	0.07	0.32

[1] There were 4 to 8 replicates per treatment.

[2] MUC2 = mucin; GLUT2 = glucose transporter 2; TNF-α = tumor necrosis factor alpha; CL-1 = claudin 1; OC = occludin; ZO-2 = zonula occludens 2.

[3]Gene expression relative to housekeeping gene GAPDH.

[4] SEM= standard error of the mean.

[5] Data was transformed to meet model assumptions.

CHAPTER 4. GARLIC DIALLYL DISULFIDE AND DIALLYL TRISULFIDE MITIGATES EFFECTS OF PRO-OXIDANT INDUCED CELLULAR STRESS AND HAS IMMUNE MODULATORY FUNCTION IN LPS-STIMULATED PORCINE EPITHELIAL CELLS

4.1. Abstract

The objective of the current study was to determine if garlic-derived diallyl disulfide (DADS) and diallyl trisulfide (DATS) could mitigate oxidative and endotoxin stress, using an intestinal porcine epithelial cell (IPEC-J2) model. The experiment was arranged as a 2 × 2 × 2 factorial of DADS + DATS (0 or 18 μM), pro-oxidant stressor (hydrogen peroxide at 0 or 100 μM), and endotoxin stressor (lipopolysaccharide (LPS) at 0 or 10 μg/mL) with a minimum of 4 to 8 replicates per treatment per variable. Cells were incubated with DADS + DATS for 18 h, LPS for 6 h, then with hydrogen peroxide for 3 h. Gene expression was measured by RT-PCR for cytokines, interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α), and tight junction proteins, claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1). Trans-epithelial electrical resistance (TEER), antioxidant enzymes superoxide disumutase (SOD) and catalase, and apical secretion of IL-8 protein into the incubation medium was also measured. There was an increase ($P < 0.01$) in TNF-α and IL-8 gene expression due to LPS, although there was no effect of hydrogen peroxide or DADS + DATS. Furthermore, there was a tendency for an increase ($P = 0.08$) in ZO-1 gene expression due to DADS + DATS.

Treatment with DADS + DATS and hydrogen peroxide did not affect TEER although there was a decrease ($P = 0.02$) in TEER with LPS incubation. Treatment of cells with hydrogen peroxide reduced SOD and catalase activity ($P < 0.01$) which was restored with pre-incubation of DADS + DATS ($P < 0.10$). There was an increase ($P < 0.01$) in IL-8 secretion due to LPS which was further augmented ($P < 0.01$) by pre-incubation with DADS + DATS. Based on the results from the current study, DADS + DATS can ameliorate oxidative effects of hydrogen peroxide, as well as alter IL-8 secretion in LPS-treated IPEC-J2 cells.

4.2. Introduction

Nursery pig gastrointestinal health and function can be influenced by several factors including dietary components, environmental stress, and intestinal microbiology. Recent literature shows weaning disrupts cellular oxidative status and leads to an increase in markers of oxidative tissue damage in pigs (Yin et al., 2014). Furthermore, pathogenic *E.coli* is a common diarrhea-causing agent in the post-weaned pig leading to significant mortality, morbidity, and economic losses (Fairbrother et al., 2005). Endotoxins, such as LPS, present on the outer membrane of gram negative *E.coli* elicit inflammatory responses in gastrointestinal cells largely through activation of the NFκB pathway in a TLR-dependent manner leading to changes in cellular oxidative status (Burkey et al., 2007). Gastrointestinal dysfunction is linked to cellular oxidative damage and inappropriate stimulation of immune mechanisms which translates into long-lasting impacts on pig health, performance, and digestive function (Pluske et al.,1997; Lambert, 2009; Smith et al., 2010). Therefore, nutritional strategies exists to mitigate the aforementioned oxidation-associated maladies which include, but are not limited to, dietary supplementation of plasma proteins, milk carbohydrates, prebiotics, probiotics, acidifiers, antioxidant vitamins and enzymes, and botanicals.

Bioactive components of garlic have been shown to have hepatoprotective, antioxidant, neuroprotective, immune modulatory properties, and growth-promoting benefits (Amagase et al. 2001; Amagase, 2006). Following grinding of whole garlic, downstream bioactive components such as DADS, DATS, and S-allyl cysteine (SAC) are formed (Amagase, 2006). Aged garlic and SAC has have been shown to restore

antioxidant function and reduce lipid peroxidation (Yamasaki and Lau, 1997; Wei and Lau, 1998) and garlic bioactive components, such as DADS and DATS, have been reported to have radical scavenging properties (Lang et al., 2004), and may reduce NFκB-induced production of inflammatory cytokine through inhibition of TLR-4 interaction with endotoxins (Zhou et al., 2011). In weaned pigs, improvements in gastrointestinal morphology and performance and reduced incidence of diarrhea with supplementation of crude garlic preparations have been shown (Tatara et al., 2008; Huang et al., 2011). Furthermore, Liu et al. (2013 and 2014) showed in weaned pigs that dietary supplementation of garlic bioactive components influence markers of innate immune function and cellular oxidative status in the intestinal mucosa and alter pathogenic *E.coli*-associated recruitment of innate immune cells to the ileum.

The IPEC-J2 cell line, derived from the jejunum of neonatal pigs, has been shown to be morphologically similar to in-vivo cells and elicits a robust response to endotoxin and oxidative stressors (Geens and Niewold, 2010; Paszti-Gere et al., 2012), making this cell line an appropriate in-vitro model for studying epithelia cell response to inflammatory and oxidant stress. We sought to determine the impact of a garlic-derived DADS + DATS in oxidative- and endotoxin-stressed cells and hypothesized the DADS + DATS would mitigate oxidative and endotoxin associated effects. To achieve these objectives, TEER, antioxidant enzymes, cytokine, and tight junction gene expression, and apical chemokine secretion in IPEC-J2 cells were measured in response to treatment with DADS + DATS.

4.3. Materials and methods

Cell culture and reagents

Intestinal porcine epithelial cells (IPEC-J2) are derived from the jejunum of a newborn, unsuckled pig (Paszti-Gere et al., 2012) and were kindly provided as a gift from Dr. Nicholas Gabler (Iowa State University, Ames, IA). Cells were grown and maintained in a humidified incubator (37 ˚C, 5 % CO_2) in 25 cm^2 flasks. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) with 5% Fetal Bovine Serum (FBS, Sigma-Aldrich, St. Louis, MO), 10 µg/ml insulin, 5.5 µg/ml transferrin, 5.0 ng/ml selenium (Sigma-Aldrich, St. Louis, MO), 5 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO) and 100 IU/ml and 100 µg/ml penicillin and streptomycin, respectively (Sigma-Aldrich, St. Louis, MO) (growing media). For the experiment, cells were maintained in growing media until confluent and in differentiation media which was similar to growing media except devoid of FBS. The garlic-derived DADS + DATS was obtained from Stanford Chemicals (Irvine, Ca) and consisted of 45% DADS and DATS which was verified by HPLC analysis at the Purdue University Metabolite Profiling Facility (West Lafayette, IN). LPS used in the current experiment was derived from *E. coli* O55:B5 (Sigma-Aldrich, St. Louis, MO). Immediately prior to experimental treatment the DADS + DATS, hydrogen peroxide, and LPS were solubilized with DMEM media. All treatments were administered on the cellular apical surface.

Experimental design

There were 8 treatments and 4 to 8 replicates per treatment arranged as a 2 × 2 × 2 factorial for the factors of DADS + DATS (0 or 18 µM), oxidative stressor (hydrogen peroxide at 0 or 100 µM), and endotoxin stressor (LPS at 0 or 10 µg/mL). Cells were incubated with DADS + DATS for 18 h prior to a 3 or 6 h hydrogen peroxide or LPS incubation, respectively. Pilot studies were conducted to identify the optimal treatment level and duration of exposure for DADS + DATS, hydrogen peroxide, and LPS based on lactate dehydrogenase release. Furthermore, lactate dehydrogenase release (Sigma-Aldrich, St. Louis, MO) was used to measure cytotoxicity of experimental reagents for the current experiment and was below 10%.

Measurement of gene expression by RT-PCR

As previously mentioned, cells were seeded into 24-well plates, differentiated, and the experiment was conducted. Cells were lysed with 5 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C until RNA isolation. RNA was extracted according to the Trizol protocol (Invitrogen, Grand Island, NY). Reverse transcription was carried out using the MMLV reverse transcription system of Promega and real-time PCR was performed on a Bio-Rad iCyler using a reaction mix that consisted of 0.50 µg cDNA, 0.075 nmol of each forward and reverse primers, SYBR Green Master (Roche, Basel, Switzerland), and nuclease-free water (Ambion, Austin, TX) to reach a reaction volume of 20 µL per well. Porcine-specific primers are reported in Supplemental Table 1. Gene expression was conducted for TNF-α, IL-8, CL-1, OC, and ZO-1 with 18S used as a housekeeping gene for normalization. Primers used are presented in Table 4-1.

Measurement of SOD and catalase activity

For SOD and catalase measurements, cells were seeded in 24-well plates (Sigma-Aldrich, St. Louis, MO) at 10^5 cells and the experiment was conducted 7-d post confluence. Superoxide dismutase activity was determined by the water-soluble tetrazolium salt method (Sigma-Aldrich, St. Louis, MO) and catalase activity was determined by colorimetric assay (Sigma-Aldrich, St. Louis, MO).

Measurement of TEER

Cells were seeded at 10^5 cells in culture media into Transwell-COL inserts with a pore size of 0.40 µm (Sigma-Aldrich, St. Louis, MO). The experiment was conducted 14-d post confluence and TEER was measured before and after the experimental treatment and expressed as percent change relative to initial value. Initial TEER readings of 300 ohms/cm^2 were detected before the experiment proceeded.

Measurement of cytokine secretion

Following the aforementioned experimental treatments, 100 µL of apical media was collected and immediately frozen at -80 ˚C. Protein analysis was conducted for IL-8 by sandwich enzyme immunoassay technique (R and D systems, Inc., Minneapolis, MN).

Statistical analysis

Data was analyzed using the Mixed procedure of SAS as a factorial experiment with main effects and subsequent interactions. If an interaction existed, simple effects were separated using the Tukey's mean separation test. Assumptions were validated using the Shapiro-Wilks Test for normality and Bartlett's-Levene's test for equal

variances (Proc Univariate). Any data plus or minus three standard deviations from the mean was considered an outlier and removed from analysis.

4.4. Results

Gene expression data are presented in Table 4-2. There was an increase in IL-8 and TNF-α gene expression due to LPS incubation ($P < 0.01$) but no impact of DADS + DATS or hydrogen peroxide. Pre-incubation of cells with DADS + DATS tended to increase ($P = 0.08$) ZO-1 gene expression although there were no other main effects. There was a reduction in TEER due to LPS (Fig. 4-1, $P = 0.02$) but no impact of DADS + DATS or hydrogen peroxide (Fig. 4-1). Relative to control cells, the 6-h LPS incubation reduced TEER values by approximately 9 percentage points.

There was an increase ($P = 0.02$) in SOD inhibition rate due to hydrogen peroxide (Fig. 4-2) and a tendency ($P = 0.08$) for a DADS + DATS × hydrogen peroxide interaction (Fig. 4-2). Relative to control cells there was a 3.7 fold increase in SOD inhibition rate due to hydrogen peroxide and pre-incubation with DADS + DATS mitigated approximately 68% of the hydrogen peroxide-associated increase in inhibition rate. Similarly, there was a reduction in catalase activity due to hydrogen peroxide (Fig. 4-3, $P < 0.01$) which was restored with DADS + DATS pre-incubation (Fig. 4-3, $P < 0.01$). Relative to control cells there was a 54% reduction in catalase activity due to hydrogen peroxide incubation and pre-incubation of cells with DADS + DATS allowed for a 96% recovery of the hydrogen peroxide-associated reduction of catalase activity.

Apical IL-8 secretion is presented in Figure 4-4. There was an impact of DADS + DATS, hydrogen peroxide, and LPS on IL-8 secretion ($P < 0.01$). Furthermore, there was a DADS + DATS and LPS interaction ($P < 0.01$) that shows augmentation of IL-8 secretion due to pre-incubation with DADS + DATS in LPS-treated cells. Specifically, incubation with LPS increased apical IL-8 secretion by 4.60 fold whereas pre-incubation of LPS-treated cells with DADS + DATS resulted in a 5.4 fold increase. Additionally, a hydrogen peroxide and LPS interaction ($P = 0.04$) existed which shows a decrease in IL-8 secretion with LPS and hydrogen peroxide incubation compared to cells treated with LPS only.

4.5. Discussion

The gastrointestinal epithelial cell layer serves as a key communication intermediate between intestinal luminal contents and physiological systems in addition to nutrient absorption functions (Paszti-Gere et al., 2014). Luminal oxidants and endotoxins are detected by epithelial cells and elicit a series of immune and protective responses that can impact nutrient uptake and metabolism and immune status. Human adenocarcinoma cells (Caco-2) are commonly used for in-vitro studies although concerns still exist in regard to the ability of these cells cells to elicit a response to luminal contents similar to that of in-vivo GI epithelial cells (Geens and Niewold, 2010). Porcine-derived cells lines, such as IPEC-J2, were isolated from the pig small intestine, are non-transformed, and have the ability to elicit a robust immunological response making them a viable in-vitro model to study oxidative- and inflammatory-associated

cellular stressors (Geens and Niewold, 2010; Paszti-Gere et al., 2012). Bioactive components in garlic, such as SAC, dially sulfides, and isolated proteins have been shown to be anti-inflammatory, anti-oxidative, and immune modulatory (Borek, 2001; Amagase, 2006). In-vitro studies show altered immune status and improved antioxidant status in stressed cells (Yamasaki and Lau, 1997; Wei and Lau, 1998; Lang et al., 2004) and in-vivo studies in pigs and rats show improved growth and intestinal function (Tatara et al., 2008; Huang et al., 2011; Liu et al., 2013; Badr et al., 2014). Therefore, the IPEC-J2 cell model was used to investigate the ability of garlic-derived DADS + DATS to mitigate hydrogen peroxide oxidant stress and LPS-induced endotoxin stress in the current study.

Oxidative stress occurs when cellular antioxidant systems do not sufficiently mitigate cellular and extracellular oxidants, such as ROS and hydrogen peroxide, leading to damage of cellular lipid membranes, protein, and DNA (Cai et al., 2013). Reactive oxygen species and hydrogen peroxide are generated during aerobic respiration and immune activation and from extracellular sources such as dietary products and endotoxins present in the gut lumen (Paszti-Gere et al., 2010). Innate antioxidant enzyme systems such as SOD, catalase, and the glutathione complex exist to breakdown ROS and hydrogen peroxide to water and oxygen. For the current experiment there was a reduction in activity of antioxidant enzymes SOD and catalase due to hydrogen peroxide that was largely mitigated by pre-treatment with DADS + DATS. Garlic bioactive components SAC, DADS, and DATS have been shown to have radical scavenging properties (Borek, 2001) and Wei and Lau (1998) showed that a garlic preparation mitigated hydrogen peroxide-induced increases in ROS and improved

antioxidant enzyme activity using a bovine endothelial cell model. Badr and Al-Mulhim (2014) showed that in rats with indomethacin-induced gastric damage, there were recoveries in SOD and glutathione activity and a reduction in malondialdhyde with supplementation of 100 and 200 mg/kg garlic product. More recently, Kohda (2013) showed that aged garlic acts directly to mitigate ROS and indirectly by stimulating gene expression of cellular antioxidant systems in a nueroblastoma cell line. A dearth of information exists on the impact of garlic products on porcine antioxidant cellular functions. The antioxidant effects observed in the current experiment are likely due to direct radical scavenging properties of diallyl sulfides present in high concentrations (45%) in the garlic product used.

E.coli pathogenesis is common in nursery pigs and is responsible for considerable post-weaning associated diarrhea, morbidity, and mortality (Fairbrother et al., 2005). Endotoxins, such as LPS, present on the outer membrane of pathogenic *E.coli* are responsible for TLR-dependent activation of immunological pathways. Likewise, ROS from exogenous or endogenous sources serves as a key intermediate in the oxidative-stress signaling mechanism for inflammation (Mittal et al., 2014). In the current experiment there was an increase in IL-8 and TNF-α gene expression due to LPS incubation and no effect of hydrogen peroxide or DADS + DATS. The effect of LPS on IL-8 and TNF-α gene expression is similar as reported in other studies using IPEC cells (Arce, 2010; Liu et al., 2012; Paszti-Gere et al., 2014). Paszti-Gere et al. (2012) showed an increase in IL-8 and TNF-α gene expression due to 1 mM hydrogen peroxide in IPEC-J2 cells. The discrepancy in results between the current experiment and previous literature may be due to experimental methods including hydrogen

peroxide dose and incubation time. There was an increase in IL-8 secretion due to LPS and DADS + DATS further modulated the LPS-induced effects as demonstrated by the LPS × garlic interaction. The literature shows conflicting data on the effects of garlic products on immune modulation. Kyo et al. (2001) showed aged-garlic products modulate the immune system, enhance natural killer cell activity, and augment concavalin A stimulation. Clemmens et al. (2010) showed proteins QR-1 and QR-2 isolated from raw garlic have mitogenic capabilities. A dearth of information exists on immune modulatory properties of garlic in vivo although Jafari et al. (2012) showed garlic extract enhanced complement activity in broiler chickens and Kuo et al. (2011) showed that supplementation of 100 mg garlic product per kg body weight increased nuetrophilia in endotoxin-injected rats. Conversely, Lang et al. (2004) showed that a synthetic allicin product reduced inflammatory cytokine secretion in unstimulated and TNF-α stimulated Caco-2 cells and Shih et al. (2010) showed that in a neutrophil-like cell model there was reduced chemotactic response and cell migration due to a garlic product. Furthermore, Lee et al. (2012) showed that dially sulfides decreased neutrophil infiltration in endotoxin-exposed rats and Liu et al. (2013) showed a decrease in neutrophils and macrophages in weaned pigs exposed to *E. coli* and supplemented with a garlic product. Discrepancies exist in the literature in regards to the effects of garlic products on chemokine dynamics which could be due to variation in bioactive components, concentration of garlic product supplemented, and duration of supplementation. Therefore, further research is warranted to better understand the mechanisms of garlic bioactive components on immune dynamics.

Previous research shows that endotoxins and oxidative stress can lead to changes in tight junction (TJ) protein structure and gene expression leading to GI dysfunction (Seth et al., 2009; Qin et al., 2009). In the current experiment only LPS decreased TEER but there was a tendency for DADS + DATS incubation to increase Z0-1 gene expression. Paszti-Gere et al. (2012) showed no changes in TJ gene expression or TEER due to hydrogen peroxide in IPEC-J2 cells. Changes in TEER due to LPS in the current experiment are consistent as reported by Geens and Niewold (2010). Recently, a mucosal gene expression profile study in pigs by Liu et al. (2014) showed that supplementation of garlic product did not impact TJ gene expression and largely impacted genes related to innate defense mechanisms. Park et al. (2011) reported changes in metalloprotease and claudin gene expression due to dially sulfides and Roselli et al. (2007) showed an improved in TEER due to synthetic allicin in *E. coli*-infected IPEC-J2 cells that was not related to antimicrobial effects. The ZO proteins are an intracellular component of the tight junction complex that are in contact with actin cytoskeleton components (Nusrat et al., 2000; Gonzalez-Mariscal et al., 2003). Previous research shows that inflammation-associated changes in myosin light chain kinase phosphorylation leads to cytoskeleton-induced changes in tight junction protein expression that is associated with loss of cellular integrity (Shen et al., 2006). An increase in ZO-1 gene expression could translate into improved epithelial cell integrity although the aforementioned gene expression change was not associated with a change in TEER and needs further investigation before conclusions can be drawn.

In conclusion, supplementation of DADS + DATS mitigated hydrogen-peroxide induced oxidant effects in IPEC-J2 cells and augmented secretion of IL-8 in LPS-

stimulated cells. Further investigation of the impact of garlic bioactive components on immune modulation and innate immune function is needed and additional research is needed to understand the functional impact of DADS + DATS on ZO-1 gene and protein expression. Taken together, results from the current experiment show that supplementation of DADS + DATS can improve mucosal antioxidant status and impact IL-8 dynamics using an IPEC-J2 model.

4.6. Acknowledgments

We would like to thank Hui Yan (Purdue University, Animal Sciences) for his assistance with cell culture, Dr. Arun Bhunia (Purdue University, Food Science) for his assistance with laboratory analyses, and Aly Gautier (Purdue University, Animal Sciences) for her assistance with gene expression analysis.

4.7. References

Amagase, H., B.L. Petesch, H. Matsuura, S. Kasuga, and Y. Itakura. 2001. Intake of garlic and its bioactive components. J. Nutr. 131: 955S–962S.

Amagase, H. 2006. Clarifying the real bioactive constituents of garlic. J. Nutr. 136: 716S–725S.

Arce, C., M. Ramirez-Boo, C. Lucena, and J.J. Garrido. 2010. Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from *Salmonella typhimurium*. Comp. Immunol. Microbiol. Infect. Dis. 33:161-174.

Badr, G.M., and J.A. Al-Mulhim. 2014 The protective effect of aged garlic extract on nonsteroidal anti-inflammatory drug-induced gastric inflammations in male albino rats. Evid. Based Complement. Alternat. Med. 2014: 759642.

Borek, C. 2001. Antioxidant health effects of aged garlic extract. J. Nutr. 131: 1010S–1015S.

Burkey, T.E., K.A. Skjolaas, S.S. Dritz, J.E. Minton. 2007. Expression of toll-like receptors, interleukin 8, macrophage migration inhibitory factor, and osteopontin in tissues from pigs challenged with *Salmonella enterica* serovar Typhimurium or serovar Choleraesuis. Vet. Immunol. Immunopathol. 115:309-319.

Cai, X., X. Chen, X. Wang, C. Xu, Q. Guo, L. Zhu, S. Zhu, and J. Xu. 2013. Pre-protective effect of lipoic acid on injury induced by H2O2 in IPEC-J2 cells. Mol. Cell Biochem. 378:73-81.

Clemmens, F., S.N. Pramod, and Y.P. Venkatesh. 2010. Identity of the immunomodulatory proteins from garlic (Allium sativum) with the major garlic lectins or agglutinins. Int. Immunopharmacol. 10: 316–324.

Fairbrother, J.M., E. Nadeau, and C.L. Gyles. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Res. Rev. 6: 17–39.

Geens, M., and T.A. Niewold. 2010. Preliminary characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enterotoxigenic *Escherichia coli*, *Escherichia coli*, and *E. coli* lipopolysaccharide. Comp. Funct. Genomics. 2010: 469583.

Gonzalez-Mariscal, L., A. Betanzos, N. Nava, and B.E. Jaramillo. 2003. Tight junction proteins. Prog. Biophys. Mol. Biol. 1: 1-44.

Huang, R.H., X.S. Qiu, F.X. Shi, C.L. Hughes, Z.F. Lu, and W.Y. Zhu. 2011. Effects of dietary allicin on health and growth performance of weanling piglets and reduction in attractiveness of faeces to flies. Animal. 5: 304–311.

Jafari, R.A., M. Ghorbanpoor, and M. Makkei. 2012. Effect of garlic supplementation on complement activity in broiler chickens. Comp. Clin. Pathol. 21:183–185.

Kohda, K., H. Goda, K. Itoh, K. Samejima, and T. Fukuuchi. 2013. Aged garlic extract reduces ROS production and cell death induced by 6-hydroxydopamine through activation of the Nrf2-ARE pathway in SH-SY5Y cells. J. Pharm. Pharmacol. 4: 31-40.

Kuo, C.H., S.H Lee, K. M. Chen, C.L. Lii, and C.T. Liu. 2011. Effect of garlic oil on neutrophil infiltration in the small intestine of endotoxin-injected rats and its association with levels of soluble and cellular adhesion molecules. J. Agric. Food Chem. 59:7717-7725.

Kyo, E., N. Uda, S. Kasuga, and Y. Itakura. 2001. Immunomodulatory effects of aged garlic extract. J. Nutr. 131:1075S–1079S.

Lang, A., M. Lahava, E. Sakhninia, I. Barshack , H.H. Fiddera, B. Avidana, E. Bardana, R. Hershkovizc, S. Bar-Meira, andY. Chowersa. 2004. Allicin inhibits spontaneous and TNF-α induced secretion of proinflammatory cytokines and chemokines from intestinal epithelial cells. Clin. Nutr. 23: 1199–1208.

Lambert, G.P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J Anim Sci. 87 (E suppl.): E101-E108

Lee, J.S., E.G. Awki, S.J. Lee, D.D. Tassew, Y.B. Park, K.S. Park, M.K. Park, B. Kim, and S.C. Park. 2012. Effect of *Lactobacillus plantarum* CJLP243 on the growth performance and cytokine response of weaning pigs challenged with enterotoxigenic *Escherichia coli*. J. Anim. Sci. 90: 3709-3717.

Liu, Y., M. Song, T.M. Che, D. Bravo, and J.E. Pettigrew. 2012. Anti-inflammatory effects of several plant extracts on porcine alveolar macrophages in vitro. J. Anim. Sci. 90: 2774-2783.

Liu, Y., M. Song, T.M. Che, A.S. Almeida, J.J. Lee, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2013. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs. J. Anim. Sci. 91:5294-5306.

Liu, Y., M. Song, D. Che, C. Bravo, C.W. Maddox, and J.E. Pettigrew. 2014. Effects of capsicum oleoresin, garlic botanical, and turmeric oleoresin on gene expression profile of ileal mucosa in weaned pigs. J Anim Sci. 92:3426-3440.

Mittal, M., M.R. Siddiqui, K. Tran, S.P. Reddy, and A.B. Malik. 2014. Reactive oxygen species in inflammation and tissue injury. Antioxid. Redox Signal. 20: 1126–1167.

Nusrat, A., J.R. Turner, and J.L. Madara. 2000. Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am. J. Gastrointest. Liver Physiol. 279:G851-G857.

Park, H.S., G.Y.Kim, I.W. Choi, N.D. Kim, H.J.Hwang, Y.W. Choi, and Y.H.Choi. 2011. Inhibition of matrix metalloproteinase activities and tightening of tight junctions by diallyl disulfide in AGS human gastric carcinoma cells. J. Food Sci. 76: T105-T111.

Paszti-Gere, E., E. Csibrik-Nemeth, S. Krisztina, R. Csizinszky, J. Csaba, and P. Galfi. 2012. Acute Oxidative Stress Affects IL-8 and TNF-α Expression in IPEC-J2 Porcine Epithelial Cells. Inflammation. 35: 994-1004.

Paszti-Gere, E., G. Matis, O. Farkas, A. Kulcsar, O. Palocz, G. Csiko, Z. Neogrady, P. Galfi. 2014. The effects of intestinal LPS exposure on inflammatory responses in a porcine enterohepatic co-culture system. Inflammation. 37: 247-260.

Pluske, J.R., D.J. Hampson, and I.H. William. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51: 215–236.

Qin, H., Z. Zhang, X. Hang, and Y.L. Jiang. 2009. *L. plantarum* prevents Enteroinvasive *Escherichia coli*-induced tight junction proteins changes in intestinal epithelial cells. BMC Microbiol. 9:63-72.

Roselli, M., M.S. Britti, I. Lerou-Luron, H. Marfaing, W.Y. Zhu, E. Mengheri. 2007. Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic *Escherichia coli* K88 in pig intestinal cells. Toxicol. in Vitro. 21: 224–229.

Seth. P., G. Samak, J.A. Shull, A. Seth, and R. Rao. 2009. Protein phosphatase 2A plays a role in hydrogen peroxide-induced disruption of tight junctions in Caco-2 cell monolayers. Biochem. J. 421:59-70.

Shen, L., E.D. Black, E.D. Witkowski, W.J. Lencer, V. Guerriero, E.E. Schneeberger, and J.R. Turner. 2006. Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. J. Cell Sci. 119: 2095-2106.

Shih, P.C., C.H. Kuo, J.Y. Juang, C.H. Liu, L. Hsu, C.T. Liu. 2010. Effects of garlic oil on the migration of neutrophil-like cell studied by using a chemotactic gradient labchip. J Biomed. Biotech. 2010: 319059.

Smith, F., J.E. Clark, B.L. Overman, C.C. Tozel, J.H. Huang, J.E. Rivier, A.T. Blikslager, and A.J. Moeser. 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am J Physiol Gastrointest Liver Physiol. 298(3):G352-G363

Tatara, M.R., E. Śliwa, K. Dudek, A. Gawron, T. Piersiak, P. Dobrowolski, J. Mosiewicz, A.K. Siwicki, and T. Studziński. 2008. Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared on artificial sow. Ann Agric Environ Med. 15: 63–69.

Wei, Z., and B.H. Lau. 1998. Garlic inhibits free radical generation and augments antioxidant enzyme activity in vascular endothelial cells. Nutr. Res. 18:61–70.

Yamasaki, T., and B.H. Lau. 1997. Garlic compounds protect vascular endothelial cells from oxidant injury. Folia Pharmacol Jpn. 110: Suppl. 1:138–141.

Yin, J., M.M Wu, H. Xiao, W.K. Ren, J.L. Duan, G. Yang, T.J. Li, and Y.L. Yin. 2014. Development of an antioxidant system after early weaning in piglets. J. Anim. Sci. 92: 612-19.

Zhao, L., J.Y. Lee, and D.H. Hwang. 2011. Inhibition of pattern recognition receptor-mediated inflammation by bioactive phytochemicals. Nutr. Rev. 69:310–332.

Table 4-1. Primers used for RT-PCR[1]

Gene	Primer (5`-3`)
18S (forward)	ATCCCTGAGAAGTTCCAGCA
18S (reverse)	CCTCCTGGTGAGGTCGATGT
IL-8 (forward)	TTTCTGCAG CTCTCTGTGAGG
IL-8 (reverse)	CTGCTGTTGTTGTTGCTTCTC
TNF-α (forward)	CGTCGCCGT TGTAGC
TNF-α (reverse)	GCCCATCTGTCGGCACCACC
CL-1 (forward)	AGAAGATGCGGATGGCTGTC
CL-1 (reverse)	CCCAGAAGGCAGAGAGAAGC
OC (forward)	TCCTGGGTGTGATGGTGTTC
OC (reverse)	CGTAGAGTCCAGTCACCGCA
ZO-1 (forward)	GAGTTTGATAGTGGCGTT
ZO-1 (reverse)	GTGGGAGGATGCTGTTGT

[1]IL-8 = interleukin 8, TNF-α = tumor necrosis factor alpha, CL-1 = claudin 1; OC = occludin, and ZO-1 = zonula occludens 1.

Table 4-2. Main effects of garlic diallyl disulfide (DADS) and diallyl trisulfide (DATS), hydrogen peroxide, and LPS on cytokine and tight junction gene expression in IPEC-J2 cells[1]

Gene	IL-8	TNF-α	CL-1	OC	ZO-1
		DADS + DATS level			
No DADS + DATS	1.60	2.37	1.02	1.51	1.09
DADS + DATS	2.12	4.87	1.20	1.43	1.82
SEM[2]	0.35	1.02	0.17	0.25	0.27
		Hydrogen peroxide level			
No hydrogen peroxide	1.91	3.30	0.97	1.39	1.46
Hydrogen peroxide	1.85	3.94	1.25	1.56	1.45
SEM[2]	0.35	1.28	0.17	0.26	0.27
		LPS level			
No LPS	0.65	1.67	0.92	1.39	1.35
LPS	3.10	5.57	1.30	1.57	1.56
SEM[2]	0.35	1.02	0.18	0.26	0.27
		P-values[3]			
DADS + DATS	0.58	0.33	0.33	0.96	0.08
Hydrogen peroxide	0.55	0.70	0.19	0.29	0.99
LPS	< 0.01	< 0.01	0.33	0.19	0.31

[1]Expression of the following genes relative to house keeping gene 18S: IL-8 = interleukin 8, TNF-α = tumor necrosis factor alpha, CL-1 = claudin 1; OC = occludin, and ZO-1 = zonula occludens 1.

[2]SEM= standard error of the mean.

[3] Data did not meet model assumption of normality, therefore *P*-values are from log-transformed data.

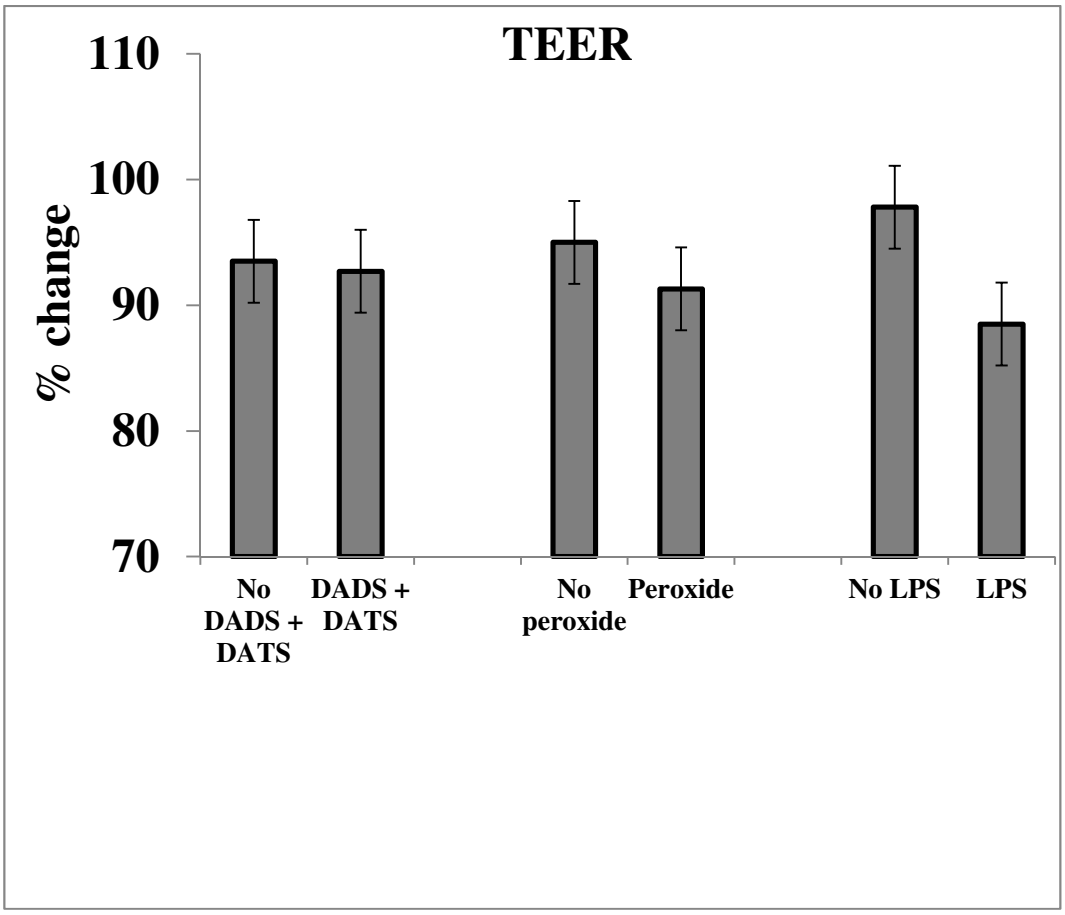

Figure 4-1. Main effects of garlic diallyl disulfide (DADS) and diallyl trisulfide (DATS), hydrogen peroxide, and LPS on trans-epithelial electrical resistance (TEER). *P*-values were 0.77, 0.22, and 0.01 for main effects of DADS + DATS, hydrogen peroxide, and LPS, respectively, with no significant interactions.

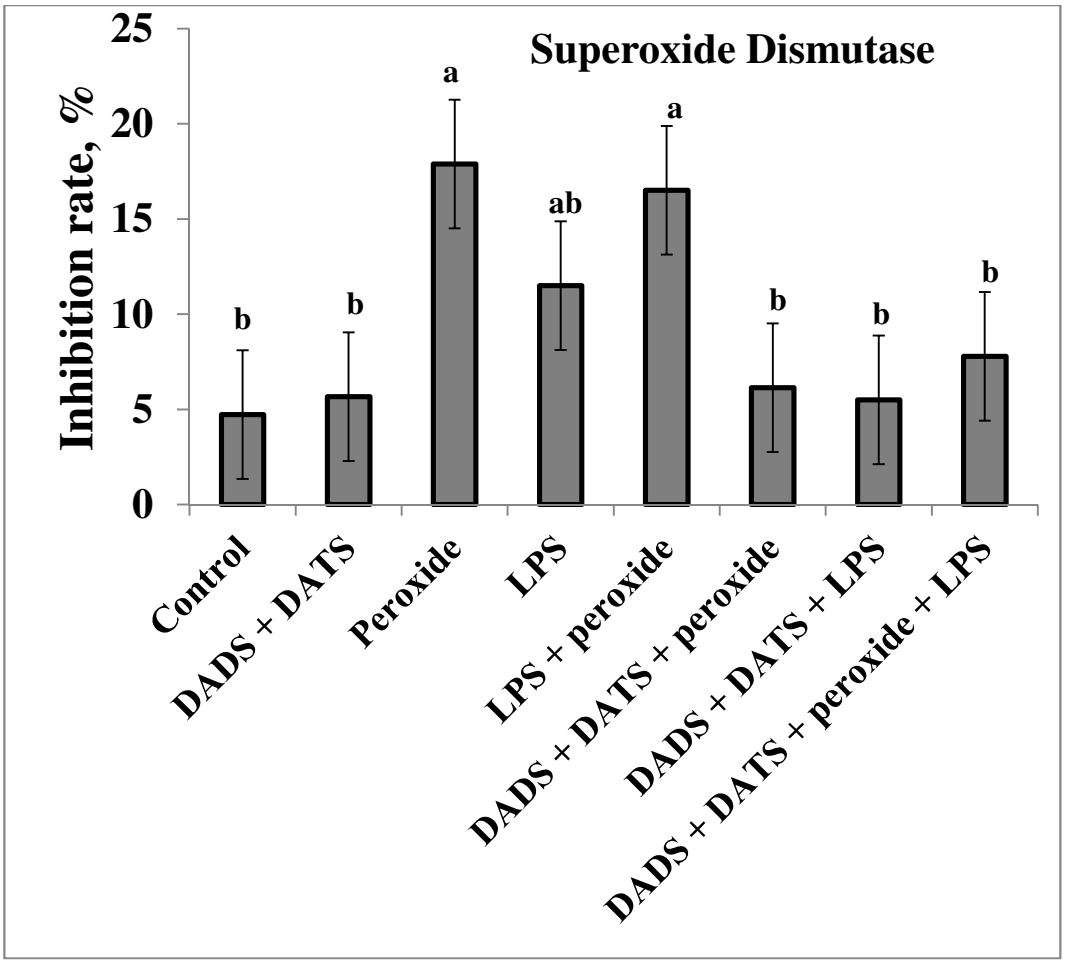

Figure 4-2. Simple effects of garlic diallyl disulfide (DADS) and diallyl trisulfide (DATS), hydrogen peroxide, and LPS on SOD activity. Main effect *P*-values were < 0.01 for DADS + DATS and hydrogen peroxide, and 0.40 for LPS; $P = 0.08$ for DADS + DATS and hydrogen peroxide interaction; $P > 0.10$ for all other interactions. Superoxide dismutase activity expressed as percent inhibition rate.

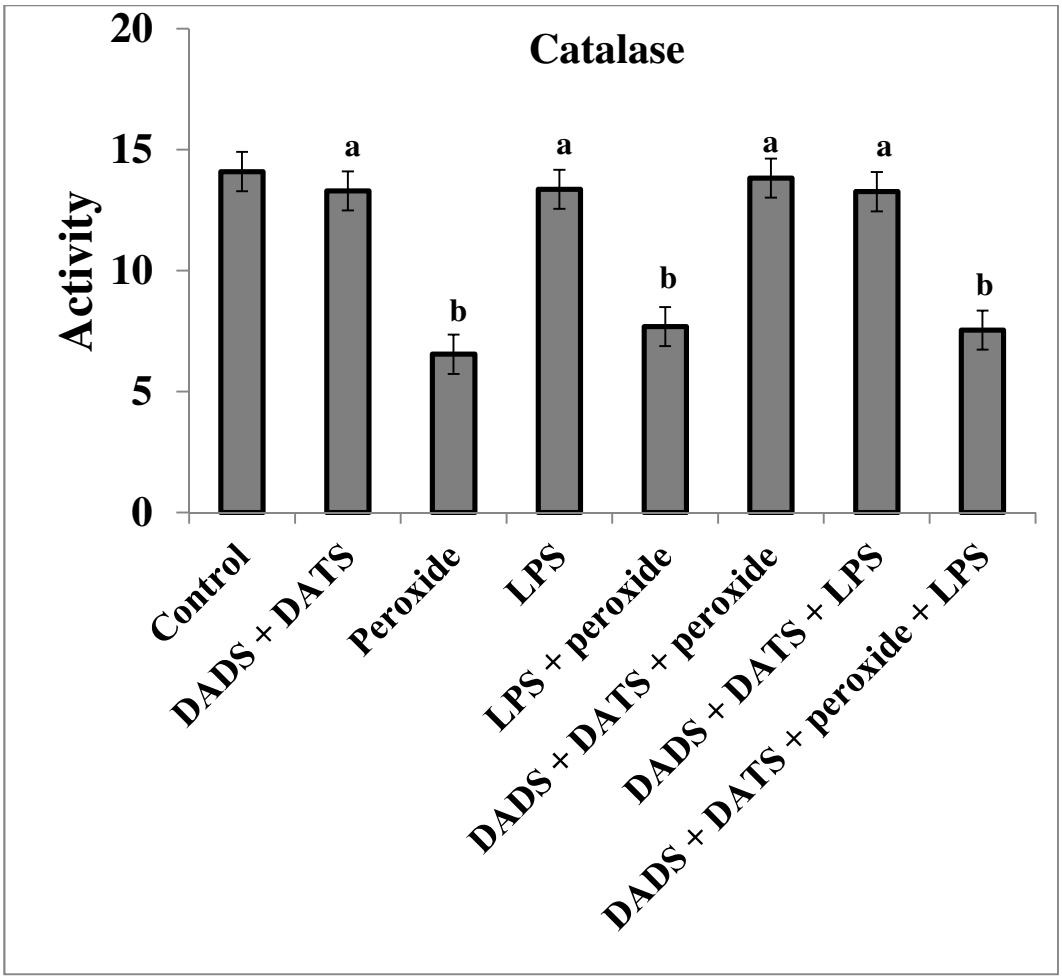

Figure 4-3. Simple effects of garlic diallyl disulfide (DADS) and diallyl trisulfide (DATS), hydrogen peroxide, and LPS on catalase activity. $P < 0.05$ for main effects of DADS + DATS, hydrogen peroxide, and LPS, $P < 0.01$ for DADS + DATS and hydrogen peroxide interaction, hydrogen peroxide and LPS interaction, and DADS + DATS and hydrogen peroxide and LPS interaction, and $P = 0.17$ for DADS + DATS and LPS interaction. Catalase activity expressed as mMol H_20_2 degraded per mL per min.

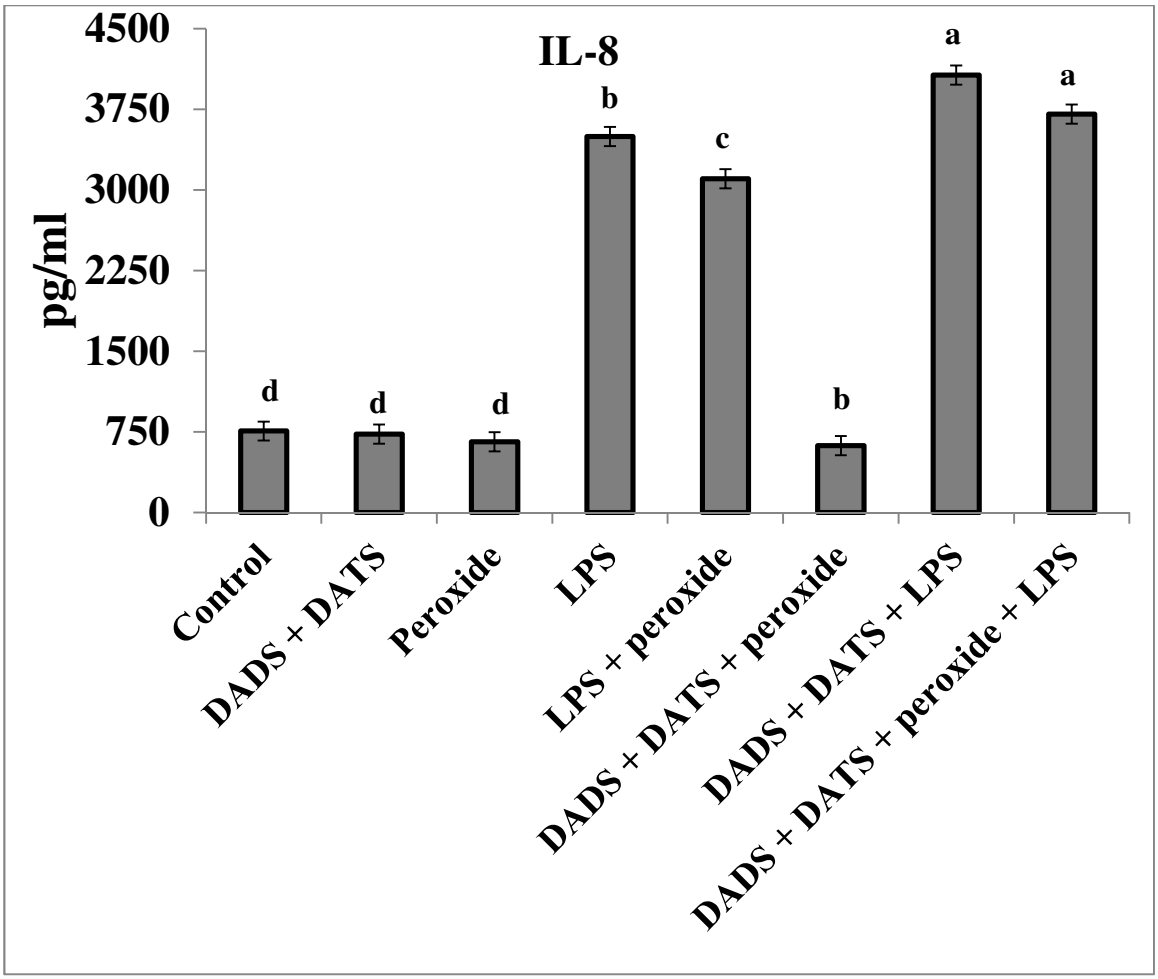

Figure 4-4. Simple effects of garlic diallyl disulfide (DADS) and diallyl trisulfide (DATS), hydrogen peroxide, and LPS on IL-8 secretion. $P < 0.01$ for main effect of DADS + DATS, hydrogen peroxide, LPS, and interaction of DADS + DATS and LPS, and $P = 0.04$ for interaction of hydrogen peroxide and LPS, respectively, with no other significant interactions.

CHAPTER 5. EXPRESSION OF CYTOKINE AND TIGHT JUNCTION GENES AND ILEAL MORPHOLOGY IN NURSERY PIGS IN RESPONSE TO GARLIC DIALLYL DISULFIDE AND DIALLYL TRISULFIDE COMPOUNDS

5.1. Abstract

Bioactive components in garlic have anti-inflammatory, antioxidant, and immune modulatory properties that may improve gastrointestinal function in pigs. The current experiment was conducted to identify the optimal dosage of garlic-derived bioactive components diallyl disulfide (DADS) and diallyl trisulfide (DATS) and to evaluate the effect of graded doses of DADS and DATS on nursery pig performance and gastrointestinal function. Eight replicate pens of barrows were allotted on the basis of initial BW (12.45 ± 1.37 kg) in a RCBD to one of six treatments that consisted of administration of 0, 0.45, 0.90, 1.80, 3.60, or 7.20 mg DADS + DATS per kg bodyweight. The DADS + DATS was administered daily by oral gavage to overnight-fasted pigs for a period of 6 d with soybean oil as the carrier and vehicle control. Growth performance and daily stool consistency scores were recorded. At the end of the experiment, pigs were euthanized and the mid ileum was excised for morphological and gene expression measurements. Mucosal gene expression was conducted by RT PCR for mucin (MUC2), interleukin 8 (IL-8), tumor necrosis factor (TNF-α), claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1). Supplementation with DADS +

DATS did not affect growth performance or stool consistency, although there was a tendency ($P = 0.06$) for increased villous height. Expression of the IL-8 gene was linearly increased ($P = 0.01$) and there was a tendency for a decrease ($P = 0.07$) in ZO-1 gene expression with garlic supplementation. There was no effect of treatment on expression of MUC2, TNF-α, CL-1, and OC genes. Results from the current study show that DADS + DATS supplementation improves ileal villus height and the broken-line regression-derived adequate dose to optimize villus height response was 1.71 mg DADS + DATS per kg BW. Furthermore, DADS + DATS supplementation impacts IL-8 and ZO-1 gene expression, which may have implications on mucosal immune function and cellular integrity.

5.2. Introduction

Nursery pig gastrointestinal health and function can be influenced by several factors including dietary components, environmental stress, and intestinal microbiology. Gastrointestinal dysfunction can lead to cellular oxidative damage and over-stimulation of inflammatory pathways with long-lasting implications on pig health, performance, and digestive function (Pluske et al., 1997; Lambert 2009; Smith et al., 2010). Bioactive phytochemicals in garlic products have been shown to have hepatoprotective, antioxidant, and immune enhancing properties. Following grinding of fresh garlic, the bioactive phytochemical alliin is rapidly broken down to thiosulfinates, such as allicin, that have been shown to be antimicrobial (Ankri and Mirelman, 1999; Amagase et al., 2001; Amagase, 2006). Dially disulfide, DATS, and S-allyl cysteine (SAC) are downstream bioactive components from extremely unstable and spontaneously degrading garlic thiosulfinates (Amagase et al., 2001; Amagase, 2006). Meta-analysis shows downstream bioactive components of garlic may mitigate oxidant-induced cellular dysfunction and influence innate immune dynamics (Borek, 2001; Kyo et al., 2001).

Previous research shows enhanced growth performance and intestinal function and reduced morbidity with supplementation of crude preparations of garlic products to young pigs (Tatara et al., 2008; Huang et al., 2011; Yan et al., 2012). The exact mode of action of garlic bioactive components is not fully understood although in-vitro research suggests that garlic-derived organosulfur compounds enhance intestinal cell integrity, reduce cellular oxidative stress by mitigation of reactive oxygen species (ROS) and

increase antioxidant enzyme function (Yamasaki and Lau 1997; Wei and Lau 1998; Roselli et al., 2007). There is a dearth of information in the literature on the impact of garlic bioactive components DADS and DATS on nursery pig performance and gastrointestinal health. We hypothesized that an adequate dose of orally administered downstream bioactive components DADS and DATS would enhance ileal mucosal morphology and integrity in pigs. To achieve our objectives we determined an effective dose as well as the effect of various doses of DADS + DATS on growth performance, ileal mucosal morphology, and cytokine and tight junction gene expression.

5.3. Materials and methods

All animal procedures were approved by the Purdue University Animal Care and Use Committee.

Animals and treatments

A total of 48 PIC 337 × C29 barrows (initial BW = 12.45 ± 1.37 kg, 34 d old) were allotted to treatment on the basis of initial BW in a randomized complete block design. The barrows were individually housed in cement-floor pens containing one nipple drinker and feed trough per pen. Room temperature was maintained at 24.4°C and there was 14 h of light per day. Pigs were allowed a 6-d acclimation period prior to initiation of experimental treatment. There were 6 treatments that consisted of daily administration of 0, 0.45, 0.90, 1.80, 3.60, or 7.20 mg of garlic-derived DADS + DATS per kg BW by oral gavage for a period of 6 d. There were 8 replicates per treatment with 1 barrow per pen. The oral gavage was prepared fresh daily with

soybean oil as the carrier and vehicle control and administered at 0800 h to overnight-fasted barrows. Barrows were allowed free access to water and were fed a mash-based grower diet that met or exceeded NRC requirements (NRC, 2012; Table 5-1). The garlic product was obtained from Stanford Chemicals (Irvine, Ca) and consisted of 22% DADS and 23% DATS with the remaining 55% consisting of a methanol-based carrier. Barrows and feeders were weighed at the start and end of the experiment to calculate ADG, ADFI, and G:F. Scours were subjectively monitored by the same person twice daily (0800 and 1600 h) and recorded corresponding to a scale of 1 = no scour, 2 = moderate fecal looseness, or 3 = severe diarrhea. On d 6 of the experiment, barrows were euthanized by intramuscular injection with ketamine (Fort Dodge Laboratories Inc., Fort Dodge, IA), Telazol (Fort Dodge Laboratories), and xylazine (Bayer Corp., Shawnee Mission, KS) at doses of 4, 2, 2, and 2.2 mg/kg BW, respectively, followed by asphyxiation with CO_2. Immediately following euthanasia a 10-cm segment of the distal ileum was excised for histological and gene expression measurements.

Histological measurements

Excised intestinal segments were flushed in ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA), stapled to a cardboard background, and fixed in 10% buffered formalin (VWR International, Radnor, PA) for approximately 30 d. Samples were subsequently dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). Segments were sliced (5 µm) and stained with hematoxylin and eosin by the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth

were measured and villus height to crypt depth ratio was calculated. Means from at least 4 villi per segment were analyzed for differences. Villus length is defined as the length from the villus tip to the valley between villi and crypt depth is defined as the length between the crypt opening and base.

RNA isolation, cDNA Synthesis, and real-time PCR

Immediately after removal, intestinal segments were flushed with ice-cold PBS (VWR International, Radnor, PA), cut in half exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C until RNA isolation. RNA was extracted according to the Trizol protocol (Invitrogen, Grand Island, NY). Reverse transcription was carried out using the MMLV reverse transcription system of Promega (Promega, Madison, WI) and real-time PCR was performed on a Bio-Rad iCyler (Bio-Rad Laboratories, Hercules, CA) using a reaction mix that consisted of 0.50 µg cDNA, 0.075 nmol of each forward and reverse primers, SYBR Green Master (Roche, Basel, Switzerland), and nuclease-free water (Ambion, Austin, TX) to reach a reaction volume of 20 µl per well. Following a 5-min incubation period at 95ºC, reactions were cycled 50 times using the following protocol: 10s at 95ºC, 20 s at 55ºC, and 72ºC for 30 s. Expression of mucin (MUC2), tumor necrosis factor alpha (TNF-α), interleukin 8 (IL-8), claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1) genes were measured using 18S as a housekeeping control. Primers used are presented in Table 5-2.

Statistical analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) with the fixed effect of treatment and the random effect of block. The following statistical model was used in the analysis: $Y_{ij} = \mu + W_i + B_j + \varepsilon_{ij}$ where Y is the response criterion; μ is the overall mean; W_i is the effect of i^{th} treatment (i = 1,2,3, …,6); B_j is the effect of j^{th} block (k = 1, 2, 3,… 8); and ε_{ij} is the error term. If there was an effect of treatment, single-degree-of-freedom linear and quadratic contrasts were conducted with coefficients for unequally spaced levels that were derived using the IML procedure of SAS. Incidence of diarrhea (scour scores) was analyzed as non-parametric data using the NPAR1WAY procedure of SAS. Broken-line regression analysis was conducted using the NLIN procedure of SAS as described by Robbins et al. (2006). Significant difference was defined as $P \leq 0.05$ and $0.05 \leq P \leq 0.10$ was considered a tendency. Model assumptions for normality and equal variances were validated by the Shapiro-Wilks test (UNIVARIATE procedure of SAS) and Brown and Forsythe test (GLM procedure of SAS), respectively.

5.4. Results

Pigs were healthy throughout the experiment and there were no incidences of morbidity, feed refusal, or aversion to receipt of the DADS + DATS gavage. Growth performance and stool consistency are reported in Table 5-3. There was no effect of treatment on growth performance or stool consistency during the experiment ($P > 0.05$). The average initial BW, final BW, and G:F were 12.45 ± 1.37 kg, 15.90 ± 1.82 kg, and

0.65 ± 0.17, respectively. Moderate stool looseness (scour score = 2) was reported throughout the experiment and there were isolated incidences of severe diarrhea (scour score = 3) observed that were not treatment specific and did not correspond with changes in growth performance.

The average villus height and crypt depth were 350 ± 69.6 μm and 134 ± 40.5 μm, respectively (Table 5-3). There was a tendency for an effect of treatment on villus height ($P = 0.06$) but not crypt depth ($P = 0.50$). Pigs that received a daily oral gavage of 0.90, 1.80, 3.60, or 7.20 mg DADS + DATS per kg BW had a 12, 17, 34, or 15 % increase in villi length, respectively, in comparison with pigs receiving the vehicle control gavage. The villus height plateaued at 381 μm which was with daily supplementation of 1.71 mg DADS + DATS per kg BW (Figure 5-1).

Gene expression data are reported in Table 5-4. There was no effect of treatment on housekeeping gene expression. Daily oral gavage of DADS + DATS linearly increased ($P = 0.01$) IL-8 gene expression and there was a tendency for decreased ZO-1 ($P = 0.07$) gene expression. In pigs that received daily oral gavage of 7.2 mg DADS + DATS per kg BW, there was approximately a 100% increase in IL-8 gene expression and a 56 % decrease in ZO-1 gene expression relative to pigs receiving the vehicle control gavage. There was no impact ($P > 0.10$) of experimental treatment on MUC2, TNF-α, CL-1, and OC gene expression. The expression of the ZO-1 gene plateaued at 0.90 arbitrary units (AU) which was with daily supplementation of 0.90 mg DADS + DATS per kg BW (Figure 5-2).

5.5. Discussion

During the nursery phase, pigs are exposed to various environmental, social, and nutritional stress events that are linked to growth performance and gastrointestinal function (Pluske et al., 1997; Lalles et al., 2007; Smith et al., 2010). A clear link has been established between structure and function of the small intestine and pig growth performance (Pluske et al., 1997) and nutritional and management strategies have been shown to mitigate nursery pig gastrointestinal dysfunction (Lalles et al., 2007). The aforementioned strategies include supplementation of specific ingredients including plasma protein, milk carbohydrates, prebiotics, probiotics, and botanicals. Garlic bioactive components have been shown to be antimicrobial (Ankri and Mirelman, 1999), antioxidant (Borek, 2001), immune modulatory (Kyo et al., 2001), and anti-inflammatory (Lang et al., 2004). Recent literature from in-vitro and rodent experiments suggests supplementation of garlic may be a viable option to improve animal performance and gastrointestinal function (Lang et al., 2004; Roselli et al., 2007; Badr and Al-Mulhim 2014). In pigs, Tatara et al. (2008) and Huang et al. (2011) showed supplementation of a crude garlic product improved performance and intestinal morphology and Liu et al. (2013) showed garlic product supplementation reduced *E.coli*-induced diarrhea and impacted innate immune parameters in sham- and *E.coli*-infected animals. Therefore, the objective of the current experiment was to determine an effective dose as well as the effect of graded doses of DADS + DATS on nursery pig performance, stool consistency, and ileal mucosa gene expression of immune and cellular integrity markers.

Previous research showed enhanced growth performance and intestinal function and reduced morbidity with supplementation of garlic products to young pigs (Tatara et al., 2008; Huang et al., 2011; Yan et al., 2012). Tatara et al. (2008) showed improved growth performance in artificially-raised piglets that corresponded to greater villous height and crypt depth throughout the small intestine and Huang et al. (2011) similarly showed improved bodyweight gain and reduced diarrhea incidence in weaned pigs supplemented with 0.10 to 0.25 g/kg garlic product in the feed. The exact mode of action of garlic bioactive components is not fully understood although in-vitro research suggests that garlic-derived organosulfur compounds reduce cellular oxidative stress by mitigation of reactive oxygen species (ROS) and increase antioxidant enzyme function (Yamasaki and Lau 1997; Wei and Lau, 1998). Furthermore, DADS and DATS have been shown to have radical scavenging properties and reduce TNF-α- and hydrogen peroxide-induced activation of NFκB and subsequent inflammatory cytokine release (Lang et al., 2004; Zhou et al., 2011). Garlic bioactive components have been shown to enhance cellular integrity in a porcine epithelial cell model exposed to *E.coli* (Roselli et al., 2007) although a dearth of information exists on the effects of garlic on tight junction proteins. Additionally, garlic components have been shown to influence both immune modulation and suppression (Kyo et al., 2001). Liu et al. (2013) showed reduced ileal infiltration of neutrophils and macrophages and serum inflammatory cytokines in *E.coli*-infected nursery pigs fed a garlic product and more recently Lui et al. (2014) showed by gene expression profile that nursery pigs fed a garlic product had increased expression of genes associated with innate immune defense and oxidative

reduction and decreased expression of genes associated with antigen presentation and iron binding.

The literature contains conflicting evidence for the effects of garlic products on nursery and grow-finish pig growth performance. Huang et al. (2011) showed supplementation of 0.10 to 0.25 g garlic oil per kg diet improved nursery pig growth performance over a 28-d feeding period and Tatara et al. (2008) reported a 3 to 5 % improvement in BW in young pigs fed diets supplemented with 1 to 2 mg/kg garlic or allicin supplemented by oral gavage. Likewise, Yan et al. (2012) showed supplementation of 2 to 4 g garlic product per kg diet improved growth performance over an extended duration. Conversely, Chen et al. (2008) showed no effect of garlic product supplementation in growing pigs and Holden (1998) showed garlic product supplementation to grow-finish pigs impaired gain in a feed intake-dependent manner. In the current study there was no impact of DADS + DATS supplementation on nursery pig growth performance, and given the short duration of the study, this observation was not unexpected. The feed efficiency values reported for the current experiment are similar to values reported by Horn et al. (2014) and Cromwell et al. (2008) although ADG and ADFI were slightly higher than previously reported by our lab using pigs from the same genetic source which is likely due to the high health status and experimental design (individually-penned pigs). A previous experiment by Huang et al. (2011) showed the effects of garlic supplementation were not realized during the first week of supplementation. Furthermore, most in-vivo and in-vitro studies demonstrating garlic-product efficacy contain an experimental challenge event such as early weaning, *E.coli* infection, or oxidative damage (Tatara et al., 2008; Badr and Al-Mulhim 2014;

Liu et al., 2014). Therefore, the lack of growth performance response due to DADS + DATS for the current experiment is likely related to the health status of the pigs and duration of supplementation. Previous studies in nursery pigs show that garlic product supplementation may reduce pig morbidity by reducing incidence of diarrhea (Huang et al., 2011; Liu et al. 2013). Huang et al. (2011) reported a linear decrease in overall incidence of diarrhea with most drastic effects during the early period of supplementation. Similarly, Liu et al. (2013) reported that supplementation of garlic product to *E.coli*-infected or sham pigs reduced diarrhea score throughout the experiment or during the first 5 d post weaning, respectively. It is hypothesized that garlic products may act to reduce nursery pig post-weaning incidence of diarrhea though enhancing innate immune function (Liu et al., 2014) or radical scavenging properties of organo-sulfur bioactive components acting to reduce gastrointestinal inflammation (Borek, 2001; Huang et al., 2011). Furthermore, Huang et al. (2011) suggested that garlic products affect gastrointestinal motility and secretion though agonistic effects on TRPA-1 receptors. For the current experiment there was no effect of DADS + DATS supplementation on diarrhea incidence. Discrepancies between the current study and previous literature are likely due to the age and health status of the pigs.

A clear distinction should be made between the effects of fresh garlic and aged garlic bioactive components on gastrointestinal function. Generally, fresh garlic bioactive components, such as allicin, are associated with gastrointestinal damage (Amagase et al., 2001) whereas evidence exists that aged garlic bioactive components are associated with improved gastrointestinal function (Horie et al., 1997; Tatara et al.,

2008; Peinado et al., 2012). In artificially-reared neonatal pigs, supplementation with garlic products improved intestinal morphology throughout the gastrointestinal tract (Tatara et al., 2008). Conversely, garlic product supplementation had no impact on intestinal morphology in *E.coli*-infected nursery pigs supplemented with garlic (Liu et al., 2013). Discrepancies in the literature are likely due to differences in product bioactive components, animal health status, and experimental design. For the current pig experiment, villus height increased 11, 15, 25, or 13 % with supplementation oral gavage of 0.90, 1.80, 3.60, and 7.20 mg DADS + DATS per kg BW, respectively relative to pigs that did not receive the garlic product gavage. The broken-line regression-derived adequate dose to optimize villus height response was 1.71 mg DADS + DATS per kg BW, which for a 10- to 15-kg pig consuming 1.2 kg feed translates to approximately 0.04 g DADS + DATS per kg feed. Ileal morphology data reported in the current experiment are within range of values reported in the literature for nursery pigs (Liu et al., 2008; Rubio et al., 2010). The exact mechanism of action of garlic bioactive components on GI morphology is not fully understood, although it may be related to microbial and immune actions. Tatara et al. (2008) showed a garlic product modulated GI antimicrobial factors such as lysozyme and Peinado et al. (2012) showed a garlic bioactive component reduced broiler chicken enteropathogen colonization that was associated with improved gastrointestinal morphology. The aforementioned studies suggest morphological benefits of garlic are related to microbial modulation and innate immune function although further research is needed to better understand the impact of garlic bioactive components on gastrointestinal microbial ecology and enterocyte function.

Garlic products have been shown to be immune modulatory in in-vitro and in-vivo research models (Kyo et al., 2001; Lang et al., 2004; Liu et al., 2014). Using a rodent cell model, Kyo et al. (1997 and 2001) showed aged garlic decreased antigen-specific histamine release from basophils and increased natural killer cell phagocytic activity suggesting aged garlic has anti-allergic and anti-tumor properties, respectively. Furthermore, Kyo et al. (2001) showed aged-garlic increased spleen cell proliferation and augmented concavalin A stimulation further suggesting an immune stimulatory role of garlic bioactive components. Likewise, Clemmens et al. (2010) identified two distinct immunomodulatory proteins in an ex-vivo experiment using human immune cells and Alma et al. (2014) showed increased urinary excretion of IL-12 and IFN-γ in humans supplemented with a garlic product. Conversely, Lang et al. (2004) showed a synthetic garlic product reduced spontaneous and TNF-α stimulated inflammatory cytokine secretion in Caco-2 cells. Recent literature in pigs showed reduced ileal macrophage and neutrophil infiltration in sham and *E.coli*-infected animals with garlic product supplementation (Liu et al., 2013). Furthermore, Liu et al. (2014) showed that supplementation of garlic to nursery pigs for 9 d increased genes associated with defense response and complement components and decreased genes associated with antigen presentation and processing. For the current experiment there was a linear increase in IL-8 gene expression due to garlic supplementation. To the author's knowledge there are currently no published studies showing the impact of garlic bioactive components on ileal IL-8 gene expression. Interleukin 8 is a chemokine that is involved in recruitment and activation of innate immune cells such as neutrophils and macrophages and a change in IL-8 gene expression may be associated with changes in

innate immune function. There was no impact of treatment on TNF-α gene expression, which is consistent with the literature (Liu et al., 2014). Taken together, results from the current study and previous in-vitro and in-vivo studies, suggest a complex relationship between garlic bioactive components and immune modulation. Further studies should investigate the impact of innate immune cell activation in intestinal tissues along with measurement of innate-immune cell molecular markers to more clearly understand the immunological effects of garlic product supplementation. One of the major constituents of the mucus layer is mucin which plays a critical intestinal barrier function (Montagne et al., 2004). For the current experiment there was no impact of garlic supplementation on MUC2 gene expression, and this is consistent with results reported by Liu et al. (2014) in nursery pigs. Tight junction proteins serve to control epithelial cell permeability and integrity (Gonzalez-Mariscal et al., 2003). In the current experiment there was no effect of treatment on CL or OC gene expression, although there was a tendency for a decrease in ZO-1 gene expression as oral gavage dose increased from 0 to 7.20 mg DADS + DATS per kg BW with a breakpoint at 0.90 mg DADS + DATS per kg BW which roughly translates to 0.02 g DADS + DATS per kg feed for a 10- to 15-kg pig consuming 1.20 kg feed per day. Roselli et al. (2007) showed enhanced membrane barrier function in *E.coli*-infected IPEC cells due to supplementation with a synthetic garlic product that was not due to antimicrobial effects and Park et al. (2011) showed DADS increased trans-epithelial electrical resistance, decreased CL gene expression, and increased metalloprotease inhibitor gene expression. In contrast, Liu et al. (2014) showed no impact of a garlic product on ileal mucosa tight junction gene expression profile in nursery pigs. For the current study, it is possible that

the change in ZO-1 gene expression is related to increases in IL-8 chemokine gene expression and innate immune function, although further research is needed to better understand the relationship between immune modulation and intestinal barrier function.

Previous research shows that garlic product supplementation improves growth performance and health (Tatara et al., 2008; Huang et al., 2011) which may be due to immune modulatory (Kyo et al., 2001) and antioxidant functions (Borek, 2001) of bioactive components. In the current study there was no impact of the garlic product on pig growth which may be due to duration of feeding and pig health status. Oral gavage of DADS + DATS to pigs influences ileal morphology, and IL-8 and ZO-1 gene expression in nursery pigs. Further research is warranted to better understand the relationship between changes in IL-8 and ZO-1 gene expression and innate immune function in nursery pigs. Overall, results from the current study show that DADS + DATS in garlic influences gastrointestinal dynamics and may be strategy to enhance nursery pig health and digestive function. Furthermore, approximately 0.08 g DADS + DATS per kg feed is adequate to improve ileal villus height in nursery pigs.

5.6. Acknowledgements

The authors would like to express their appreciation to Biomatrix and JBS United for their financial and technical support of this trial. Further appreciation is given to Jason Fields, Purdue Animal House, for support with pig husbandry. The authors would also like to express appreciation to Aly Gautier and Daniel Miranda, Purdue University Department of Animal Sciences, for their assistance with treatment

administration and Donna Brooks, Purdue University Veterinary School, for her assistance with histological measurements.

5.7. References

Alma, E., A. Eken, H. Ercil, K. Yelsel, and N. Daglioglu. 2014. The effect of garlic powder on human urinary cytokine excretion. Urology J. 11(1): 1309-1315.

Amagase, H., B.L. Petesch, H. Matsuura, S. Kasuga, and Y. Itakura. 2001. Intake of garlic and its bioactive components. J. Nutr. 131: 955S–962S.

Amagase, H. 2006. Clarifying the real bioactive constituents of garlic. J. Nutr. 136: 716S–725S.

Ankri, S., and D. Mirelman. 1999. Antimicrobial properties of allicin from garlic. Microb. Infect. 2: 125–129.

Badr, G.M., and J.A. Al-Mulhim. 2014. The Protective effect of aged garlic extract on nonsteroidal anti-inflammatory drug-induced gastric inflammations in male albino rats. Evid. Based Complement. Alternat. Med. 2014: 759642.

Borek, C. 2001. Antioxidant Health Effects of Aged Garlic Extract. J. Nutr. 131: 1010S–1015S.

Chen, Y.J., I.H. Kim, J.H. Cho, J.S. Yoo, Q. Wang, Y. Wang, and Y. Haung. 2008. Evaluation of dietary L-carnitine or garlic powder on growth performance, dry matter and nitrogen digestibilities, blood profiles and meat quality in finishing pigs. Anim. Feed Sci. Tech. 141(1): 141-152.

Clemmens, F., S.N. Pramod, and Y.P. Venkatesh. 2010. Identity of the immunomodulatory proteins from garlic (Allium sativum) with the major garlic lectins or agglutinins. Int. Immunopharmacol.10: 316–324.

Cromwell, G.L., G.L. Allee, and D.C. Mahan. 2008. Assessment of lactose level in the mid- to late- nursery phase on performance of weanling pigs. J. Anim. Sci. 86: 127-133.

Gonzalez-Mariscal, L., A. Betanzos, P. Nava, and B.E. Jaramillo. 2003. Tight junction proteins. Progr. Biophys. Molec. Biol. 81: 1-44.

Horie, T., S. Awazu, Y. Itakura, and T. Fuwa. 1997. Alleviation by garlic of antitumor drug-induced damage to the intestine. J. Nutr. 131(3s): 1071S-1074S.

Holden, P.J., J. McKean, and E. Franzenburg. 1998. Botanicals for pigs-garlic. Iowa State University, Swine Research Report.

Horn, N., F. Ruch, G. Miller, K. M. Ajuwon, and O. Adeola. 2014. Impact of acute water and feed deprivation events on growth performance, intestinal characteristics, and serum stress markers in weaned pigs. J. Anim. Sci. 92:4407-4416.

Huang, R.H., X.S. Qiu, F.X., Shi, C.L. Hughes, Z.F. Lu, and W.Y. Zhu. 2011. Effects of dietary allicin on health and growth performance of weanling piglets and reduction in attractiveness of faeces to flies. Animal. 5: 304–311.

Kyo, E., N. Uda, M. Kakimoto, K. Yokoyama, M. Ushijima, I. Sumioka, S. Kasuga, and Y. Itakura, Y. 1997. Anti-allergic effects of aged garlic extract. Phytomedicine.4: 335–340.

Kyo, E., N. Uda, S. Kasuga, and Y. Itakura. 2001. Immunomodulatory effects of aged garlic extract. J. Nutr. 131:1075S–1079S.

Lalles J.P., P. Bosi, H. Smidt, and C.R. Stokes. 2007. Nutritional management of gut health in pigs around weaning. Proc. Nutr. Soc. 66: 260–268.

Lambert, G.P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J. Anim. Sci. 87 (E suppl.) E101-E108.

Lang, A., M. Lahava, E. Sakhninia, I. Barshack, H.H. Fiddera, B. Avidana, E. Bardana, R. Hershkovizc, S. Bar-Meira, and Y. Chowersa. 2004. Allicin inhibits spontaneous and TNF-α induced secretion of proinflammatory cytokines and chemokines from intestinal epithelial cells. Clin. Nutr. 23: 1199–1208.

Liu P., X.S. Piao, S.W. Kim, L. Wang, Y.B. Shen, H.S. Lee, and S.Y. Li. 2008. Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of Escherichia coli and Lactobacillus in weaning pigs. J. Anim. Sci. 86(10): 2609-2618.

Liu, Y., M. Song, T.M. Che, A.S. Almeida, J.J. Lee, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2013. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs. J. Anim. Sci. 91:5294-5306.

Liu, Y., M. Song, D. Che, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2014. Effects of capsicum oleoresin, garlic botanical, and turmeric oleoresin on gene expression profile of ileal mucosa in weaned pigs. J. Anim. Sci. 92:3426-3440.

Montagne, L., C. Piel, and J. P. Lalles. 2004. Effect of diet on mucin kinetics and composition:nutrition and health implications. Nutr. Rev. 62:105–114.

NRC, 2012. Nutrient requirements of Swine. 11th rev ed. Natl. Acad. Press, Washington, D.C.

Olukosi, O.A., and N. D. Dono. 2014. Modification of digesta pH and intestinal morphology with the use of benzoic acid or phytobiotics and the effects on broiler chicken growth performance and energy and nutrient utilization. J. Anim. Sci. 92:3945–3953.

Park, H.S., G.Y. Kim, I.W.Choi, N.D. Kim, H.J. Hwang, Y.W. Choi, and Y.H. Choi. 2011. Inhibition of matrix metalloproteinase activities and tightening of tight junctions by diallyl disulfide in AGS human gastric carcinoma cells. J. Food Sci.76: T105-T111.

Peinado, M.J., R. Ruiz, A. Echavarri , and L. A. Rubio. 2012. Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. Poult. Sci. 91:2148–2157.

Pluske, J.R., D.J. Hampson, and I.H. William. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review, Livest. Prod. Sci. 51, 215–236.

Robbins, K. R., A. M. Saxton, and L. L. Southern. 2006. Estimation of nutrient requirements using broken-line regression analysis. J. Anim. Sci. 84:E155–E165.

Roselli, M., M.S. Britti, I. Lerou-Luron, H. Marfaing, W.Y. Zhu, and E. Mengheri. 2007. Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic Escherichia coli K88 in pig intestinal cells. Toxicol. in Vitro. 21: 224–229.

Rubio, L.A., R. Ruiz, M.J. Peinado, and A. Echavarri. 2010. Morphology and enzymatic activity of the small intestinal mucosa of Iberian pigs as compared with a lean pig strain. J. Anim. Sci. 88(11): 3590-3597.

SAS Institute. 2006. SAS/STAT User's Guide. Release 9.1. SAS Institute Inc., Cary, NC.

Smith, F., J.E. Clark, B.L. Overman, C.C. Tozel, J.H. Huang, J.E. Rivier, A.T. Blikslager, and A.J. Moeser. 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 298(3):G352-G363.

Tatara, M.R., E. Śliwa, K. Dudek, A. Gawron, T. Piersiak, P. Dobrowolski , J. Mosiewicz, A. K. Siwicki, and T. Studziński. 2008. Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared on artificial sow. Ann. Agric. Environ. Med. 15: 63–69.

Wei, Z., and B.H., Lau. 1998. Garlic inhibits free radical generation and augments antioxidant enzyme activity in vascular endothelial cells. Nutr. Res. 18:61–70.

Yan, L., Q.W. Meng, and I.H. Kim. 2012. Effects of fermented garlic powder supplementation on growth performance, nutrient digestibility, blood characteristics, and meat quality in grow-finish pigs. Anim. Sci. J. 83: 411-417.

Yamasaki, T., and B.H. Lau. 1997. Garlic compounds protect vascular endothelial cells from oxidant injury. Folia Pharmacol. Jpn.110 (Suppl.1):138–141.

Zhao, L., J.Y. Lee, and D.H. Hwang. 2011. Inhibition of pattern recognition receptor-mediated inflammation by bioactive phytochemicals. Nutr. Rev. 69:310–320.

Table 5-1. Ingredient composition of common diet

Item	Common diet
Ingredients, g/kg	
Corn	664.3
Soybean meal	300
Limestone[1]	10.0
Monocalcium phosphate[2]	9.0
Salt	6.5
L-Lys HCL	4.4
Vitamin premix[3]	1.5
DL-Met	1.5
Mineral premix[4]	1.2
Selenium premix[5]	0.5
Total	1,000
Calculated nutrients and energy, as-fed basis	
ME, kcal/kg	3243
CP, g/kg	203.0
Ca, g/kg	6.45
P, g/kg	5.79
Nonphytate P, g/kg	3.32
Ca:P	1.11
SID Lys, g/kg	12.45

[1] Contained 38% Ca.

[2] Contained 17% Ca and 21.1 % P.

[3] Vitamin premix per kilogram of diet: vitamin A, 5,090 IU; vitamin D3, 1,270 IU ; vitamin E, 22 IU, vitamin K activity, 7.2 mg; menadione, 2,412 µg; vitamin B12, 22.4 µg; riboflavin, 8.11 mg; d-pantothenic acid, 61.20; and niacin, 106.59 mg.

[4] Mineral premix supplied per kilogram of diet: Cu (as copper chloride), 18 mg; I (as ethylenediamine dihydroiodide), 0.95 mg; Fe (as iron carbonate), 334 mg; Mn (as manganese oxide), 60 mg; and zinc (as zinc oxide), 150 mg.

[5] Supplied as 300 µg of Se per kilogram of diet.

Table 5-2. Primers used for RT-PCR[1]

Gene	Primer (5`-3`)
18S (forward)	ATCCCTGAGAAGTTCCAGCA
18S (reverse)	CCTCCTGGTGAGGTCGATGT
MUC2 (forward)	CAACGGCCTCTCCTTCTCTGT
MUC2 (reverse)	GCCACACTGGCCCTTTGT
TNF-α (forward)	CGTCGCCGT TGTAGC
TNF-α (reverse)	GCCCATCTGTCGGCACCACC
IL-8 (forward)	TTTCTGCAG CTCTCTGTGAGG
IL-8 (reverse)	CTGCTGTTGTTGTTGCTTCTC
CL-1 (forward)	AGAAGATGCGGATGGCTGTC
CL-1 (reverse)	CCCAGAAGGCAGAGAGAAGC
OC (forward)	TCCTGGGTGTGATGGTGTTC
OC (reverse)	CGTAGAGTCCAGTCACCGCA
ZO-1 (forward)	GAGTTTGATAGTGGCGTT
ZO-1 (reverse)	GTGGGAGGATGCTGTTGT

[1] MUC2 = mucin 2; TNF-α = tumor necrosis factor alpha; IL-8 = interleukin 8; CL-1 = claudin 1; OC = occluding; and ZO-1 = zonula occludens 1.

Table 5-3. Growth performance and ileal histology responses of pigs that received oral gavage of DADS + DATS [1]

DADS + DATS orally administered, mg /kg BW[2]	0	0.45	0.90	1.80	3.60	7.20	SEM[3]	*P*-value
Item								
IBW, kg	12.5	12.4	12.5	12.4	12.5	12.4	0.50	0.99
FBW, kg	16.4	15.3	15.7	16.0	15.9	16.0	0.65	0.65
ADG, g	629	526	522	604	564	563	54.72	0.54
ADFI, g	955	814	800	907	1021	959	96.76	0.39
G:F, g:g	0.71	0.63	0.67	0.70	0.55	0.62	0.06	0.41
Scour scores[4]	1.51	1.63	1.56	1.50	1.86	1.74	0.14	0.55
Villus height, µm (VH)[5]	310	293	348	363	415	357	32.04	0.06
Crypt depth, µm (CD)[5]	129	141	133	123	170	126	20.46	0.50
VH:CD	2.53	2.24	2.86	3.26	2.80	2.98	0.45	0.58

[1] Pigs were administered DADS + DATS by oral gavage daily with soybean oil as the carrier for 6 d. For growth performance data there were 6 to 8 replicate pens per treatment and for histology data there were 4 to 6 replicate pens per treatment with 1 pig per pen. Pigs were allowed a 6-d adaptation period prior to the trial.

[2] BW=body weight.

[3] SEM = standard error of the mean. The highest SEM is reported.

[4] Analyzed as non-parametric data. Scour scores were recorded once daily on a scale from 1 to 3, with 1 = normal stools and 3 = severe diarrhea.

[5] Distal ileum was sampled at the end of the trial for histological analysis.

Table 5-4. Ileal mucosal gene expression responses of pigs that received oral gavage of DADS + DATS [1]

DADS + DATS orally administered, mg /kg BW[2]	0	0.45	0.90	1.80	3.60	7.20	SEM[3]	*P*-value
Item								
MUC2	1.72	1.19	1.21	1.33	1.41	1.41	0.42	0.95
IL-8[4,5]	2.08	0.75	0.91	2.54	2.60	4.15	0.96	0.02
TNF-α	1.44	1.46	1.34	1.21	1.08	1.43	0.31	0.93
IL-6[4]	1.38	1.59	1.46	1.85	1.75	1.07	0.76	0.87
CL-1[4]	3.48	1.45	2.75	1.45	0.95	0.46	1.15	0.49
OC	1.89	0.81	1.41	1.19	1.01	1.18	0.36	0.27
ZO-1	1.78	0.87	1.33	0.92	1.02	0.78	0.25	0.07

[1]Pigs were administered DADS + DATS by oral gavage daily with soybean oil as the carrier for 6 d. There were 4 to 6 pigs per treatment. Pigs were allowed a 6-d adaptation period prior to the trial. BW= body weight, MUC2 = mucin 2, IL-8 = interleukin 8, TNF-α = tumor necrosis factor alpha, IL-6 = interleukin 6, CL-1 = claudin 1, OC = occludin, ZO-1 = zonula occludens 1. 18S was used as the housekeeping gene.

[2] BW=body weight.

[3] SEM = standard error of the mean. The highest SEM is reported.

[4] *P*-values derived from log transformed data.

[5] Linear effect at $P < 0.01$.

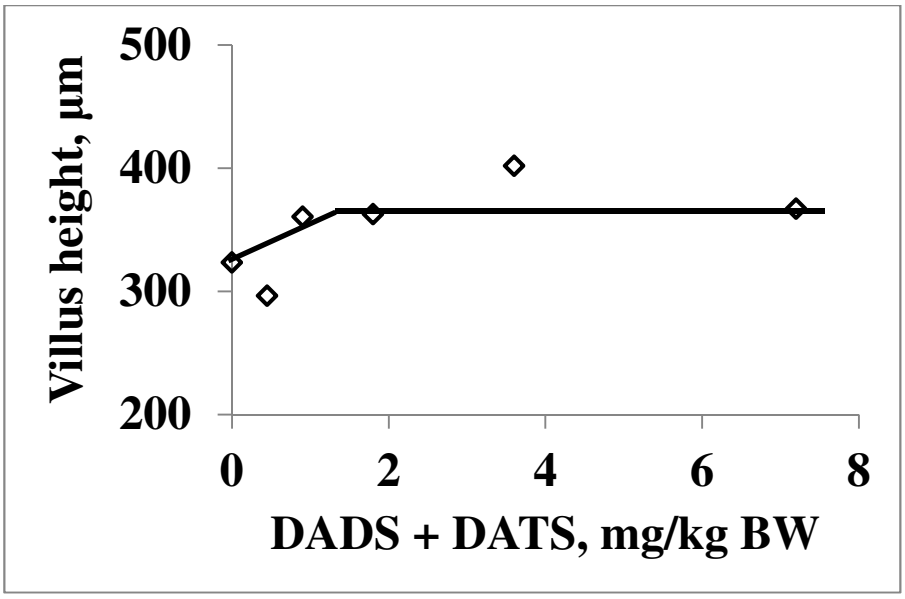

Figure 5-1. Fitted broken-line plot of villus height as a function of DADS + DATS intake in nursery pigs. The adequate DADS + DATS intake determined was 1.71 ± 0.49 mg/kg BW (P = 0.10).

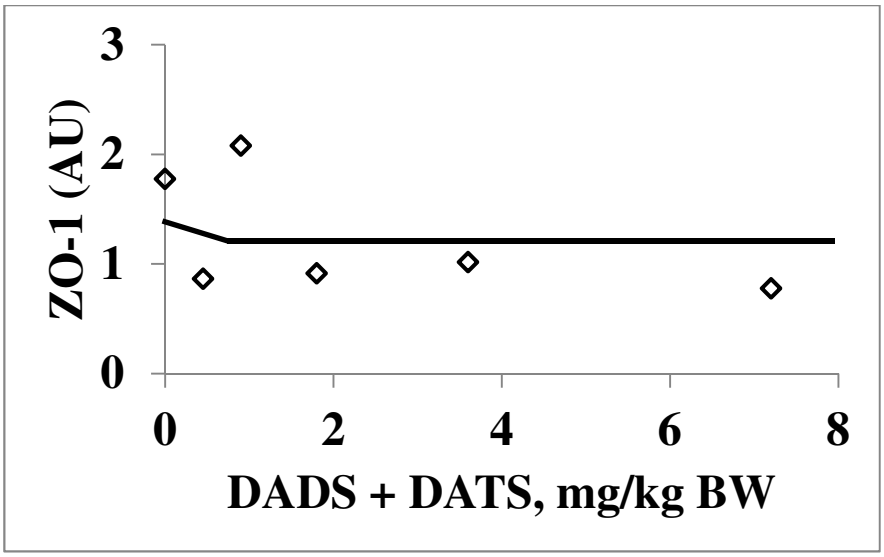

Figure 5-2. Fitted broken-line plot of ZO-1 gene expression as a function of DADS + DATS intake in nursery pigs. The adequate DADS + DATS intake determined was 0.90 ± 0.06 mg/kg BW (P = 0.08).

CHAPTER 6. DETERMINATION OF THE ADEQUATE DOSE OF GARLIC DIALLYL DISULFIDE AND DIALLYL TRISULFIDE FOR EFFECTING CHANGES IN GROWTH PERFORMANCE, NUTRIENT AND E DIGESTIBILITY, ILEAL CHARACTERISTICS, AND SERUM IMMUNE PARAMETERS IN BROILER CHICKENS

6.1. Abstract

The objective of the current experiment was to determine the adequate dose and impact of graded concentrations of garlic diallyl disulfide (DADS) and diallyl trisulfide (DATS) on growth performance, total-tract nutrient and E digestibility, serum immune parameters, and ileal characteristics in broiler chickens. At 28-d post-hatch, male broiler chickens were allotted on the basis of initial bodyweight (BW) (1.34 ± 0.106 kg) in a RCBD to one of six treatments that consisted of an oral gavage of 0, 0.45, 0.90, 1.80, 3.6, or 7.2 mg of DADS + DATS per kg BW with 8 replicate cages per treatment and 4 birds per cage. The DADS + DATS was administered to birds by daily oral gavage for a period of 6 d. Growth performance was recorded and excreta were collected for analysis of DM, N, and E digestibility and on the last day of the experiment, the median bird in each cage was euthanized and the mid ileum was excised for morphological and gene expression measurements and blood was collected for serum natural antibody and complement assays. Body weight gain and villus height were linearly increased ($P <$ 0.01) with oral gavage of DADS + DATS. There was a quadratic effect ($P < 0.01$) of

the oral gavage on digestibility of DM, N, and E that corresponded to an average broken-line regression-derived adequate dose of 1.16 mg DADS + DATS per kg BW. Supplementation of DADS + DATS by oral gavage had no impact on gene expression markers although there was a tendency for an increase ($P = 0.10$) in serum natural antibody activity due to treatment. Results from the current study indicate that supplementation of a gavage containing DADS + DATS improves BW gain, ileal morphology, and digestibility of DM, N, and E and affects serum immune parameters in broiler chickens. The average broken-line regression-derived adequate dose to optimize BW gain and villus height response was 2.51 mg DADS + DATS per kg BW.

6.2. Introduction

Broiler chickens are exposed to various dietary, environmental, and microbial factors that can influence gastrointestinal function during their lifecycle. Gastrointestinal dysfunction can lead to oxidative cellular damage and inappropriate immune activation with long-lasting implications on health, performance, and digestive function (Yegani and Korver, 2008). Bioactive phytochemicals in garlic have been shown to contain antioxidant, immune modulatory, antimicrobial, and anti-parasitic properties (Amagase et al., 2001; Amagase 2006). The intact garlic bulb contains compartmentalization of bioactive AA precursors, such as alliin, and enzymes, such as allinase. Following crushing or grinding of the garlic bulb, alliin comes in contact with allinase and the unstable intermediate allicin is formed, which is rapidly and spontaneously degraded to stable bioactive phytochemicals such as the oil-soluble derivatives diallyl sulfide (DAS), DADS, and DATS and water-soluble derivatives such as s-allyl-cys (SAC). Previous research using in-vitro and rodent models shows bioactive garlic components may be able to improve gastrointestinal function by mitigation of oxidative intermediates, and immune and microbial modulation (Borek, 2001; Kyo et al. 2001; Ramiah et al., 2014).

Variable results on the impact of garlic product supplementation to poultry have been shown. Cross et al. (2011) showed garlic product supplementation improved broiler chick growth performance for the first 7-d post hatch, although Olukosi and Dono (2014) showed no improvement in broiler chicken performance with dietary supplementation of a crude garlic preparation. Garlic-associated improvements in

growth performance may be due to enhanced gastrointestinal morphology (Tatara et al., 2008) and nutrient digestibility (Yan et al., 2012). Additionally, garlic bioactive components have been shown to modulate poultry immune function by influencing complement activity (Jafari et al., 2010), increasing lymphocyte proliferation (Hanieh et al., 2012), and reducing parasitic-associated intestinal inflammation (Kim et al., 2013) which may have implications on broiler pathogen resistance, morbidity, mortality, gastrointestinal function, and growth performance. However, a dearth of information still exists on the impact of these garlic-derived compounds in poultry. Therefore, we hypothesized that an adequate dose of orally administered downstream garlic bioactive components DADS and DATS would enhance growth performance, ileal morphology, and markers of cellular integrity and immune dynamics in broiler chickens. To achieve our objectives, we determined the effective dose as well as the effect of various doses of DADS + DATS on growth performance, total-tract digestibility of DM, N, and E, ileal morphology, ileal mucosa cytokine and tight junction gene expression, and serum natural antibody and complement activity.

6.3. Materials and methods

All procedures used in the current study were approved by the Purdue University Animal Care and Use Committee.

Experimental design

Male broiler chicks (Ross 708) were purchased from a local hatchery on d of hatch, weighed, and tagged for identification. Birds were housed in electrically-heated

battery cages (Alternative Design and Manufacturing and Supply, Inc., Siloam Springs, AR) and allowed ad-libitum access to feed and water throughout the experiment. Brooder temperature was kept at 35.5, 31, and 27, and 25 ˚C from 0 to 7 d, 8 to 14 d, and 15 to 34 d post-hatch, respectively. Birds were allowed ad-libitum access to feed and water throughout the experiment and a fed standard broiler starter and grower diets that met or exceeded NRC requirements (NRC, 1994) from 0 to 21 d and 22 to 34 d post-hatch, respectively. The broiler grower diet formula and specifications are presented in Table 6-1. Chromic oxide was included in the grower diet (5 g/kg) and served as an indigestible marker. At 28-d post-hatch, birds were allotted on the basis of initial BW (1.34 kg ± 0.106) in a RCBD to one of six treatments that consisted of administration of 0, 0.45, 0.90, 1.80, 3.60, or 7.20 mg DADS + DATS product per kg bodyweight with 8 replicates per treatment and 4 birds per cage. Birds were administered the freshly prepared DADS + DATS solution by daily oral gavage at approximately the same time each day for a period of 6 d with soybean oil as the carrier and vehicle control. The garlic-derived DADS + DATS product was obtained from Stanford Chemicals (Irvine, Ca) and consisted of 45% DADS and DATS.

Sample collection

Body weight and feed intake were measured at the start and conclusion of the 6-d experiment. On 32 d post-hatch excreta trays were emptied, cleaned and lined with waxed paper. Excreta was collected on 33 and 34 d post-hatch, pooled by cage, and stored at -20˚C until processed for analysis. At the end of the experiment the median bird in each cage was selected and euthanized by CO_2 asphyxiation. Blood was collected for serum analysis and the distal ileum was excised and flushed with ice-cold

phosphate buffered saline (VWR International, Radnor, PA) for gene expression and histology. The excised ileal segment was cut in half exposing the lumen, scraped with a glass slide, and contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C for real-time quantitative PCR analysis. For histological measurements a 10-cm segment of the excised ileum was stapled to a cardboard background and fixed in 10% buffered formalin (VWR International, Radnor, PA) for approximately 30 d prior to processing.

Serum natural antibody and complement analysis

Serum was separated by centrifuging collected blood samples at 1,400 × g at 8˚C for 15 minutes and preserved at -20˚C until analysis. Extracted serum samples were subsequently analyzed for natural antibody and complement activity by Cotter Laboratories (Arlington, MA) as described by Cotter (2012). Briefly, the sera were tested by microtiter analysis for capacity to agglutinate rabbit erythrocytes by two agglutination types, strong (HA1) and weak (HA2) for natural antibody activity; and 2 degrees of lysis, complete (L_{100}) and half (L_{50}) and visually evaluated using end-point dilutions for complement activity.

Chemical analysis of diet and excreta

Pooled excreta samples were dried in a forced-air oven for 1 wk at 56˚C and the diet and excreta were subsequently ground to a homogenous mixture by passing through a 0.50-mm screen (Retsch ZM 100, GmbH & Co. K.C., Haan, Germany). Dry matter content of the diet and excreta were determined by overnight drying at 105˚C. Diet and excreta gross energy was assessed by adiabatic bomb calorimetry (Model 1261, Parr Instrument Co., Moline, IL, USA) using benzoic acid as the calibration standard and

nitrogen content was determined by the combustion method (Leco Model FP 2000, Leco Corp., St. Joseph, MI, USA) using ethylenediaminetetraacetic acid as the standard. Furthermore, diets and excreta were wet-ashed (nitric/perchloric wet ash, AOAC Method 935.13A) and chromium concentration was determined by using a spectrophotometer (Spectronic 21D, Milton Roy Co., Rochester, NY) according to methods described by Fenton and Fenton (1979). Apparent total-tract digestibility was calculated using the formula:

$$ND, \% = 100 \times [\, 1 - (Cr_i/Cr_o \times N_o/N_i)]$$

where ND represents percent nutrient digestibility; C_i and C_o are concentration of chromium in the diet or excreta, respectively; N_i and N_o are concentration of nutrient or energy in the diet or excreta, respectively.

Histological measurements

Following tissue fixation, samples were dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). Segments were sliced (5 µm) and stained with hematoxylin and eosin by the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth were measured and villus height to crypt depth ratio was calculated. Means from at least 4 villi per segment were analyzed for differences. Villus length is defined as the length from the villus tip to the valley between villi and crypt depth is defined as the length between the crypt opening and base.

Ileal mucosa gene expression by quantitative real-time PCR

Expression of ileal mucosa MUC2, IL-8, TNF-α, IL-6, TLR-4, OC, CL-2, ZO-1, and ZO-2 genes were determined using quantitative real-time PCR. Ribonucleic acid was extracted according to the Trizol protocol (Invitrogen), and reverse transcription was performed using the MMLV reverse transcription system of Promega (Promega, Madison, WI, USA). Quantitative real-time PCR was performed on a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, Ca, USA) using a reaction mix that consisted of 0.50 μg cDNA, 0.075 nmol each of forward and reverse primers, SYBR Green Master (Roche, Basel, Switzerland), and nuclease-free water (Ambion, Austin, TX) to reach a reaction volume of 20 μL per well. Following a 5-min incubation period at 95°C, reactions were cycled 50 times using the following protocol: 10 s at 95°C, 20 s at 55°C, and 30 s at 72°C. The standard curve was determined using pooled samples, and water served as a negative control. All reactions were analyzed in duplicate and formation of a single PCR product was confirmed by melting curves. Gene expression level was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) and normalized using glyceraldehyde phosphate dehydrogenase (GAPDH) expression. Primers used are presented in Table 6-2.

Statistical analysis

Data was analyzed using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC) with the fixed effect of treatment and the random effect of block. The following statistical model was used in the analysis: $Y_{ij} = \mu + W_i + B_j + \varepsilon_{ij}$ where Y is the response criterion; μ is the overall mean; W_i is the effect of i^{th} treatment (i = 1,2,3, …,6); B_j is the effect of j^{th} block (k = 1, 2, 3,… 8); and ε_{ij} is the error term. If there was

an effect of treatment, single-degree-of-freedom linear and quadratic contrasts were conducted with coefficients for unequally spaced levels generated using the IML procedure of SAS. Broken-line regression analysis was conducted for BW gain, villus height, and nutrient digestibility measurements using the NLIN procedure of SAS as described by Robbins et al. (2006). Significant difference was defined as $P \leq 0.05$; and $0.05 \leq P \leq 0.10$ was considered a tendency. Model assumptions for normality and equal variances were validated by the Shapiro-Wilks test (UNIVARIATE procedure of SAS) and Brown and Forsythe test (GLM procedure of SAS), respectively.

6.4. Results

The garlic product was analyzed at the Purdue University Metabolite Profiling Facility (West Lafayette, IN) by HPLC and the two bioactive components were determined to be DADS (22%) and DATS (23%) with the remaining 55% of product consisting of a methanol-based solvent.

There was no mortality or morbidity during the experiment and initial BW of broiler chickens 28-d post-hatch was 1.34 ± 0.106 kg. Overall means for final body weight, BW gain, ADG, ADFI, and G:F were 1.74 ± 0.102 kg, 399 ± 48.6 g, 66.6 ± 8.10 g, 79.4 ± 14.0 g, and 0.85 ± 0.14, respectively. Final body weight, BW gain, and ADG were linearly increased ($P < 0.01$) due to treatment (Table 6-3). Relative to birds receiving the vehicle control gavage, there was a 5.4, 6.9, 7.6, 8.5, and 17.8 % increase in BW gain for broiler chickens receiving the oral gavage containing 0.45, 0.90, 1.80, 3.60, and 7.2 mg DADS + DATS per kg BW, respectively. The breakpoint for BW gain

was a DADS + DATS intake level of 3.48 mg per kg BW (Figure 6-1A). There was no effect of DADS + DATS supplementation on ADFI or G:F. The overall means for villus height, crypt depth, and villus height to crypt depth ratio (VH:CD) were 714 ± 107.7 µm, 290 ± 64.3 µm, and 2.59 ± 0.62. Villus height was increased linearly ($P = 0.01$) with increasing concentrations of DADS + DATS (Table 6-3). Relative to birds receiving the vehicle control gavage, there was a 6.6, 11.0, 20.4, 12.4, and 20.5 % increase in villus height for birds receiving a gavage containing 0.45, 0.90, 1.80, 3.60, and 7.20 mg DADS + DATS per kg BW, respectively. Furthermore, there was a tendency ($P = 0.07$) for an increase in VH:CD due to treatment. The breakpoint for villus height was a DADS + DATS intake level of 1.54 mg DADS + DATS per kg BW (Figure 6-1 B). There was no effect ($P > 0.10$) of treatment on crypt depth.

The effect of oral administration of DADS + DATS on total-tract DM, N, E, and ME digestibility is reported in Table 6-3. Overall means for DM, N, and E digestibility (%) were 74.3 ± 3.9, 66.3 ± 4.4, and 75.0 ± 3.1, respectively. Furthermore, the overall mean for ME was 3.08 ± 0.13 kcal/g. There was a quadratic effect of treatment ($P < 0.01$) on all variables measured. Relative to birds that received a gavage of the vehicle control, there were 3.9, 9.4, 9.7, 9.4, and less than 1 % increases in DM digestibility with an oral gavage of 0.45, 0.90, 1.80, 3.60, and 7.20 mg DADS + DATS per kg BW, respectively. Likewise, there were 3.3, 7.4, 4.9, 7.7, and less than 1% increases in N digestibility and 4.8, 6.0, 3.7, 6.7, and 1.3 % increases in E digestibility, relative to birds that received an oral gavage of the vehicle control. Breakpoint analyses for total-tract digestibility of DM, N, and E revealed adequacy at 1.02, 1.59, and 0.87 mg DADS + DATS per kg BW, respectively (Figure 6-2).

There was no response in the expression of MUC2, IL-8, TNF-α, IL-6, TLR4, OC, CL-1, ZO-1, ZO-2 genes to oral gavage with DADS + DATS (Table 6-4). Expression of the housekeeping gene (GAPDH) was not affected oral gavage with DADS + DATS.

Natural antibody and serum complement activity are reported in Table 6-4. There was a tendency for a treatment effect ($P = 0.10$) on natural rabbit strong agglutination (Rb HA1), which is an indicator of natural antibody production. Relative to birds receiving the vehicle control gavage, there was a -4.7, 0, 8.8, 3.6, and 10% change in natural antibody production with supplementation of an oral gavage containing 0.45, 0.90, 1.80, 3.60, and 7.20 mg DADS + DATS per kg BW. Rabbit complete and partial lysis titers (Rb L_{100} and Rb L_{50}) represent the classical and alternative complement pathways, respectively. There was no effect of treatment ($P > 0.10$) on serum complement markers.

6.5. Discussion

Nutritional strategies exist to optimize the broiler chicken gastrointestinal environment allowing for improved efficiency and health and include dietary supplementation of probiotics, prebiotics, organic acids, enzymes, polyunsaturated fatty acids, and phytochemicals (Sugiharto, 2014). Phytochemicals present in garlic have been shown to be antimicrobial (Ankri and Mirelman, 1999), antioxidant (Borek, 2001), immune modulatory (Kyo et al., 2001), and anti-inflammatory (Lang et al., 2004). In broiler chickens, Ramiah et al. (2014) and Peinado et al. (2012) showed improved

growth and intestinal morphology with supplementation of garlic bioactive components, respectively, whereas Olukosi and Dono (2014) showed garlic product supplementation improves utilization of E and N. Furthermore, several studies show garlic bioactive components have immune modulatory functions in poultry and swine (Kim et al., 2013; Liu et al., 2013 and 2014). Taken together, the aforementioned literature suggests that garlic bioactive components may be a nutritional strategy to optimize broiler chicken gastrointestinal function. The literature shows conflicting information for the effects of garlic products on broiler chicken growth performance. Ramiah et al. (2014) showed a periodical improvement in broiler growth performance when birds were fed diets containing 0.50% garlic product, whereas Cross et al. (2011) showed a 6% improvement in broiler BW gain only during the first 7 d post-hatch, and Olukosi and Dono (2014) reported no change in broiler chicken growth performance with supplementation of 10 g garlic meal/kg diet. For the current experiment there was a linear improvement in BW gain with supplementation of increasing doses of the DADS + DATS-containing gavage. The growth performance values reported from the current experiment are similar to values reported by Cross et al. (2011) and Ramiah et al. (2014). The improvement in growth performance is perhaps related to improvements in ileal morphology, nutrient digestibility, and change in immune status, as discussed below. Generally, fresh garlic bioactive components, such as allicin, are associated with gastrointestinal damage (Amagase et al., 2001), whereas evidence exists that aged garlic bioactive components are associated with improved gastrointestinal function (Horie et al., 1997, Tatara et al., 2008, and Peinado et al., 2012). Conflicting evidence exists on the effects of garlic on gastrointestinal morphology in poultry. Olukosi and

Dono (2014) showed no impact of garlic meal on jejunal morphology, whereas Peinado et al. (2012) showed increased ileal villus height and mucosa thickness in broiler chickens fed a diet supplemented with the garlic bioactive component propane thiosulfinate. Discrepancies in the literature are likely due to differences in product bioactive components, animal health status, and experimental design. For the current experiment there was a linear increase in villus height and a tendency for an increase in villus height to crypt depth ratio with supplementation of an oral gavage containing increasing doses of DADS + DATS. Ileal morphology data reported in the current experiment are within range of values reported in the literature for 35-d-old broiler chickens (Gao et al., 2008; Boroojeni et al., 2011). Improvements in villus height suggest improved absorptive potential and gut secretory function. The mechanism by which garlic bioactive components improve ileal morphology is not fully understood although it could be related to changes in microbial ecology and mucosal immune function. Peinado et al. (2012) showed that garlic-derived bioactive components reduced gastrointestinal enteropathogen load and were associated with improved gastrointestinal morphology and Olukosi and Dono (2014) showed supplementation of garlic meal to broiler chickens altered gastrointestinal pH and fatty acid composition. Furthermore, Tatara et al. (2008) showed that supplementation of a garlic product to neonatal pigs resulted in increased serum antimicrobial factors such as lysozyme that was associated with improved morphology throughout the small intestine. The average broken-line regression-derived adequate dose to optimize BW gain and villus height response was 2.51 mg DADS + DATS per kg BW, which for a 1.75-kg broiler chicken

consuming 80 g feed per day translates to approximately 0.06 g DADS + DATS per kg feed.

Recent literature shows garlic product supplementation improves nutrient retention and digestibility utilization in swine and poultry. Yan et al. (2012) showed an improvement in DM and N in grow-finish pigs supplemented with a fermented garlic product, and Olukosi and Dono (2014) reported garlic meal supplementation increased AME, AME_n, N digestibility, and ileal digestible energy. Conversely, Cross et al. (2011) showed no change in DM, OM, and N digestibility when broiler chickens were fed a garlic product. In the current experiment, there was a quadratic effect of treatment on digestibility of DM, N, and E. More specifically, nutrient digestibility increased with supplementation of an oral gavage containing 0.45 to 3.6 mg DADS + DATS per kg BW and then decreased with an oral gavage containing 7.20 mg DADS + DATS per kg BW. The average broken-line regression-derived adequate dose to optimize total-tract nutrient and E digestibility was 1.16 mg DADS + DATS per kg BW, which for a 1.75-kg broiler chicken consuming 80 g feed per day translates to approximately 0.03 g DADS + DATS per kg feed. Olukosi and Dono (2014) reported a probiotic-like effect of garlic product supplementation that was associated with an increase in VFA production and a decrease in cecal pH, and although Cross et al. (2011) did not report any changes in nutrient digestibility with garlic product supplementation to broiler chickens, they did report changes in cecal isovaleric and isobutyric acid concentrations that are indicative of microbial modulation. The discrepancy between a quadratic effect of treatment on nutrient digestibility and a linear effect of treatment on growth and ileal morphology is not clear, although it suggests that DADS + DATS has a complex and

multi-faceted mode of action. Previous research shows that garlic product supplementation can impact animal lumen microbial by-products, immune function, and ileal morphology (Tatara et al., 2008; Cross et al., 2011; Kim et al., 2013). We hypothesize that the bioactive garlic component-induced microbial and immune changes are concentration specific; hence nutrient utilization and energy partitioning are dependent on the concentration of the bioactive component fed and subsequent physiological response. Further research is needed to investigate the aforementioned hypothesis.

Garlic bioactive components have been demonstrated to be immune modulatory using in-vivo and in-vitro research models (Kyo et al., 2001; Lang et al., 2004; Hanieh et al., 2012; Kim et al., 2013; Liu et al., 2014). Kyo et al. (2001) showed aged garlic was mitogenic and augmented concavalin A using a murine-cell model and Clement et al. (2010) identified two distinct immune stimulatory proteins from garlic using an ex-vivo human cell culture model. Similarly, using white leghorn lymphocytes in-vitro, Hanieh et al. (2012) showed garlic bioactive components stimulate proliferation of splenocytes and lymphocytes, increased gene expression of mitogenic cytokines IL-2 and INF-γ, and increased killing activity of macrophages. Kim et al. (2013) showed garlic-derivatives, propyl thiosulphinate and propyl thiosulphinate oxide enhanced the immune response to an Eimeria infection in broiler chickens. For the current experiment there was a tendency for an increase in Rb HA1 with increasing supplementation of the DADS + DATS-containing gavage, which is indicative of an increase in pentameric IgM concentrations (Cotter, 2012). A dearth of information exists on the impact of garlic bioactive products on serum antibody dynamics, although

Kim et al. (2013) reported enhanced serum antibody levels in Eimeria-infected broiler chickens. In broiler chickens Jafari et al. (2010) showed fresh-garlic components influence serum complement activity and Liu et al. (2014) showed garlic product supplementation increases genes related to complement function in the mucosa of nursery pigs. In the current experiment there was no impact of treatment on serum complement activity. The discrepancy between the current results and the literature could be due to broiler health status, age, or product bioactivity. Conflicting evidence exists on the impact of garlic bioactive components on cytokine gene and protein expression. Lang et al. (2004) showed reduced expression and secretion of inflammatory cytokines and chemokines in TNF-α stimulated and unstimulated Caco-2 cells and Liu et al. (2014) showed no impact of a garlic product on ileal mucosa cytokine gene expression in nursery pigs. In the current study there was no impact of treatment on mucosal gene expression of MUC2, IL-8, TNF-α, IL-6, and TLR4. The tight junction protein complex comprises of intra- and extra-cellular proteins that form a selective paracellular pore and serve to control epithelial cell integrity and permeability (Gonzalez-Mariscal et al., 2003). Previous research by Park et al. (2011) shows DADS increases trans-epithelial electrical resistance, decreases claudin gene expression, and increases metalloprotease inhibitor gene expression. Furthermore, Roselli et al. (2007) reported improved cellular integrity in *E.coli*-infected epithelial cells treatment with a garlic product that was not related to an antimicrobial effect. In this study there was no impact of treatment on tight junction gene expression which is consistent with recently-published results investigating garlic product supplementation to nursery pigs (Liu et al., 2014).

Results from the current study show that DADS + DATS supplementation by daily oral gavage for a period of 6 d improves broiler chicken growth performance, ileal morphology, and total-tract nutrient and E digestibility. There tends to be an increase in serum pentameric IgM due to oral supplementation of a DADS + DATS-containing gavage, which is indicative of improved humoral immune function. The improvement in broiler performance, ileal morphology, and nutrient and E digestibility, is not linked to changes in mucosal gene expression markers measured for the current study. Additional studies are needed to understand the relationships among garlic bioactive components, microbial ecology, and immune modulation. Overall, results from the current study show that garlic-derived DADS + DATS influences broiler performance and gastrointestinal function and that 0.06 g DADS + DATS per kg feed is adequate to improve BW gain and ileal morphology, whereas 0.03 g DADS + DATS per kg feed is adequate to improve apparent total-tract nutrient and E digestibility.

6.6. Acknowledgements

The authors would like to express their appreciation to Biomatrix and JBS United for their financial and technical support of this trial. Further appreciation is given to Jason Fields, Purdue Animal House, for support with chicken husbandry. The authors would also like to express appreciation to Jamie Purrington and Catalina Stefanello, Purdue University Department of Animal Sciences, for their assistance with treatment administration, Donna Brooks, Purdue University Veterinary School, for her

assistance with histological measurements, Dr. Paul Cotter for his assistance with serum analysis.

6.7. References

Amagase, H., B.L. Petesch, H. Matsuura, S. Kasuga, and Y. Itakura. 2001. Intake of garlic and its bioactive components. J. Nutr. 131: 955S–962S.

Amagase, H. 2006. Clarifying the real bioactive constituents of garlic. J. Nutr. 136: 716S–725S.

Ankri, S., and D. Mirelman. 1999. Antimicrobial properties of allicin from garlic. Microb. Infect. 2: 125−129.

Borek, C. 2001. Antioxidant health effects of aged garlic extract. J. Nutr. 131: 1010S–1015S.

Boroojeni, F.G., A. H. Samie, M. A. Edriss, M. Khorvash, G. Sadeghi, A. Van Kessel, and J. Zentek. 2011. Replacement of corn in the diet of broiler chickens using foxtail millet produced by 2 different cultivation strategies Poult. Sci. 90: 2817-2827.

Cross, D.E., R.M. Mcdevitt, and T. Acamovic. 2011. Herbs, thyme essential oil and condensed tannin extracts as dietary supplements for broilers, and their effects on performance, digestibility, volatile fatty acids and organoleptic properties. Br. Poult. Sci. 52: 227-337.

Clement, F., S.N. Pramod, and Y.P. Venkatesh. 2010. Identity of the immunomodulatory proteins from garlic (*Allium sativum*) with the major garlic lectins or agglutinins. Int. Immunopharmacol.10: 316–324.

Cotter, P.F. 2012. The contrasting properties of 2 xenogenic erythrocyte-reactive natural antibodies in commercial ducks. Poult. Sci. 91: 653-659.

Fenton, T. W., and M. Fenton. 1979. An improved procedure for the determination of dietary chromic oxide in feed and feces. Can. J. Anim. Sci. 59:631–634.

Gao J., H. J. Zhang, S. H. Yu, S. G. Wu, I. Yoon, J. Quigley, Y. P. Gao, and G. H. Qi. 2008. Effects of yeast culture in broiler diets on performance and immunomodulatory functions. Poult. Sci. 87: 1377-1384.

Gonzalez-Mariscal, L., A. Betanzos, P. Nava, and B.E. Jaramillo. 2003. Tight junction proteins. Progr. Biophys. Molec. Biol. 81: 1-44.

Hanieh, H., K. Narabara, Y. Tanaka, Z. Gu, A. Abe, and Y. Kondo. 2012. Immunomodulatory effects of *Allium*s and *Ipomoea batata* extracts on lymphocytes and macrophages functions in White Leghorn chickens. Anim. Sci. J. 83: 68-76.

Horie, T., S. Awazu, Y. Itakura, and T. Fuwa. 1997. Alleviation by garlic of antitumor drug-induced damage to the intestine. J. Nutr. 131(3s): 1071S-1074S.

Jafari, R. A., M. Ghorbanpoor, and M. Makkei. 2012. Effect of garlic supplementation on complement activity in broiler chickens. Comp. Clin. Pathol. 21: 183-185.

Kim, D., H.S. Lillehoj, S.H. Lee, E. P. Lillehoj, and D. Bravo. 2013. Improved resistance to Eimeria acervulina infection in chickens due to dietary supplementation with garlic metabolites. Br. J. Nutr. 109: 76-88.

Kyo, E., N. Uda, S. Kasuga, and Y. Itakura. 2001. Immunomodulatory effects of aged garlic extract. J. Nutr. 131:1075S–1079S.

Lang, A., M. Lahava, E. Sakhninia, I. Barshack, H.H. Fiddera, B. Avidana, E. Bardana, R. Hershkovizc, S. Bar-Meira, and Y. Chowersa. 2004. Allicin inhibits spontaneous and TNF-α induced secretion of proinflammatory cytokines and chemokines from intestinal epithelial cells. Clin. Nutr. 23: 1199–1208.

Liu, Y., M. Song, T.M. Che, A.S. Almeida, J.J. Lee, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2013. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs. J. Anim. Sci. 91:5294-5306.

Liu, Y., M. Song, D. Che, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2014. Effects of capsicum oleoresin, garlic botanical, and turmeric oleoresin on gene expression profile of ileal mucosa in weaned pigs. J. Anim. Sci. 92:3426-3440.

Livak, K. J., and D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCt method. Methods. 25:402–408.

NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC.

Olukosi, O.A., and N. D. Dono. 2014. Modification of digesta pH and intestinal morphology with the use of benzoic acid or phytobiotics and the effects on broiler chicken growth performance and energy and nutrient utilization. J. Anim. Sci. 92:3945–3953.

Park, H.S., G.Y. Kim, I.W.Choi, N.D. Kim, H.J. Hwang, Y.W. Choi, and Y.H. Choi. 2011. Inhibition of matrix metalloproteinase activities and tightening of tight junctions by diallyl disulfide in AGS human gastric carcinoma cells. J. Food Sci.76: T105-T111.

Peinado, M.J., R. Ruiz, A. Echavarri, and L. A. Rubio. 2012. Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. Poult. Sci. 91:2148–2157.

Ramiah, S. K., I. Zulkifli, N. Asyikin, A. Rahim, M. Ebrahimi, and G. Y. Meng. 2014. Effects of two herbal extracts an virginiamycin supplementation on growth performance, intestinal microflora population and fatty acid composition in broiler chickens. Austr. Asian J. Anim. Sci. 27: 375-382.

Robbins, K. R., A. M. Saxton, and L. L. Southern. 2006. Estimation of nutrient requirements using broken-line regression analysis. J. Anim. Sci. 84:E155–E165.

Roselli, M., M.S. Britti, I. Lerou-Luron, H. Marfaing, W.Y. Zhu, and E. Mengheri. 2007. Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic Escherichia coli K88 in pig intestinal cells. Toxicol. in Vitro. 21: 224–229.

Sugiharto, S. 2014. Role of nutraceuticals in gut health and growth performance of poultry. J. Saudi Soc. Agric. Sci. 2014.

SAS Institute. 2006. SAS/STAT User's Guide. Release 9.1. SAS Institute Inc., Cary, NC.

Tatara, M.R., E. Śliwa, K. Dudek, A. Gawron, T. Piersiak, P. Dobrowolski , J. Mosiewicz, , A. K. Siwicki, and T. Studziński T. 2008. Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared on artificial sow. Ann. Agric. Environ. Med. 15: 63–69.

Yan, L., Q.W. Meng, and I.H. Kim. 2012. Effects of fermented garlic powder supplementation on growth performance, nutrient digestibility, blood characteristics, and meat quality in grow-finish pigs. Anim. Sci. J. 83: 411-417.

Yegani, M. and D. R. Korver. 2008. Factors affecting intestinal health in poultry. Poult. Sci. 87: 2052-2063.

Table 6-1. Composition of common grower diet

Ingredient	g/kg
Corn	572.0
Soybean meal (47% CP)	310.0
Soybean oil	50.0
Monocalcium phosphate	15.0
Limestone (38% Ca)	15.0
Salt	4.0
Vitamin-mineral premix[1]	3.0
DL-Methionine	3.0
Chromic oxide premix[2]	25.0
Threonine	1.0
Lysine	2.0
Total	1000.0

Nutrient content, formulated	
Protein, g/kg	200
ME, kcal/kg	3252
Ca, g/kg	9.1
P, g/kg	6.7
Ca:P	1.3
Non-phytate P, g/kg	4.3

[1]Supplied the following per kg diet:Vitamin A, 5484 IU; vitamin D3, 2643 ICU; vitaminE,11 IU; menadione sodium bisulfite,4.38 mg; riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitaminB12, 13.2 ug; biotin, 55.2 ug; thiamine mononitrate, 2.2 mg; folic acid, 990 ug; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug.

[2]20% Chromic oxide, 80% fine ground corn.

Table 6-2. Primer sequences (5' to 3') used in real-time PCR

Gene[1]	Forward primer	Reverse primer
GAPDH	TCCTAGGATACACAGAGGACCA	CGGTTGCTATATCCAAACTCA
MUC2	GCTACAGGATCTGCCTTTGC	AATGGGCCCTCTGAGTTTTT
IL-8	GCGGCCCCCACTGCAAGAAT	TCACAGTGGTGCATCAGAATTGAGC
TNF-α	AGATGGGAAGGGAATGAACC	CTGGTTACAGGAAGGGCAAC
IL-6	GAATGTTTTAGTTCGGGCACA	TTCCTAGAAGGAAATGAGAATGC
TLR-4	GTTCCTGCTGAAATCCCAAA	TATGGATGTGGCACCTTGAA
OC	TCATCGCCTCCATCGTCTAC	TCTTACTGCGCCTCTTCTGG
CL-1	CTGATTGCTTCCAACCAG	CAGGTCAAACAGAGGTACAAG
ZO-1	CTTCAGGTGTTTCTCTTCCTCCTC	CTGTGGTTTCATGGCTGGATC
ZO-2	CGGCAGCTATCAGACCACTC	CACAGACCAGCAAGCCTACAG

[1] GAPDH = glyceraldehyde 3-phosphate dehydrogenase; MUC2 = mucin 2; IL-8 = interleukin 8; TNF-α = tumor necrosis factor alpha; IL-6 = interleukin 6; TLR4 = toll-like receptor 4; OC = occludin; CL-1 = claudin 1; ZO-1 = zonula occludens 1; and ZO-2 = zonula occludens 2.

Table 6-3. Growth performance, ileal morphology, and total-tract nutrient and energy digestibility of broiler chickens that received an oral gavage of graded levels of garlic diallyl disulfide (DADS) and diallyl trisulfide (DATS)

DADS + DATS orally administered, mg /kg BW[1]	0	0.45	0.90	1.80	3.60	7.20	SEM[2]	*P*-value		
								Treatment	Linear	Quadratic
Growth performance										
Initial weight, kg	1.34	1.35	1.33	1.36	1.34	1.33	0.039	0.67	-	-
Final weight, kg	1.71	1.73	1.74	1.74	1.74	1.79	0.161	0.03	< 0.01	0.87
Weight gain[3], g	367	388	394	397	401	446	6.05	0.03	< 0.01	0.87
ADG[3], g	61	65	66	66	67	74	2.67	0.03	< 0.01	0.86
ADFI[3], g	72	79	81	76	83	85	5.11	0.40	-	-
G:F[3]	0.88	0.83	0.82	0.90	0.83	0.88	0.06	0.85	-	-
Ileal morphology										
Villus height (VH), µm	632	677	710	794	722	795	39.1	0.03	0.01	0.22
Crypt depth (CD), µm	283	325	263	324	245	297	24.8	0.11	-	-
VH:CD[4]	2.40	2.12	2.61	2.56	3.02	2.71	0.26	0.07	-	-
Total-tract nutrient and energy digestibility										
DM, %	71.8	74.7	76.0	74.2	76.4	72.2	0.97	0.04	0.42	< 0.01
N, %	63.6	65.8	68.7	66.9	68.9	63.6	1.40	0.04	0.49	< 0.01
E, %	72.0	75.6	76.6	74.8	77.2	73.0	0.92	<0.01	0.63	< 0.01
ME, kcal/g	2.96	3.10	3.14	3.07	3.17	3.00	0.04	<0.01	0.63	< 0.01

[1] Birds were fed a common grower diet and received the garlic DADS + DATS by daily oral gavage based on body weight (BW) for 6 d starting at 28 d post-hatch.

[2] Standard error of the mean.

[3] Data analyzed using initial body weight as a covariate.

[4] Data did not meet model assumptions therefore the *P*-value is from log-transformed data.

Table 6-4. Ileal mucosal gene expression and serum natural antibody and complement profile of broiler chickens that received an oral gavage of graded levels of garlic diallyl disulfide (DADS) and diallyl trisulfide (DATS)

DADS + DATS product orally administered, mg /kg BW[1]	0	0.45	0.90	1.80	3.60	7.20	SEM[2]	*P*-value
Ileal gene expresion[3]								
MUC2[4]	1.22	1.03	0.92	1.64	1.43	1.21	0.31	0.16
IL-8[4]	1.34	1.37	1.95	1.40	1.02	1.11	0.52	0.82
TNF-α[4]	1.04	1.12	0.96	0.94	1.04	1.04	0.12	0.90
IL-6	1.32	1.29	2.62	1.06	1.39	3.19	1.12	0.32
TLR4	1.00	1.17	0.98	0.93	1.08	0.99	0.15	0.89
OC	1.15	1.32	1.01	1.08	1.24	0.79	0.21	0.45
CL-1[4]	1.22	1.19	1.43	1.06	1.66	0.77	0.45	0.87
ZO-1	1.00	1.32	1.01	0.90	1.38	1.19	0.19	0.27
ZO-2[4]	0.91	0.98	0.87	0.90	1.16	1.09	0.21	0.64
Natural antibody and serum complement profile[5]								
Rb HA1	5.63	5.37	5.63	6.17	5.84	6.25	0.28	0.10
Rb HA2	6.88	8.00	7.38	7.12	7.45	7.13	0.74	0.85
Rb L_{100}	1.25	2.13	2.13	1.33	1.86	1.85	0.36	0.25
Rb L_{50}	3.50	4.00	4.25	4.17	3.86	4.50	0.38	0.37

[1] Birds were fed a common grower diet and received the garlic DADS + DATS by daily oral gavage based on body weight (BW) for 6 d starting at 28 d post-hatch.

[2] Standard error of the mean.

[3] Expression of the following genes relative to housekeeping gene GAPDH: MUC2= mucin 2; IL-8= interleukin 8; TNF-α= tumor necrosis factor alpha; IL-6= interleukin 6; TLR4= toll-like receptor 4; OC= occluden; CL-1= claudin 1; ZO-1= zonula occludens 1; ZO-2= zonula occludens 2.

[4] Data did not meet model assumptions therefore *P*-values are from log-transformed data.

[5] Analyzed as non-parametric data: HA1 = strong hemagglutination, HA2 = weak hemagglutination, L_{100} = complete lysis and L_{50} = incomplete lysis titers, representing the classical and alternative lytic pathways, respectively.

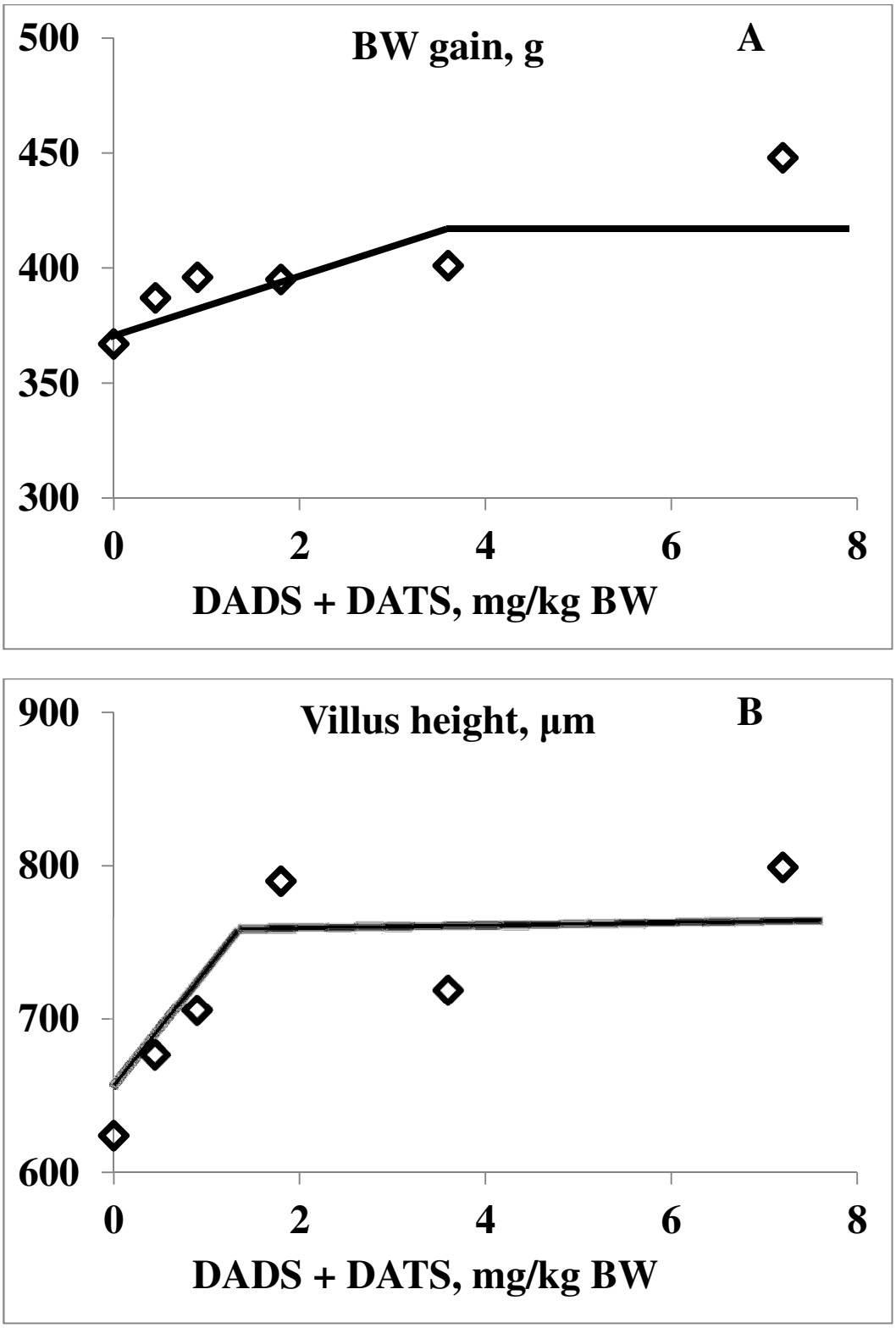

Figure 6-1. Fitted broken-line plot of BW gain (A) and villus height (B) as a function of DADS + DATS intake in broiler chickens. The adequate DADS + DATS intake determined was 3.48 ± 2.67 ($P = 0.02$) and 1.54 ± 0.79 ($P < 0.01$) mg/kg BW for gain and villus height, respectively.

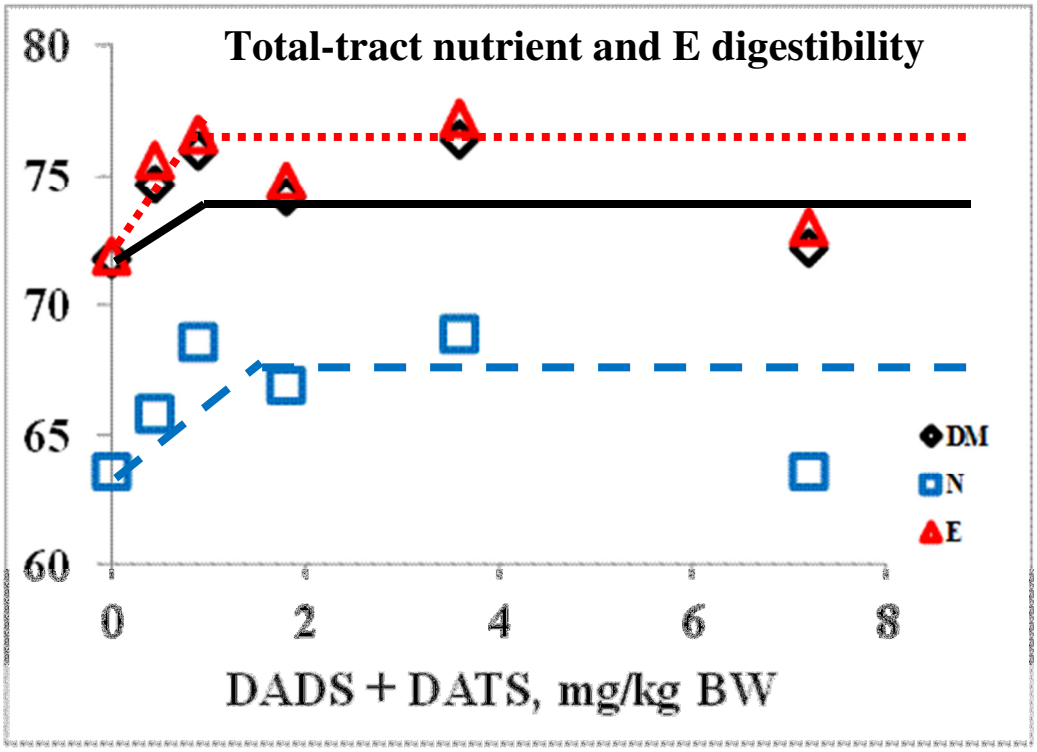

Figure 6-2. Fitted broken-line plot of apparent total-tract digestibility of DM, N, and E as a function of DADS + DATS intake in broiler chickens. The adequate DADS + DATS intake determined was 1.02 ± 0.18(P = 0.05), 1.59 ± 0.57(P = 0.12), and 0.87± 0.04 (P < 0.01) mg/kg BW for DM, N, and E digestibility, respectively.

CHAPTER 7. ABILITY OF GARLIC-DERIVED DIALLY DISULFIDE AND DIALLYL TRISULFIDE SUPPLEMENTED BY ORAL GAVAGE TO MITIGATE EFFECTS OF AN ACUTE POST-WEANING FEED AND WATER DEPRIVATION EVENT IN NURSERY PIGS

7.1. Abstract

During the post-weaning phase, nursery pigs encounter environmental and dietary stressors that impact growth performance and intestinal function. Compounds in garlic have been shown to contain anti-inflammatory, antioxidant, and immune modulatory properties that may be able to mitigate the effects of nursery pig stressors. The objective of the current experiment was to determine if oral gavage of garlic-derived diallyl disulfide (DADS) and diallyl trisulfide (DATS) could mitigate the effects of a 24-h post-weaning feed + water deprivation event in nursery pigs. Pigs (6.0 ± 0.05 kg, 21 d old) were allotted to 4 treatments in a RCBD at weaning. There were 8 replicate pens per treatment with 6 mixed-sex pigs per pen. Treatments were arranged as a 2 × 2 factorial and consisted of with or without a 24-h post-weaning feed + water deprivation event and with or without an oral gavage containing 3.6 mg DADS + DATS per kg BW. The DADS + DATS gavage was administered daily for a period of 6 d with soybean oil as the carrier and vehicle control. Growth performance and morbidity were recorded throughout the experiment and on 1, 6, and 21 d post-weaning 1 pig per pen was selected, blood was collected for measurement of serum stress markers, and the

pig was euthanized. A segment of the mid ileum was subsequently excised for morphological, and gene and protein expression measurements. Mucosal gene expression was conducted by RT PCR for interleukin 8 (IL-8), tumor necrosis factor (TNF-α), superoxide dismutase (SOD), glutathione peroxidase (GPX), claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1). Furthermore, activity of mucosal SOD was measured by colorimetric assay and ZO-1 protein expression was measured by western blot analysis. Immediately following the feed + water deprivation event there was a decrease ($P < 0.01$) in growth performance and an increase ($P = 0.01$) in serum cortisol. There was no effect of the DADS + DATS-containing gavage on growth performance or serum stress markers throughout the experiment. The feed + water deprivation event tended ($P = 0.10$) to decrease ileal villus height and supplementation of DADS + DATS by oral gavage increased ($P = 0.03$) villus height 1 d post-weaning. Supplementation of DADS + DATS by oral gavage decreased ($P = 0.03$) and tended to decrease ($P = 0.08$) gene expression of SOD on 6 and 21 d post-weaning, respectively. Furthermore, at 1 d post-weaning, ileal mucosa SOD activity was decreased ($P = 0.01$) by the feed + water deprivation and increased ($P = 0.04$) by oral supplementation of DADS + DATS although there was no effect of treatment on SOD activity on 6 or 21 d post-weaning. Expression of the tight junction genes ZO-1 and OC were reduced ($P \leq 0.05$) due to the feed + water deprivation event 1 d post-weaning. There was an effect ($P = 0.05$) of feed + water deprivation and a tendency of an effect ($P = 0.08$) for an interaction between the feed + water deprivation event and oral gavage of DADS + DATS on 6 and 21 d post-weaning, respectively, on the expression of the tight junction genes ZO-1 and OC. Results from the current study show that an acute feed + water

deprivation event can impact growth performance, intestinal characteristics, and antioxidant status in nursery pigs, which can be partially mitigated by oral supplementation of garlic compounds DADS + DATS.

7.2. Introduction

Following weaning, pigs are exposed to environmental and dietary stressors that can limit digestive potential and ultimately growth performance. Post-weaning stress-induced lethargy has been linked to poor feed and water consumption which subsequently leads to atrophy of intestinal morphology and increased incidence of mortality and morbidity (Pluske et al., 1997; Lambert 2009; Smith et al., 2010; Horn et al., 2014). Furthermore, recent literature in pigs shows weaning leads to increased oxidative stress which subsequently causes intestinal dysfunction (Zhu et al., 2012). Nutritional strategies including dietary supplementation of plasma proteins, lactose, probiotics, prebiotics, and plant-derived compounds exist to mitigate weaning-associated gastrointestinal dysfunction (Lalles et al., 2007). Bioactive compounds in garlic have been shown to contain antioxidant, antimicrobial, and immunomodulatory properties (Amagase et al., 2001; Amagase, 2006). Furthermore, in-vitro studies show garlic compounds mitigate oxidant-induced cellular dysfunction, modulate innate immune function, and stimulate proliferation of lymphocytes. Recent studies show improved growth performance and intestinal function with supplementation of crude garlic preparations to young pigs although the mode of action is poorly understood (Tatara et al., 2008; Huang et al., 2011). The mechanism of action for the ability of

garlic compounds to improve cellular oxidative status and improve animal gastrointestinal characteristics has been partially attributed to the ability of some garlic compounds, such as DADS and DATS, to have radical scavenging properties (Borek, 2001; Kyo et al., 2001). Limited information exists on the ability of garlic compounds to reduce stress effects in monogastric species, although Prieto and Campo (2010) showed that dietary supplementation of a garlic extract alleviated heat stress in white leghorn chickens. For the current experiment, we hypothesized that garlic-derived DADS + DATS would mitigate the effects of a post-weaning feed + water deprivation event. To achieve our objectives we administered DADS + DATS by daily oral gavage to pigs exposed to a 24-h post-weaning feed + water deprivation event and measured growth performance, serum stress markers, ileal morphology, and gene and protein expression markers of immune and antioxidant status and cellular integrity.

7.3. Materials and methods

All animal procedures were approved by the Purdue University Animal Care and Use Committee.

Animals and treatments

A total of 192 crossbred (Hampshire × Duroc × Yorkshire × Landrace) mixed-sex pigs (initial BW = 6.0 ± 0.05 kg, 21 d old) were allotted to treatment on the basis of initial BW in a randomized complete block design. The pigs were housed in pens (1.83 × 2.44 m) with 1 cup nipple drinker and 1 feed trough. Room temperature was 34.4˚C at weaning and reduced by 0.50˚C each subsequent day and there was 14 h of artificial

light per day. There were 4 treatments arranged as a 2 × 2 factorial that consisted of: 1) no feed or water deprivation event and oral gavage of vehicle control, 2) feed and water deprivation event and oral gavage of vehicle control, 3) no feed or water deprivation event and oral gavage of DADS + DATS, and 4) feed and water deprivation event and oral gavage of DADS + DATS. There were 8 replicates per treatment with 6 mixed-sex pigs per pen and gender was balanced within replicate. The feed + water deprivation event was administered at weaning for a period of 24 h. Following the deprivation event pigs were allowed feed and water ad libitum and fed a 3-phase mash-based feeding program that met or exceed NRC requirements (NRC, 2012; Table 7-1). The oral gavage contained 3.6 mg DADS + DATS per kg BW, was prepared fresh daily with soybean oil as the carrier and vehicle control, and administered daily to each pig at 0800 h for a period of 6 d post-weaning. The garlic product was obtained from Stanford Chemicals (Irvine, Ca) and consisted of 22% DADS and 23% DATS with the remaining 55% consisting of a methanol-based carrier. Garlic compound activity was validated by the Purdue University Metabolite Profiling Laboratory (Purdue University, West Lafayette, IN). Pigs and feeders were weighed on 1, 6, and 21 d post-weaning to calculate ADG, ADFI, and G:F. Scours were subjectively monitored by the same person twice daily (0800 and 1600 h) and recorded corresponding to a scale of 1 = no scour, 2 = moderate fecal looseness, or 3 = severe diarrhea. Incidence of injectable medications and visual morbidity (fall-off pigs) were also monitored and recorded. On 1, 6, and 21 d post-weaning the median pig in each pen was selected, blood was collected into serum collection tubes (CORVAC, serum separator tube, Tyco Healthcare Group LP, Mansfield, MA) and euthanized by intramuscular injection with ketamine (Fort Dodge

Laboratories Inc., Fort Dodge, IA), Telazol (Fort Dodge Laboratories), and xylazine (Bayer Corp., Shawnee Mission, KS) at doses of 4, 2.2, and 2.2 mg/kg BW, respectively, followed by asphyxiation with CO_2. Immediately following euthanasia a 10-cm segment of the distal ileum was excised for histological and gene and protein expression measurements.

Histological measurements

Excised intestinal segments were flushed with ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA), stapled to a cardboard background, and fixed in 10% buffered formalin (VWR International, Radnor, PA) for approximately 30 d. Samples were subsequently dehydrated with ethanol (VWR International, Radnor, PA), cleared with Sub-X® (Polysciences, Inc., Warrington, PA) and placed in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO). Segments were sliced (5 µm) and stained with hematoxylin and eosin by the Purdue Histology and Phenotyping Laboratory (Purdue University, West Lafayette, IN USA). Villus height and crypt depth were measured and villus height to crypt depth ratio was calculated. Means from at least 4 villi per segment were analyzed for differences. Villus length is defined as the length from the villus tip to the valley between villi and crypt depth is defined as the length between the crypt opening and base.

RNA isolation, cDNA Synthesis, and real-time PCR

Immediately after removal, intestinal segments were flushed with ice-cold PBS (VWR International, Radnor, PA), cut in half exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at -80°C until RNA

isolation. Extraction of RNA was according to the Trizol protocol (Invitrogen, Grand Island, NY). Reverse transcription was carried out using the MMLV reverse transcription system of Promega (Promega, Madison, WI) and real-time PCR was performed on a Bio-Rad iCyler (Bio-Rad Laboratories, Hercules, CA) using a reaction mix that consisted of 0.50 µg cDNA, 0.075 nmol of each forward and reverse primers, SYBR Green Master (Roche, Basel, Switzerland), and nuclease-free water (Ambion, Austin, TX) to reach a reaction volume of 20 µl per well. Following a 5-min incubation period at 95ºC, reactions were cycled 50 times using the following protocol: 10 S at 95ºC, 20 s at 55ºC, and 72ºC for 30 s. Expression of interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), superoxide disumutase (SOD), glutathione peroxidase (GPX), claudin 1 (CL-1), occludin (OC), and zonula occludens 1 (ZO-1) genes were measured using GAPDH as a housekeeping control.

Protein analysis

An additional mucosal scraping was taken from the ileum, immediately frozen in liquid nitrogen, and stored at - 80 ˚C. Samples were thawed and protein was extracted in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO), centrifuged at 3,300 × g for 15 min, and the supernatant was recovered for protein analysis. Protein concentration was then determined by the bicinchonininc acid assay method (Pierce Endogen, Rockford, IL). Superoxide dismutase activity was determined from the protein lysate by the Dojindo's highly water-soluble tetrazolium salt assay kit (Sigma-Aldrich, St. Louis, MO) and expressed as SOD units per mg protein. For western blot analysis, proteins were denatured at 95°C in loading buffer and separated on a 12.5% SDS-polyacrylamide gel. Membranes were blotted with anti-ZO-1 (Cell Signaling,

Beverly, MA) overnight at 4˚C, and specific bands were detected with an alkaline phosphatase-conjugated secondary antibody (Upstate, Lake Placid, NY). Visualized bands were determined by autoradiographs prepared from gels, and signal intensity was quantified using a Digital Science Imaging System (V. 2.0.1, Kodak, New Haven, CT). Following blotting and visualization with the target antibody, membranes were stripped using stripping buffer (Sigma-Aldrich, St. Louis, MO) and β-actin was visualized and quantified using aforementioned procedures and used as a housekeeping protein.

Statistical analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with the fixed effects of feed + water deprivation event and DADS + DATS oral gavage and the random effect of block. The following statistical model was used in the analysis: $Y_{ijk} = \mu + W_i + F_j + (WF)_{ij} + B_k + \varepsilon_{ijk}$, where Y is the response criterion, μ is the overall mean, W_i is the effect of the i_{th} feed + water deprivation event (i = 1, 2), F_j is the effect of the j_{th} effect of DADS + DATS gavage (j = 1, 2), B_k is the effect of k_{th} block (k = 1, 2, 3,…, 8), $(WF)_{ij}$ is the interaction effect between the i_{th} feed + water deprivation event and the j_{th} DADS + DATS oral gavage, and ε_{ijk} is the error term. When an interaction effect existed, simple effect means were separated using the PDIFF option in SAS. When there was no interaction effect the interaction term was combined with the error term for statistical analysis. Significant difference was defined as $P < 0.05$, and $0.05 \leq P \leq 0.10$ was considered a tendency. Model assumptions for normality and equal variances were validated by the Shapiro-Wilks test (Proc Univariate) and Brown-Forsythe test (Proc GLM), respectively.

7.4. Results

There were 4 instances of mortality during the trial that were not treatment specific. Additionally, a moderate scour was evident for most pigs during the first week post-weaning although there was no effect of treatment (data not shown). Subsequent to the scour, there were a total of 19 and 45 instances of injectable medications and fall-off pigs, respectively (Fig. 7-2). There was a numerical, but not statistical, increase in medication injections and fall-off observations in pigs exposed to the deprivation event which was mitigated by oral gavage of DADS + DATS. Growth performance is presented in Table 7-1. There was a decrease ($P < 0.01$) in ADG, ADFI, and G:F during the first phase post-weaning that resulted in a 0.23-kg lighter pig due to the deprivation event. During the second phase (1 to 6 d post-weaning) there was an increase ($P < 0.05$) in growth performance due to the previous deprivation event that showed a 55, 30, and 48% increase in ADG, ADFI, and G:F, respectively relative to pigs not exposed to the deprivation event. There was no effect of the oral DADS + DATS-containing gavage during phases 1 or 2. During phase 3 (6 to 21 d post-weaning) and cumulatively, there was no effect of treatment on growth performance. The average FBW was 8.1 kg at 21 d post-weaning and the average cumulative ADG, ADFI, and G:F for the 21-d trial were 0.14 kg, 0.24 kg, and 0.31, respectively.

On 1 d post-weaning, the average ileal villus height was 256 ± 34 μm and crypt depth was 72 ± 4 μm (Fig.7- 2). The deprivation event tended ($P = 0.10$) to decrease villus height. Additionally, the oral gavage of DADS + DATS increased ($P = 0.03$) villus height and there was a tendency ($P = 0.09$) for a deprivation event and DADS +

DATS interaction that showed the oral gavage of DADS + DATS could partially mitigate the effects of the deprivation event on ileal villus height. There was no effect of the deprivation event on crypt depth 1 d post-weaning. On 6 d post-weaning the average villus height and crypt depth were 228 and 40 μm, respectively. The was no effect ($P < 0.10$) of the deprivation event or an interactive effect on ileal morphology 6 d post-weaning (data not shown). At the end of the 21-d trial average villus height and crypt depth were 279 and 50 μm, respectively, and there was no effect of treatment on ileal morphology (data not shown).

At 1 d post-weaning the average serum cortisol and CRF concentrations were 6.47 ± 0.06 and 0.85 ± 0.03 ng/mL, respectively (Table 7-3). Immediately following the deprivation event, at 1 d post-weaning, there was a 3% increase ($P = 0.01$) in serum cortisol and a tendency for an 8% increase ($P = 0.09$) in serum CRF. There was no effect of the DADS + DATS-containing gavage or stressor interaction 1 d post-weaning. On 6 and 21 d post-weaning the average serum cortisol concentrations were 6.42 ± 0.08 and 6.67 ± 0.05 ng/mL,respectively, and the average serum CRF concentrations were 0.62 ± 0.04 and 0.46 ± 0.02 ng/mL, respectively. There was no effect of treatment on serum cortisol or CRF concentrations on 6 and 21 d post-weaning (Table 7-3).

Gene expression data are shown in Table 7-4. There was no effect of treatment on expression of the housekeeping gene, GAPDH. Furthermore, there was no effect of treatment on expression of IL-8, TNF-α, GPX, or CL-1 genes in the ileal mucosa on 1, 6, and 21 d post-weaning (data not shown). There was a decrease ($P = 0.02$) and a tendency for a decrease ($P = 0.08$) in expression of the SOD gene in the ileal mucosa due to the oral gavage of DADS + DATS on 6 and 21 d post-weaning, respectively, but

there was no treatment effect 1 d post-weaning. There was a decrease ($P < 0.05$) in tight junction genes OC and ZO-1 in the ileal mucosa 1 d post-weaning due to the post-weaning deprivation event. However, there was no effect of treatment on expression of OC on 6 and 21 d post-weaning. At 6 and 21 d post-weaning there was an interaction ($P = 0.05$) and a tendency for an interaction ($P = 0.08$), respectively, between the deprivation event and the DADS + DATS-containing gavage for ZO-1 gene expression.

There was a decrease ($P = 0.01$) in SOD activity due to the deprivation event and an increase ($P = 0.04$) in SOD activity due to supplementation of DADS + DATS by oral gavage 1 d post-weaning (Fig. 7-3). There was no effect of treatment on ileal mucosa SOD enzyme activity on 6 and 21 d post-weaning (data not shown). Furthermore, there was no impact of treatment on ZO-1 protein expression (Fig. 7-4).

7.5. Discussion

A clear link has been established between weaning-associated stress events, feed and water consumption, gastrointestinal function, and growth performance (Pluske et al., 1997; Spreewenberg et al., 2001). Poor post-weaning feed consumption and subsequent negative effects are of major concern to the U.S. swine industry and Brooks et al. (2001) estimated that up to 10% of pigs do not consume feed for up to 48 h post-weaning. Furthermore, poor water consumption in nursery pigs is common due to transportation or weaning-associated lethargy and a recent study in our lab (Horn et al., 2014) showed that a 24-h water deprivation event has long-lasting implications on nursery pig performance and intestinal health. Therefore, it is necessary to evaluate nutritional and management strategies to minimize stress effects in nursery pigs.

Compounds derived from garlic have been well documented to have antioxidant, antimicrobial, and immune modulatory properties using in-vitro and rodent research models (Amagase et al., 2001; Amagase, 2006). Recent literature in young pigs shows that garlic compounds can reduce *E.coli*-associated diarrhea and improve intestinal morphology (Tatara et al., 2008; Lui et al., 2013) although a dearth of information exists on the mechanism of action of garlic-derived compounds. Therefore the objective of the current trial was to determine if a post-weaning oral gavage of garlic-derived compounds DADS + DATS could mitigate effects of an acute post-weaning feed + water deprivation event.

For the current study, overall pig performance was within the expected range for the Purdue University Research Farm although there was a high incidence of moderate stool looseness during the week following weaning. Furthermore, following the week-long incident of stool looseness there was a high degree of injectable medications given and several pigs that exhibited morbidity. Although not statistical, it is noteworthy to point out that pigs exposed to the deprivation event had a 6 and 3 times as much injectable medications and instances of visual morbidity as pigs not exposed to the deprivation event. Furthermore, when pigs exposed to the deprivation event were orally gavaged with DADS + DATS, the number of injectable medications and fall-off pigs were reduced to levels similar to pigs not exposed to the deprivation event. Limited information exists on the impact of garlic compounds on pig morbidity although Huang et al. (2010) showed a reduction in scour incidence in nursery pigs fed diets supplemented with dietary crude garlic compounds and Liu et al. (2013) showed dietary garlic product supplementation reduced diarrhea incidence in nursery pigs

exposed to *E.coli*. Huang et al. (2010) suggests that reduced nursery pig morbidity due to dietary garlic supplementation is due to antimicrobial mechanisms as shown by Roselli et al. (2007) and discussed by Ankri and Mirelman (1999). Furthermore, Ramiah et al. (2014) showed a reduction in gastrointestinal enteropathogens in broilers fed 0.50% crude garlic powder. Morbidity results from the current trial are noteworthy and warrant further validation in a large-scale production trial. The post-weaning feed + water deprivation event resulted in a 0.23-kg lighter pig when compared to pigs not exposed to the deprivation event 1 d post-weaning. Furthermore, there was a 55% improvement in ADG 6 d post-weaning due to the deprivation event but no further effects up to 21 d post-weaning. Deprivation event results are consistent with those reported by our lab in a previous study using a similar experimental design (Horn et al., 2014). In our previous study we showed that the post-deprivation event growth improvement was related to a 48 h period of water gorging. Conversely, our previous research shows the deprivation event has a negative impact on growth performance during the late nursery phase (Horn et al., 2014). Discrepancies between the current trial and previous research are likely due to differences in pig source and trial duration. Daily oral gavage of garlic-derived DADS + DATS for 6 d post-weaning had no impact on nursery pig growth performance. Conflicting evidence exists on the impact of garlic compounds on pig growth performance. Tatara et al. (2008) showed up to a 5% improvement in BW of pigs artificially reared with a milk replacer including garlic compounds and Yan et al. (2014) showed an improvement in growth performance in grow-finish pigs fed diets supplemented with dietary garlic. Conversely, Chen et al. (2008) and Holden (1998) showed no effect of dietary garlic supplementation on pig

growth performance and Olukosi and Dono (2014) showed no effect of supplementation of dietary crude garlic meal on broiler chicken performance. Discrepancies in the literature are likely due to differences in animal health and garlic compound bioactive components. It is possible for the current trial there was no impact of the DADS + DATS-containing gavage on growth performance due to the short (6 d) duration.

Serum stress mediators have been shown to increase at weaning, especially due to weaning stress events, and are related to poor intestinal function (Smith et al., 2010; Wang et al., 2015). For the current trial, there was a 3 and 8 % increase in serum stress markers cortisol and CRF 1 d post-weaning. This data, consistent with growth performance results, shows that the 24-h post-weaning feed + water deprivation was stressful. Furthermore, the data in the current report is consistent with data reported from a similar experiment (Horn et al., 2014). Post-weaning perception of stress results in an increase of the central stress mediator, CRF, which in turn triggers hypothalamic release of ACTH, and then adrenal release of the glucocorticoid cortisol. Cortisol has been well documented to cause stress-coping metabolic and behavioral changes (Moeser et al., 2007). Furthermore, central and peripheral CRF release leads to reduced gastrointestinal integrity and mucosal inflammatory response through stimulation and recruitment of intestinal mast cells and secretion of inflammatory cytokines (Overman et al., 2012). Recent literature shows that a spike in post-weaning CRF is associated with villus atrophy and up to a 40% increase in intestinal permeability (Wang et al., 2015). There was no impact of oral gavage of DADS + DATS on serum stress markers, or any effect of treatment on serum stress markers on 6 and 21 d post-weaning. The aforementioned is consistent with the growth performance results and is likely due to

the limited nature of the deprivation event and negative feedback mechanisms that exist to limit long-term increases in cortisol and CRF.

Poor feed intake following weaning limits energy supply to gastrointestinal tissues and leads to intestinal villus atrophy (Pluske et al., 1997). Conflicting results exist on the impact of an acute post-weaning stress event on intestinal morphology. Previous research from our lab (Horn et al., 2014) along with results reported by Moeser et al. (2007) showed there is little impact of feed + water or early weaning stress events on ileal morphology, respectively. However, Wang et al. (2015) showed a 42 % decrease in villus height which was attributed to weaning-associated stress. At 1 d post-weaning in the current study pigs exposed to the deprivation event had a 29% reduction in ileal villus height when compared to pigs not exposed to the deprivation event. The aforementioned decrease in villus height shows that the deprivation event has the potential to limit nutrient absorption and may compromise gut protective function during the first week post-weaning. Oral gavage of DADS + DATS reversed the impact of the deprivation event on ileal villus height 1 d post-weaning. Current results show that pigs exposed to the deprivation event and receiving the oral gavage of DADS + DATS had a 37% increase in villus height when compared to pigs exposed to the deprivation event but not orally gavaged with DADS + DATS. Limiting and conflicting evidence exists on the impact of garlic compounds on intestinal morphology. Tatara et al. (2008) showed that supplementation of milk replacer with a garlic product resulted in improved villus height and crypt depth in neonatal pigs whereas Lui et al. (2013) showed no improvement in intestinal morphology in nursery pigs fed diets supplemented with a dietary garlic product and challenged with pathogenic *E.coli.* The

exact mechanism of action of DADS + DATS on ileal morphology is not fully understood although it is likely related to antimicrobial, immune modulatory, or antioxidant properties of garlic compounds. Peinado et al. (2014) showed dietary supplementation of the garlic compound propyl propane thiosulfinate reduced intestinal enteropathogens in broilers, Ramiah et al. (2014) and Olukosi and Dono (2014) showed alteration of intestinal VFA concentrations with dietary supplementation of crude garlic preparations. Furthermore, dietary supplementation of a garlic product resulted in increased intestinal innate immune secretions and affected genes related to immune function in the ileal mucosa of nursery pigs (Tatara et al., 2008; Liu et al., 2014).

Post-weaning changes in markers of intestinal mucosa immune, antioxidant, and cellular integrity status have been shown to be related to short- and long-term effects on pig growth efficiency and health (Pie et al., 2005; Lambert, 2009; Zhu et al., 2012; Hu et al., 2013). Specifically, changes in post-weaning expression of mucosal cytokine gene expression have been shown to be induced by weaning stress and play a critical role in regulating mucosal immune and inflammatory status (Hu et al., 2013). For the current trial there was no impact of the feed + water deprivation event on expression of cytokines TNF-α or IL-8 in the ileal mucosa which is consistent with results reported by Horn et al. (2014) using a similar deprivation stress event. However, Hu et al. (2013) reported an increase in mucosal cytokine genes due to early weaning stress from 3 to 7 d post-weaning in pigs. Because expression of cytokines is time-specific, it is likely that there was no impact of the feed + water deprivation event due to sample timing. Using in-vitro and rodent models, garlic compounds have been shown to be immune modulatory (Kyo et al., 2001). For the current trial there was no impact of

administration of DADS + DATS by oral gavage on mucosa cytokine expression and these results are consistent with a mucosal gene expression profile study reported by Liu et al. (2014).

Under normal physiological conditions, cellular antioxidant enzyme systems, such as SOD and GPX, exist to maintain cellular oxidative balance (Zhu et al., 2012). Oxidative stress occurs when cellular antioxidant systems do not sufficiently mitigate cellular and extracellular oxidants, such as ROS and hydrogen peroxide, leading to damage of cellular lipid membranes, protein, and DNA (Cai et al., 2013). Recent literature in pigs shows that weaning-associated stress events result in changes in antioxidant status that can be ameliorated by supplementation of dietary antioxidants (Zhu et al., 2012; Yin et al., 2014). For the current experiment there was a 40% decrease in mucosal SOD activity immediately following the deprivation event, suggesting that the post-weaning deprivation event induced a short-term period of oxidative stress in nursery pigs. There was no impact of the feed + water deprivation event on SOD or GPX enzyme activity on 6 and 21 d post-weaning and no impact of the deprivation event on SOD or GPX gene expression at any time point. Consistent with this observation, Yin et al. (2014) reported an early weaning stress event caused a decrease in SOD activity 1 d post-weaning but did not impact GPX activity until 3 d post-weaning. Furthermore, Yin et al. (2014) concluded that a poor connection exists between ileal SOD gene expression and SOD enzyme activity due to exogenous factors such as Cu and Zn status and post-translational protein modification. Borek (2001) reported that garlic compounds have radical scavenging properties and potential to alter cellular oxidative status. Wei and Lau (1998) showed that a garlic product was able to

mitigate hydrogen peroxide-induced oxidation and improved antioxidant enzyme function using an endothelial cell model. More recently, Badr and Al-Mulhim (2014) showed supplementation of 200 mg garlic product per kg diet was able to mitigate indomethacin-induced gastric damage by improving SOD and GPX enzyme activity. For the current experiment, there was an increase in SOD activity due to oral gavage of DADS + DATS. This data shows that the oral gavage was able to improve SOD activity in pigs not exposed to a deprivation event and was able to partially mitigate oxidant effects of the deprivation event. Interestingly, there was a decrease in SOD gene expression due to the DADS + DATS-containing gavage on 6 and 21 d post-weaning. The reverse effect of the DADS + DATS gavage on gene expression versus enzyme activity is not clear but may be due to negative feedback mechanisms related to anti-peroxide or post-translational effects of DADS + DATS. Sadi et al. (2008) showed that antioxidant-induced effects of vitamin C and lipoic acid on rat liver SOD activity were poorly related to SOD gene expression and Pedraza-Chaverri et al. (2001) showed that a decrease in catalase activity in rat tissue due to dietary supplementation of 2% garlic was not related to garlic-induced changes in enzyme activity.

Tight junction proteins are critical for control of epithelial cell integrity (Gonzalez-Mariscal et al., 2003). Weaning associated stress events have been shown to induce changes in tight junction dynamics that are linked to poor gut function and animal efficiency (Hu et al., 2013; Horn et al., 2014). Results from the current report show that the deprivation event reduced expression of OC and ZO-1 tight junction genes in the ileal mucosa 1 d post-weaning, but not on 6 and 21 d post-weaning. Hu et al. (2013) showed that an early weaning event reduced OC and ZO-1 expression up to 7

d post-weaning and Horn et al. (2014) showed a feed + water deprivation event impacted tight junction gene expression mainly during the first week post-weaning. Limited information exists on the impact of garlic compounds on tight junction dynamics in pigs. Roselli et al. (2007) showed that a garlic product was able to mitigate the effects of *E.coli* on porcine cellular integrity and Park et al. (2011) showed DADS increased trans-epithelial resistance and increased gene expression of metalloprotease inhibitor protein. Conversely, Liu et al. (2014) reported no impact of a dietary garlic supplementation on gene expression markers of cellular integrity in the ileal mucosa of nursery pigs. There was a deprivation event by DADS + DATS interaction for ZO-1 gene expression on 6 and 21 d post-weaning in the current study that showed a reversal effect of the deprivation event and oral gavage when in combination. Perhaps the DADS + DATS-induced effects are related to microbial population shifts and further work is needed investigating the impact of DADS + DATS on intestinal microbial ecology and cellular integrity. Furthermore, the interactive effect suggests that DADS + DATS may be initiating hormesis on cellular integrity as discussed by Mattson (2008) and further work is needed investigating the impact of garlic compounds on adaptive stress signaling pathways. The effect of DADS + DATS on ZO-1 gene expression did not translate into detectable protein changes. The relationship between tight junction gene and protein expression in pigs is poorly understood and further research is warranted.

A 24-h feed + water deprivation event resulted in an increase in serum stress markers, reduction in growth performance, and atrophy of ileal villi during the first week post-weaning. Additionally, results from the current trial show the deprivation

event has negative implications on mucosal antioxidant status and cellular integrity as measured by SOD activity and tight junction gene expression, respectively. Daily oral gavage of garlic-derived DADS + DATS for 6 d post-weaning improved ileal villus height and mucosal SOD activity and partially mitigated the effects of the post-weaning deprivation event on ileal characteristics. Overall, results from the current trial show that supplementation of garlic-derived compounds DADS and DATS by oral gavage may be a viable strategy to improve post-weaning gastrointestinal dysfunction in nursery pigs.

7.6. Acknowledgments

The authors would like to express their appreciation to Biomatrix International and JBS United, Inc. for their financial and technical support of this trial. Further appreciation is given to Richard Byrd, Purdue Animal Sciences Research and Education Farm, for support with pig husbandry. The authors would also like to express appreciation to Hui Yan, Purdue University Department of Animal Sciences, for his assistance with western blot analysis, Donna Brooks, Purdue University Veterinary School, for her assistance with histological measurements, and Bruce Cooper, Purdue University Metabolite Profiling Laboratory, for his assistance with garlic compound analysis.

7.7. References

Amagase, H., B.L. Petesch, H. Matsuura, S. Kasuga, and Y. Itakura. 2001. Intake of garlic and its bioactive components. J. Nutr. 131: 955S–962S.

Amagase, H. 2006. Clarifying the real bioactive constituents of garlic. J. Nutr. 136: 716S–725S.

Ankri, S., and D. Mirelman. 1999. Antimicrobial properties of allicin from garlic. Microb. Infect. 2: 125−129.

Badr, G.M., and J.A. Al-Mulhim. 2014. The protective effect of aged garlic extract on nonsteroidal anti-inflammatory drug-induced gastric inflammations in male albino rats. Evid. Based Complement. Alternat. Med. 2014: 759642.

Borek, C. 2001. Antioxidant health effects of aged garlic extract. J. Nutr. 131: 1010S–1015S.

Brooks, P. H., C. A. Moran, J. D. Beal, V. Demeckova, and A. Campbell. 2001. Liquid feeding for the young piglet. In: M. A. Varley, and J. Wiseman, editors, The weaner pig: Nutrition and management. CAB Int., Wallingford, UK. p. 153–178.

Cai X, Chen X, Wang X, Xu C, Guo Q, Zhu L, Zhu S, Xu J. 2013. Pre-protective effect of lipoic acid on injury induced by H2O2 in IPEC-J2 cells. Mol. Cell Biochem. 378:73-81.

Chen, Y.J., I.H. Kim, J.H. Cho, J.S. Yoo, Q. Wang, Y. Wang, and Y. Haung. 2008. Evaluation of dietary L-carnitine or garlic powder on growth performance, dry matter and nitrogen digestibilities, blood profiles and meat quality in finishing pigs. Anim. Feed Sci. Tech. 141(1): 141-152.

Gonzalez-Mariscal, L., A. Betanzos, P. Nava, and B.E. Jaramillo. 2003. Tight junction proteins. Progr. Biophys. Molec. Biol. 81: 1-44.

Holden, P.J., J. McKean, and E. Franzenburg. 1998. Botanicals for pigs-garlic. Iowa State University, Swine Research Report.

Horn, N., F. Ruch, G. Miller, K. M. Ajuwon and O. Adeola. 2014. Impact of acute water and feed deprivation events on growth performance, intestinal characteristics, and serum stress markers in weaned pigs. J. Anim. Sci. 92:4407-4416.

Hu, C. H., K. Xiao, Z. S. Luan, and S. Jong. 2013. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. J. Anim. Sci. 91:1094–1101.

Huang, R.H., X.S. Qiu, F.X., Shi, C.L. Hughes, Z.F. Lu, and W.Y. Zhu. 2011. Effects of dietary allicin on health and growth performance of weanling piglets and reduction in attractiveness of faeces to flies. Animal. 5: 304–311.

Kyo, E., N. Uda, S. Kasuga, and Y. Itakura. 2001. Immunomodulatory effects of aged garlic extract. J. Nutr. 131:1075S–1079S.

Lalles J.P., P. Bosi, H. Smidt, and C.R. Stokes. 2007. Nutritional management of gut health in pigs around weaning. Proc. Nutr. Soc. 66: 260–268.

Lambert, G.P. 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J. Anim. Sci. 87 (E suppl.): E101-E108.

Liu, Y., M. Song, T.M. Che, A.S. Almeida, J.J. Lee, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2013. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs. J. Anim. Sci. 91:5294-5306.

Liu, Y., M. Song, D. Che, D. Bravo, C.W. Maddox, and J.E. Pettigrew. 2014. Effects of capsicum oleoresin, garlic botanical, and turmeric oleoresin on gene expression profile of ileal mucosa in weaned pigs. J. Anim. Sci. 92:3426-3440.

Mattson, M.P. 2008. Hormesis defined. Ageing Res. Rev. 7(1): 1-7.

Moeser, A. J., C. V. Klok, K. A. Ryan, J. G. Wooten, and D. Little. 2007. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am. J. Physiol. Gastrointest. Liver Physiol. 292:G173–G181.

NRC, 2012. Nutrient requirements of Swine. 11th rev ed. Natl. Acad. Press, Washington, D.C.

Olukosi, O.A., and N. D. Dono. 2014. Modification of digesta pH and intestinal morphology with the use of benzoic acid or phytobiotics and the effects on broiler chicken growth performance and energy and nutrient utilization. J. Anim. Sci. 92:3945–3953.

Overman, E. L., J. E. Rivier, and A. J. Moeser. 2012. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-a. PLoS ONE 7(6):e39935.

Paszti-Gere, E., E. Csibrik-Nemeth, S. Krisztina, R. Csizinszky, J. Csaba, and P. Galfi. 2012. Acute oxidative stress affects IL-8 and TNF-α expression in IPEC-J2 porcine epithelial cells. Inflammation. 35: 994-1004.

Park, H.S., G.Y. Kim, I.W.Choi, N.D. Kim, H.J. Hwang, Y.W. Choi, and Y.H. Choi. 2011. Inhibition of matrix metalloproteinase activities and tightening of tight junctions by diallyl disulfide in AGS human gastric carcinoma cells. J. Food Sci.76: T105-T111.

Pedraza-Chaverrí, J., M. Granados-Silvestre, O. N. Medina-Campos, P. D. Maldonado, I. M. Olivares-Corichi, and M. E. Ibarra-Rubio. 2001. Post-transcriptional control of catalase expression in garlic-treated rats. Mol. Cell Biochem. 216: 9-19.

Peinado, M.J., R. Ruiz, A. Echavarri, and L. A. Rubio. 2012. Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. Poult. Sci. 91:2148-2157.

Pie, S., J.P. Lalles, F. Blazy, J. Laffitte, B. Seve, and I.P. Oswalk. 2004. Weaning Is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. J. Nutr. 134: 641-647.

Pluske, J.R., D.J. Hampson, and I.H. William. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51, 215–236.

Prieto, M.T. and J. L. Campo. 2010. Effect of heat and several additives related to stress levels on fluctuating asymmetry, heterophil:lymphocyte ratio, and tonic immobility duration in white leghorn chicks. Poult. Sci. 89:2071-2077.

Ramiah, S. K., I. Zulkifli, N. Asyikin, A. Rahim, M. Ebrahimi, and G. Y. Meng. 2014. Effects of two herbal extracts an virginiamycin supplementation on growth performance, intestinal microflora population and fatty acid composition in broiler chickens. Austr. Asian J. Anim. Sci. 27: 375-382.

Roselli, M., M.S. Britti, I. Lerou-Luron, H. Marfaing, W.Y. Zhu, and E. Mengheri. 2007. Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic Escherichia coli K88 in pig intestinal cells. Toxicol. in Vitro. 21: 224–229.

SAS Institute. 2006. SAS/STAT User's Guide. Release 9.1. SAS Institute Inc., Cary, NC.

Sadi, G., O. Yılmaz, and T. Guray. 2008. Effect of vitamin C and lipoic acid on streptozotocin-induced diabetes gene expression: mRNA and protein expressions of Cu–Zn SOD and catalase. Mol. Cel Biochem. 309: 109-116.

Smith, F., J.E. Clark, B.L. Overman, C.C. Tozel, J.H. Huang, J.E. Rivier, A.T. Blikslager, and A.J. Moeser. 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 298(3):G352-G363.

Spreeuwenberg, M. A. M., J. M. A. J. Verdonk, H. R. Gaskins, and M. W. A. Verstegen. 2001. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. J. Nutr. 131:1520–1527.

Tatara, M.R., E. Śliwa, K. Dudek, A. Gawron, T. Piersiak, P. Dobrowolski , J. Mosiewicz, , A. K. Siwicki, and T. Studziński T. 2008. Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared on artificial sow. Ann. Agric. Environ. Med. 15: 63–69.

Valko, M., D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur, and J. Telser. 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39:44–84.

Wang, H., C. Zhang, G.Wu, Y. Sun, B. Wang,B. He, Z. Dai, and Z. Wu. 2015. Glutamine enhances tight junction protein expression and modulates corticotropin-releasing factor signaling in the jejunum of weanling piglets. J. Nutr. 145: 25-31.

Wei, Z., and B.H., Lau. 1998. Garlic inhibits free radical generation and augments antioxidant enzyme activity in vascular endothelial cells. Nutr. Res. 18:61–70.

Yin, J., M. M. Wu, H. Xiao, W. K. Ren, J. L. Duan, G. Yang,T. J. Li, and Y. L. Yin. 2014. Development of an antioxidant system after early weaning in piglets. J. Anim. Sci. 92: 612-619.

Zhu, L.H., K. L. Zhao, X. L. Chen, and J. X. Xu. 2012. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. J. Anim. Sci. 90: 2581-2589.

Table 7- 1. Ingredient composition of diets

Item	Phase 1	Phase 2	Phase 3
Ingredients, g/kg			
Corn	450.5	522.8	690.1
Soybean meal	225.0	275.0	275.0
Whey permeate	200.0	100.0	-
Plasma	60.0	20.0	-
Fish meal	38.0	63.0	-
Monocalcium phosphate[1]	9.0	6.0	9.0
Limestone[2]	7.5	5.0	10.0
Salt	2.5	3.5	6.5
Vitamin premix[3]	1.0	1.0	1.0
Mineral premix[4]	0.8	0.8	0.8
Selenium premix[5]	0.5	0.5	0.5
L-Lys HCl	2.8	1.4	4.3
DL-Met	1.7	1.0	1.5
L-Thr	0.7	-	1.3
Total	1,000	1,000	1,000
Calculated nutrients and energy, as-fed basis			
ME, kcal/kg	3317.6	3297.8	3260.6
CP, g/kg	225.0	234.0	186.0
Ca, g/kg	8.6	8.1	5.9
P, g/kg	8.0	7.4	5.3
Nonphytate P, g/kg	6.4	5.5	3.3
Ca:P	1.08	1.09	1.12
SID Lys, g/kg	13.9	13.0	12.1

[1] Contained 17% Ca and 21.1 % P.
[2] Contained 38% Ca.
[3] Vitamin premix per kilogram of diet: vitamin A, 5,090 IU; vitamin D3 1,270 IU ; vitamin E 22 IU, vitamin K activity 7.2 mg; menadione, 2,412 µg; vitamin B12, 22.4 µg; riboflavin 8.11 mg; d-pantothenic acid 61.20; and niacin 106.59 mg.
[4] Mineral premix supplied per kilogram of diet: Cu (as copper chloride), 18 mg; I (as ethylenediamine dihydroiodide) 0.95 mg; Fe (as iron carbonate) 334 mg; Mn (as manganese oxide) 60 mg; and zinc (as zinc oxide) 150 mg.
[5] Supplied as 300 µg of Se per kilogram of diet.

Table 7-2. Main effects of feed + water deprivation and an oral gavage containing diallyl disulfide (DADS) and diallyl trisulfide (DATS) on growth performance of pigs [1,2]

Item	Post-Weaning Feed + Water Deprivation		DADS + DATS			*P*-value	
	-	+	-	+	SEM[3]	Deprivation	DADS + DATS
IBW, kg	6.01	5.97	6.09	5.89	0.05	0.28	< 0.01
Phase 1, 0 to 1 d post-weaning[4]							
FBW, kg	5.83	5.60	5.73	5.70	0.02	< 0.01	0.24
ADG, kg	-0.18	-0.42	-0.28	-0.32	0.02	< 0.01	0.26
ADFI, kg	0.04	0.00	0.03	0.02	< 0.01	< 0.01	0.30
G:F	-4.19	0.00	-3.46	-4.92	0.86	< 0.01	0.30
Phase 2, 1 to 6 d post-weaning[4]							
FBW, kg	5.93	6.00	6.00	5.94	0.06	0.44	0.54
ADG, kg	0.04	0.09	0.06	0.07	0.01	< 0.01	0.76
ADFI, kg	0.07	0.10	0.09	0.09	0.01	0.03	0.81
G:F	0.46	0.88	0.62	0.73	0.12	0.01	0.47
Phase 3, 6 to 21 d post-weaning[4]							
FBW, kg	8.11	8.11	8.06	8.17	0.23	0.99	0.77
ADG, kg	0.34	0.32	0.32	0.33	0.03	0.66	0.70
ADFI, kg	0.24	0.24	0.24	0.24	0.01	0.93	0.66
G:F	0.62	0.58	0.60	0.60	0.03	0.41	0.94
Cumulative[4]							
ADG, kg	0.23	0.23	0.22	0.24	0.03	0.99	0.62
ADFI, kg	0.18	0.19	0.18	0.19	0.01	0.71	0.66
G:F	0.54	0.55	0.55	0.55	0.03	0.94	0.99

[1]Pigs exposed to a 24-h post-weaning feed + water deprivation and administered a DADS + DATS-containing oral gavage daily from 0 to 6 d post-weaning.
[2]There were 8 replicates per treatment with 6 pigs per pen at the start of the trial.
[3]SEM = standard error of the mean. The higested SEM among treatment means used.
[4]Data analyzed using IBW as a covariate.

Table 7-3. Main effects of feed + water deprivation and an oral gavage containing diallyl disulfide (DADS) and diallyl trisulfide (DATS) on serum stress markers of pigs [1,2]

Item	Post-weaning feed + water deprivation		DADS + DATS			*P*-value	
	-	+	-	+	SEM[3]	Deprivation	DADS + DATS
1 d post-weaning							
Cortisol, ng/mL	6.35	6.55	6.47	6.42	0.06	0.01	0.49
CRF, ng/mL[4]	0.81	0.88	0.85	0.85	0.03	0.11	0.89
6 d post-weaning							
Cortisol, ng/nL	6.42	6.42	6.40	6.44	0.08	0.93	0.68
CRF, ng/mL[4]	0.64	0.59	0.61	0.62	0.05	0.30	0.91
21 d post-weaning							
Cortisol, ng/mL	6.86	6.66	6.67	6.66	0.04	0.66	0.88
CRF, ng/mL[4]	0.48	0.44	0.47	0.45	0.02	0.15	0.67

[1]Pigs exposed to a 24-h post-weaning feed + water deprivation and administered a DADS + DATS-containing oral gavage daily from 0 to 6 d post-weaning.
[2]There was a minimum of 7 replicates per treatment.
[3]SEM = standard error of the mean. The higested SEM among treatment means used.
[4] CRF = corticotrophin releasing factor.

Table 7-4. Simple effects of feed + water deprivation and an oral gavage containing diallyl disulfide (DADS) and diallyl trisulfide (DATS) on ileal gene expression responses of pigs[1,2,3]

Item							*P*-value	
Post-weaning feed + water deprivation	-	+	-	+	SEM[4]	Deprivation	DADS + DATS	Interaction
DADS + DATS	-	-	+	+				
SOD, d post-weaning								
1[5]	5.5	13.4	9.15	6.10	3.81	0.52	0.76	0.14
6	2.94	2.65	1.12	1.25	0.68	0.91	0.02	0.75
21	1.81	1.84	1.14	1.11	0.41	0.99	0.08	0.94
OC, d post-weaning								
1	1.44	1.07	1.77	0.94	0.30	0.05	0.75	0.43
6	1.87	1.29	1.18	1.75	0.37	0.97	0.74	0.11
21[5]	1.09	1.84	1.00	1.17	0.36	0.49	0.50	0.54
ZO-1, d post-weaning								
1[5]	1.34	1.11	1.41	1.19	0.25	0.02	0.67	0.97
6	2.06[a]	0.99[b]	0.90[b]	1.26[ab]	0.36	0.20	0.30	0.05
21[5]	1.91[a]	0.93[b]	0.93[b]	1.37[ab]	0.42	0.47	0.48	0.08

[1]Pigs exposed to a 24-h post-weaning feed + water deprivation and administered a DADS + DATS-containing gavage daily from 0 to 6 d post-weaning.
[2]There were 5 to 8 replicates per treatment.
[3] Gene expression of superoxide dismutase (SOD), occludin (OC), and zonula occludens 1 (ZO-1) expressed relative to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
[4]SEM = standard error of the mean. The higested SEM among treatment means used.
[5]*P*-value from transformed data using a log transformation.

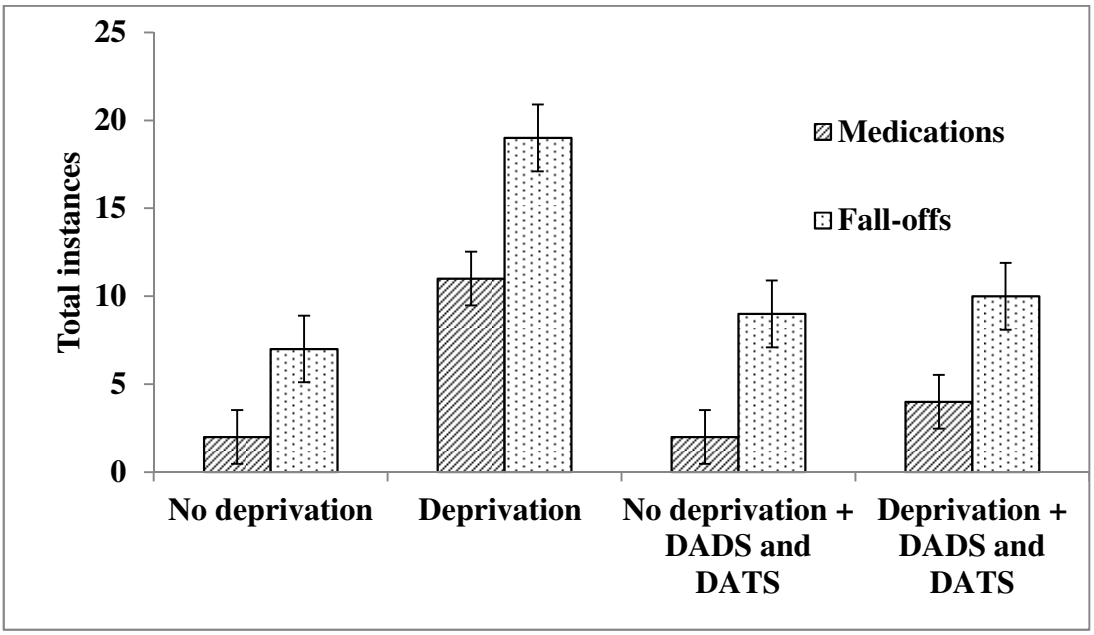

Figure 7-1. Simple effects of feed + water deprivation and an oral gavage containing diallyl disulfide (DADS) and diallyl trisulfide (DATS) on total instances of injectable medications and fall-off pig observations. Pigs exposed to a 24-h post-weaning feed + water deprivation and administered garlic-derived DADS + DATS by daily oral gavage from 0 to 6 d post-weaning. Analyzed as non-parametric data. There were 8 replicate pens per treatment with 6 pigs per pen at the start of the trial. *P*-values = 0.12 and 0.36 for medications and fall-offs, respectively

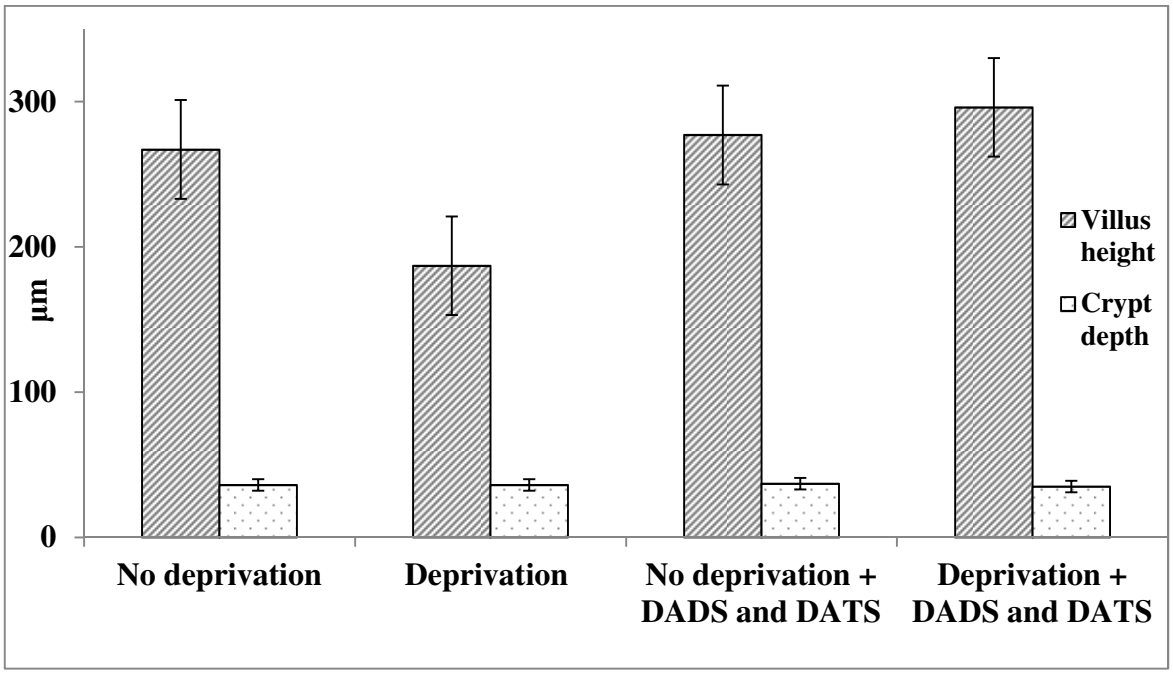

Figure 7-2. Simple effects of feed + water deprivation and an oral gavage containing diallyl disulfide (DADS) and diallyl trisulfide (DATS) on ileal morphology 1 d post-weaning. Pigs exposed to a 24-h post-weaning feed + water deprivation and administered garlic-derived DADS + DATS by daily oral gavage from 0 to 6 d post-weaning. There were 7 replicates per treatment. For villus height $P = 0.10$, 0.03, and 0.09 for main effects of deprivation, DADS + DATS gavage, and subsequent interaction, respectively. For crypt depth there was no effect of the deprivation event, DADS + DATS, or subsequent interaction.

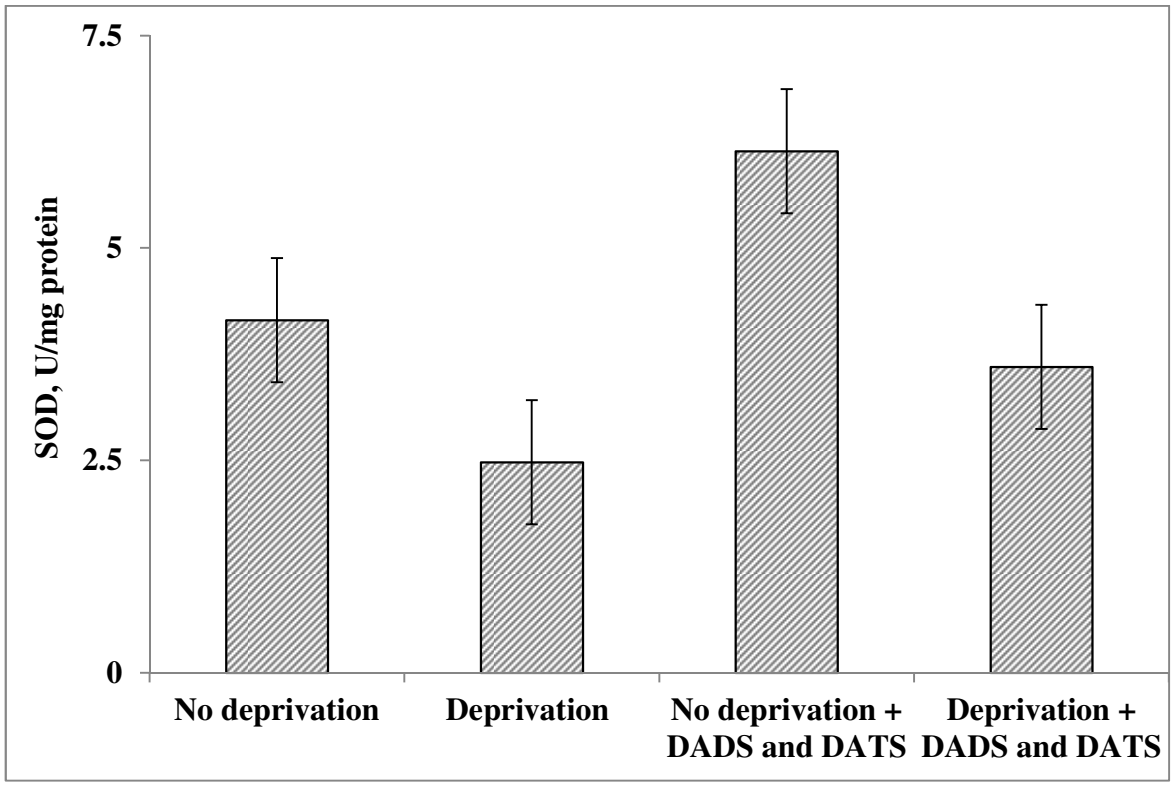

Figure 7-3. Simple effects of feed + water deprivation and an oral gavage containing diallyl disulfide (DADS) and diallyl trisulfide (DATS) on superoxide dismutase (SOD) activity in ileal mucosa 1 d post-weaning. Pigs exposed to a 24-h post-weaning feed + water deprivation and administered garlic-derived DADS + DATS by daily oral gavage from 0 to 6 d post-weaning. There was a minimum of 7 replicates per treatment. *P*-values = < 0.01, 0.04, and 0.51 for main effects of feed + water deprivation, DADS + DATS gavage, and subsequent interaction, respectively. One unit of activity is the amount of enzyme that inhibits the rate of superoxide formation reaction by 100%.

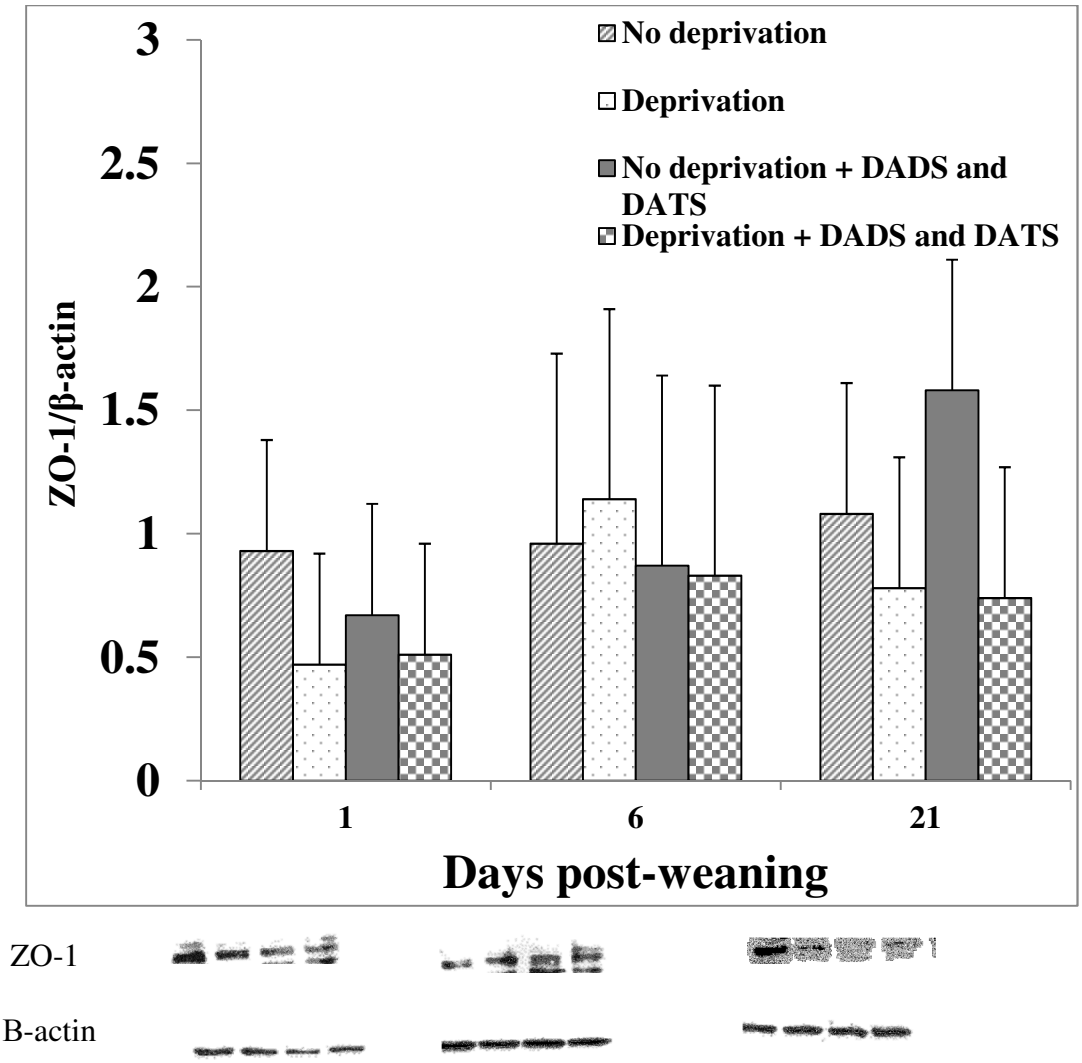

Figure 7-4. Simple effects of feed + water deprivation and an oral gavage containing diallyl disulfide (DADS) and diallyl trisulfide (DATS) on ZO-1 protein in ileal mucosa. Pigs exposed to a 24-h post-weaning feed + water deprivation and administered garlic-derived DADS + DATS by daily oral gavage from 0 to 6 d post-weaning. There was a minimum of 4 replicates per treatment. There was no effect of the deprivation event, DADS + DATS gavage, or subsequent interaction at any point.

CHAPTER 8. SUMMARY

Pigs are exposed to various stress events throughout their production cycle that have implications on gastrointestinal function. One of the most notable and unavoidable stress events is weaning. Poor feed intake, lethargy, and diarrhea are common during the post-weaning period which can lead to breakdown of the gut barrier and in turn causes chronic inflammation, poor performance, and disease susceptibility. The mucosal immune system of pigs between 18 to 28 d of age is immature and prone to over activation by stress events, which exacerbates weaning-associated gut dysfunction. Nutritional strategies exist to mitigate weaning-associated gut dysfunction and include supplementation of highly-digestible carbohydrate and AA sources, probiotics, prebiotics, and plant-derived compounds. Compounds derived from garlic have been shown to alter microbial ecology and mucosal immune function and mitigate oxidative stress using in-vitro or rodent models. The objectives of the studies presented within this dissertation were to characterize the impact of a post-weaning deprivation event on nursery pig growth and intestinal function and to determine the ability of garlic-derived bioactive components diallyl disulfide (DADS) and diallyl trisulfide (DATS) to mitigate weaning-associated gastrointestinal dysfunction.

The first experiment (Chapter II) was conducted to characterize the impact of a feed + water deprivation on nursery pig growth and gut function and the second experiment (Chapter III) was conducted to determine if an interaction exists between a post-weaning deprivation event and subsequent heat stress event. In the first experiment pigs were exposed to a 24-h feed, water, or feed + water event immediately post-weaning. Growth performance was monitored up to 28 d post-weaning and intestinal characteristics were measured during the first wk post-weaning. The water deprivation event was more detrimental then the feed deprivation event and resulted in an approximate 0.30 kg loss immediately following which was followed by a brief water-gorging period resulting in improved performance. However, pigs exposed to the post-weaning deprivation event had reduced performance in the mid- and late-nursery periods which translated into a 0.46 kg lighter pig at 28 d post-weaning. These results clearly show that acute post-weaning water deprivation has long-term implications on pig performance. For the second experiment pigs were exposed to a 24-h post-weaning feed + water deprivation event followed by a 3-d cyclic heat stressor starting at 27 d post-weaning. The heat stress event resulted in a 14% reduction in gain which was recovered the week following. However, a post-weaning deprivation event by heat stress event interaction existed that showed pigs exposed to both stress events had the poorest overall ADG and ADFI when compared to pigs exposed to only one stress event or no stress events. Furthermore, for both experiments, the post-weaning deprivation event corresponded with a spike in serum CRF and changes in tight junction gene expression during the first week post-weaning. These data show that post-weaning gastrointestinal dysfunction may be related to neuroendocrine signaling and degradation of tight junction

proteins. However, the impact of deprivation stress on gut morphology and mucosal tight junction gene expression was not consistent across experiments.

The third experiment (Chapter IV) was to identify the mode of action of garlic-derived DADS and DATS using an IPEC-J2 cell model. Hydrogen peroxide (100 μM) was used an oxidative stressor, whereas LPS (10 μg/mL) was used as an endotoxin stressor. Epithelial cells were pre-incubated with DADS + DATS (18 μM) for 18 h and hydrogen peroxide and LPS were then exposed to the cells for 6 and 3 h, respectively. Treatment of IPEC-J2 cells with LPS induced an immunological response which showed up-regulation of IL-8 and TNF-α genes and a decrease in trans-epithelial electrical resistance (TEER). Furthermore, incubation with hydrogen peroxide decreased antioxidant enzyme activity, but did not impact TEER. These results show that IPEC-J2 cells can be used to study LPS- and hydrogen peroxide-induced effects. Pre-incubation of IPEC-J2 cells with DADS + DATS resulted in augmentation of LPS-stimulated IL-8 secretion. These results show that garlic-derived DADS + DATS has immune modulatory properties. However, due to limitations surrounding cell culture research, definitive conclusions cannot be made in regards to whether the immunological impact would be “positive” or “negative” in-vivo. Pre-incubation of IPEC-J2 cells with DADS + DATS mitigated the oxidant effects of hydrogen peroxide as measured by SOD and catalase activity. These results show that garlic-derived DADS + DATS has immune modulatory and antioxidant properties.

The fourth and fifth experiments of this dissertation (Chapters V and VI) were aimed to identify the optimal dose and effects of various doses of DADS + DATS supplemented by oral gavage on swine and broiler chicken performance and gut function.

Treatments consisted of daily oral supplementation of 0, 0.45, 0.90, 1.80, 3.60, and 7.20 mg DADS + DATS per kg BW for a period of 6 d. In pigs, the oral gavage did not affect growth performance; however there was an increase in ileal villus height due to the DADS + DATS-containing gavage. Furthermore, there was a linear increase in IL-8 gene expression and a decrease in gene expression of the tight junction gene ZO-1. The IL-8 results are consistent with the aforementioned in-vitro study and suggest that DADS + DATS has immune modulatory properties that alter tight junction dynamics. The optimal oral dosage of DADS + DATS to maximize villus height in pigs was determined to be 1.71 mg/kg BW. In broiler chickens oral supplementation of DADS + DATS linearly improved BW gain and villous height. Total-tract digestibility of DM, N, and E were also improved in a quadratic fashion. However, markers of immune and tight junction function in the mucosa were not affected. The optimal oral dose of DADS + DATS to maximize BW gain and villus height was 2.5 mg/kg BW whereas the optimal oral dose to maximize total-tract nutrient and E digestibility was 1.16 mg/kg BW. These studies show that oral supplementation of DADS + DATS impacts growth and gastrointestinal function in pigs and poultry although species-specific differences exist.

The objective of the sixth experiment (Chapter VII) was to determine if supplementation of DADS + DATS could mitigate the effects of a 24-h post-weaning feed + water deprivation event on nursery pig performance and gastrointestinal function. The DADS + DATS (3.6 mg/kg BW) was administered by daily oral gavage starting at weaning for a period of 6 d. The 24-h post-weaning deprivation event impacted growth performance similar to previous experiments. However, in the final experiment the feed + water deprivation event resulted in atrophy of ileal morphology which was not

consistent with earlier experiments. The discrepancy is likely due to differences in pig health and management between studies (JBS United vs. Purdue University pigs). Pigs from experiment six had lower weaning weights and higher incidence of post-weaning diarrhea when compared to pigs from previous experiments. The oral gavage of DADS + DATS did not affect growth performance; however it did improve ileal morphology 1 d post-weaning in all pigs. The weaning deprivation resulted in decreased activity of the antioxidant enzyme SOD which was mitigated by oral supplementation of DADS + DATS. These results show that the oral gavage of garlic-derived DADS + DATS can mitigate feed + water deprivation associated effects on ileal morphology and oxidative status.

In conclusion, the results from this dissertation show that a post-weaning feed + water deprivation event has short- and long-term implications on growth performance and gastrointestinal architecture and function and stressor interactions exist. Furthermore, garlic-derived DADS and DATS has antioxidant, immune modulatory, and gut-enhancing properties. When supplemented by oral gavage DADS + DATS can partially mitigate the effects of a post-weaning deprivation event. Further research is needed investigating the mode of action of DADS and DATS. Specifically, an understanding of how DADS + DATS influences NFκB pathway activation, up-regulation of antioxidant response elements, and intestinal microflora would give insight into the mode of action of the garlic-derived compounds. Also, better understanding of the relationship between tight junction gene and protein expression and gastrointestinal permeability is needed. Further research investigating the impact of garlic-derived compounds on microbial ecology and mucosal immunity (using immune-challenged models) may provide insight

on the mode of action and why species-specific differences exist. Lastly, validation of DADS + DATS effects on growth, nutrient and E digestibility, and gastrointestinal function by dietary supplementation are needed before these results can be practically applie

APPENDICES

Appendix A Propagating IPEC-J2 cells[1]

Resurrecting cells

1. Thaw cells in a 37˚C water bath
2. Transfer contents into a conical tube and suspend in 10 mL of growing media
3. Pellet cells by centrifugation
4. Re-suspend cells in 20 mL of fresh growing media and plate cells
5. Do not disturb cells for 1 to 2 d.

Initial cell maintenance

1. Grow cells in growing media
2. Replace media every 2 d
3. Split cells at 90% confluence

Splitting of cells

1. Wash cells with 10 mL PBS
2. Add 10 mL of 1X trypsin for 15 min until cells become detached
3. Add an equal amount of growing media to stop trypsin reaction
4. Centrifuge and discard supernatant
5. Re-suspend pellet in growing media by gentle rocking
6. Plate cells

Maintaining cells

1. Feed cells growing media until 100% confluence
2. Change out growing media every 2 d
3. Feed cells differentiation media starting at 100% confluence
4. Change out differentiation media every 3 to 4 d
5. Conduct experiment 7 to 14 d post confluence

Preserving cells

1. Conduct steps 1,2, and 3 under splitting of cells
2. Re-suspend pellet in 20% FBS and 5% DMSO
    a. 1 ml FBS/DMSO mixture per 2×10^6 cells
    b. Mix suspension gently and aliquot to freezer vials
    c. Freeze at -80˚C overnight then move to liquid N for long-term storage

[1]All liquids should be sterile and all steps should be conducted under aseptic conditions

Appendix B IPEC-J2 cell media recipes[1]

IPEC-J2 growing media recipe

1. Glucose-free DMEM/Ham's F12 (1:1) (add 3.70 g sodium bicarbonate and adjust to pH 7.2)
2. 5% FBS
3. Penicillin/Streptomycin (100 IU/mL and 100 µg/mL, respectively)
4. 10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL Se
5. 5 ng/mL epidermal growth factor

IPEC differentiation media recipe

1. Make IPEC Growing Media minus FBS

[1]Media should be mixed and maintained under sterile conditions.

Appendix C Western Blot Protocol

Antibody used:

Rabbit Anti-ZO-1, Gene Accession number: 097758, P3944, Q07157

Protocol:

Protein Extraction

1. Prepare RIPA lysis buffer mix

1x RIPA lysis buffer	500 µL/sample
Protease inhibitor	10 µl/ml (Store at -20 ℃)
Phosphatase inhibitor 3	10 µl/ml (Store at 4 ℃)
Na_3VO_4 (100 uM)	10 µl/ml (Store at -20 ℃)
PMSF (10 mg/ml)	10 µl/ml (Store at -20 ℃)

2. Add 500 µl RIPA lysis mix to each tube

3. Homogenize tissue samples

4. Centrifuge (4 ℃) at 10,000 rpm for 10 min

5. Collect supernatant into fresh tubes and store at -80 ℃

6. Estimate protein content by BCA analysis

10% Acrylamide Gel preparation

Resolving gel (10%)	2 gel	Stack gel (3.75%)	2 gel
Di H2O	9.9 ml	Di H2O	3.64 ml
30% acrylamide	8.33 ml	30% acrylamide	626 ul
1.5 M Tris-HCL (pH=8.8)	6.3 ml	1.0 M Tris-HCL (pH=6.8)	626 ul
10% SDS	250 ul	10% SDS	50 ul
10% APS	250 ul	10% APS	50 ul
TEMED	10 ul	TEMED	5 ul

1. Make Resolving gel solution

2. Pool the gel and add 0.01% SDS on top

3. Wait for coagulation

4. Make Stack gel solution

5. Pool Stack gel solution and add comb

6. Wait for coagulation

7. Add a little water on the gel if comb was removed and store at 4 ℃

Prepare sample mix, Run Gel and Membrane transfer

1. Add protein, lysis buffer, and loading buffer to each well

2. Incubate at 95℃ for 5 minutes then store on ice

3. Prepare electrophoresis instrument and clean well

4. Load 3 μl marker and 50 μl sample mix

5. Run gel at 150V for 80 miutes

Membrane transfer, Stain

1. Prepare 1x transferring buffer

2. Prepare and soak nitrocellulose membranes and blot papers

3. After running gel, soak the gel in transferring buffer for 10 minutes
4. Transfer protein from the gel to the membrane with transfer instrument (15 V, 45 minutes)
5. Stain membrane with Ponceau for few seconds
6. Use d H2O water to wash Ponceau, then use 1x TBST to wash membrane on the swing table for 5 min for 4 times.

Western blotting

1. Blot with 5% BSA for 30 minutes
2. Blot with primary antibody overnight in the refridgerator
3. Pour back primary antibody, and wash membranes 4 times
4. Blot secondary antibody at room temperature for 1 hour
5. Pour out secondary antibody, and wash membranes 4 times
6. Add HRP substrate Luminal reagent for 5 minutes
7. Dry membrane
8. Put membrane between plastic membranes, and stabilize in Cassette
9. Expose and develop film

Washing and next Blot

1. Wash membranes 2 times
2. Add Blot stripping buffer for 30 minutes
3. Wash membranes 4 times.
4. Return to Western Blot steps.

VITA

VITA

Nathan Horn is from Brownsburg, IN. He graduated from Brownsburg High School in 2001 (Brownsburg, IN) and earned a Bachelor's degree from the College of Agriculture at Purdue University in 2005 with a major in Animal Science and minor in Biological Sciences. He continued his education at Purdue University and earned a Master's degree in Monogastric Nutrition in 2008. He worked as a research associated at JBS United, Inc. from 2008 to 2012 with a focus on nursery pig nutrition and livestock mycotoxicosis. During the winter of 2012 he returned to Purdue University and will earn a Ph.D. in Monogastric Nutrition in December of 2015. Nathan has accepted a scientist position at JBS United, Inc. (Sheridan, IN) following graduation.