DEVELOPMENT OF A WEANED PIG MODEL OF ENTEROTOXIGENIC *E.COLI*-INDUCED ENVIRONMENTAL ENTEROPATHY

A Thesis

by

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ABSTRACT

Environmental enteropathy is chronic inflammatory disease of the small intestine that hinders the childhood development in impoverished countries. As it assumes a large role in the malnutrition as well as stunting of growth in the early stages of life. This is correlated with an increased likelihood of contracting a chronic disease in adulthood. A specific culprit of the disease is unknown but it is assumed that an enteric pathogen plays a role as fecal-oral contamination and poor living conditions are the primary routes to infection. Weaned pigs experience similar symptoms to that of humans in a disease known as post weaning diarrhea. The aim of this study was to develop a model of environmental enteropathy that can be utilized for the research of new interventions. Twenty-four weaned piglets at approximately 14lbs were randomly assigned to one of three treatments. The pigs were housed 2 pigs per pen with a total of 4 pens per treatment. The treatments were as follows: 1) Control (sham challenged with PBS) 2) Acute (received one dose of ATCC 23545) and 3) Chronic (received a daily dose of ATCC 23545). After 2 d of acclimation, pigs were challenged with the respective treatment on d 1. Feed disappearance, body weight, blood and fecal samples were taken on d 0, 1, 3, and 6. Blood and fecal samples were analyzed for inflammatory markers. All pigs were necropsied on d 7 for the collection of intestinal samples for histology and determination of Enterotoxigenic *Eschericia coli* counts. The ETEC 23545 treatment had no effect on ADG, ADFI, or G:F throughout the duration of the trial ($P \geq .18$) A treatment interaction was seen on d 6 serum levels of IL-6 ($P<0.05$) but an effect was not seen in the d 6 serum levels of IL-8. The fecal calprotectin levels did not have a treatment ($P=.95$) day ($P=.48$) or treatment x day ($P=.95$) effect. However, the fecal colony counts experienced a treatment effect ($P<.0001$). The acutely challenged pigs most

closely mimicked environmental enteropathy in children with highest fecal shedding of *E.coli*, elevated colonization of small intestine and elevated levels of serum IL-6.

DEDICATION

So do not fear, for I am with you; do not be dismayed, for I am your God. I will strengthen you and help you; I will uphold you with my righteous right hand

~

Isaiah 41:10

To my twin pillars, Mama and Daddy, your strong foundation and unshakeable faith in me has made all of this possible. Thank you for lifting me up every time I was discouraged, thank you for listening to me rant about the struggles of grad school, and thank you for never giving up on me especially at times when I gave up on myself. I wouldn't be the woman I am today without your guidance and love. I couldn't have made it here without y'all so this one is for you. I love you.

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Don't wait until everything is just right. It will never be perfect. There will always be challenges, obstacles, and less-than-perfect conditions. So what? Get started now. With each step you take, you will grow stronger and stronger, more and more skilled, more and more self-confident, and more and more successful. – Mark Hansen

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All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
BW	Body Weight
cGMP	Cyclic Guanosine Monophosphate
CV	Coefficient of Variation
d	Day
EE	Environmental Enteropathy
ETEC	Enterotoxigenic *Escherichia coli*
G:F	Gain to Feed Ratio
IL-6	Interleukin-6
IL-8	Interleukin-8
PWD	Post-Weaning Diarrhea
TNF-α	Tumor Necrosis Factor-alpha

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CHAPTER I

INTRODUCTION

Environmental enteropathy (EE) or environmental enteric dysfunction (EED) is a chronic gut inflammatory condition that is caused by continuous fecal-oral contamination of gastrointestinal pathogens. These pathogens result in condensed small intestine villi, increased crypt depth and inflammation of the lamina propria thus resulting in insufficient nutrient uptake (Korpe and Petri, 2012). Poor sanitation along with malnutrition are both positively associated with the development of EE. The gut flora of malnourished children tend to be reduced to an immature state where there are copious amounts of *Enterobacteriaciae* affixed to the intestinal wall. Enterotoxigenic *Escherichia coli* (ETEC) is often the culprit for the development of EE in humans (George et al, 2015). It is the most common source of diarrhea in young children especially under the age of five (Gaastra et al, 1996) and accounts for twenty to forty percent of traveler's diarrhea in adults (Nataro et al, 1998.) The onset of ETEC is marked by sudden watery stool which leads to dehydration as well as lethargy, lasting approximately three to four days (Qadri et al, 2005). Though the disease results in the reduction of the gut microbiota, individuals affected by EE remain fairly asymptomatic (Mckay et al, 2010).

A major physiological problem associated with EE is the stunting of growth. For an individual to be considered "stunted", one's height must fall two standard deviations below the average that is set by the World Health Organization for a particular age. Stunting in developing countries occurs for three specific reasons: chronic nutrition deficiencies, persistent infections, or chronic inflammatory diseases such as EE (Owino et al, 2016). In a pooled analysis of nine separate countries, children who experienced several diarrheal episodes, an episode defined as

the onset of diarrhea to the last day of diarrhea with two consecutive days without diarrhea, before reaching six months of age rarely partook in compensatory gain as only six percent fully recovered from being stunted (Checkley et al, 2008). In 2010, stunting was globally affecting 171 million children from birth to five years of age. It is predicted that in 2020 approximately twenty-four percent of all pre-school aged (0-5 years) children residing in developing countries will suffer from growth stunting (de Onis et al, 2011). Stunting that occurs early on in life from an enteric disease increases the likelihood of an individual developing cardiovascular and metabolic diseases in adulthood (DeBoer et al, 2012).

Therapeutic treatments against EE have not been highly successful in the past. Antibiotics, such as rifaxmin, and probiotics, like *Lactobacillus* GG, have been tested as possible treatment methods for children with EE; however, neither treatments displayed improvement in intestinal barrier function (Trehan et al, 2009) (Galpin et al, 2005). Therefore, there is an increased interest to determine new methods to alter gastrointestinal pathogenic bacteria.

Using weaned pigs as a human model for EE is more applicable than using mice, as pigs' gut immune responses as well as ETEC symptoms closely mimic that of humans. Pigs and humans both release tryptase and TNF-α as a form of intestinal permeability regulation (Roura et al, 2016). In regard to symptomatic similarities, pigs inoculated with ETEC experience sloughing of intestinal villi, increased crypt depths and scours (Jones et al, 1972). The objective of this project is to develop an ETEC induced EE model that can be used to evaluate different therapies which can later be translated for the use in humans.

CHAPTER II

LITERATURE REVIEW

BACKGROUND

Background and Pathogenesis of Enterotoxigenic Escherichia coli in Humans

In the 1950s, enterotoxigenic *Escherichia coli* (ETEC) was discovered by De and colleagues through a clinical investigation trial of patients presented with Cholera like symptoms. The individuals experienced acute watery diarrhea and severe dehydration but possessed fecal samples that were negative for *Vibrio cholerae* culture (De et al, 1956). Later, a group of researchers confirmed the presence of an *Escherichia coli* that was responsible for producing an enterotoxin which causes homologous clinical symptoms to that of Cholera (Carpenter et al, 1965, Lindenbaum et al, 1965, Fleckenstein, 2013).

Enterotoxigenic *E. coli* plays a major role in the annual death toll of children under five in impoverished nations and is a common cause of traveler's diarrhea. This pathogen not only affects humans but can be detrimental to neonatal livestock, especially in swine, bovine, and ovine species. Enterotoxigenic *E. coli* separates itself from the other classes of diarrheagenic *E.coli* with the ability to produce enterotoxins and possessing colonization factors (Qadri et al, 2005). The enterotoxins are classified as heat-labile (LT) or heat-stable (ST), either one or both can be produced by ETEC. Enterotoxin STa is responsible for disease in both humans and animals while STb is primarily associated with causing scours in piglets. The ST enterotoxin induces diarrhea in its host by first binding to the extracellular guanylyl cyclase C, found on the intestinal brush border. This allows for accumulation of cyclic GMP (cGMP) and the activation of the cGMP dependent protein kinase. The activation leads to phosphorylation of the cystic fibrosis transmembrane regulator (CFTR) and the hindered absorption of NaCl. This generates a

net loss of water through diarrhea (Fleckenstein et al, 2010). Similar to the ST enterotoxin, the LT activates the CFTR through a chain reaction which leads to an increased secretion of water and electrolytes thus causing diarrhea in the host. However, LT will activate CFTR through the inhibition of Gsα GTPase and activation of adenylate cyclase (Fleckenstein et al, 2010). The LT enterotoxin is also broken down into two subtypes, LT-I which occurs in both human and animal disease and LT-II which is only traceable through animal diseases (Figure A1). Thus, ETEC strains that are the source for human disease will produce either STa, LT-I or both STa and LT-I (Sears and Kaper, 1996). In order for ETEC to infect the host, it must first colonize the small intestine via colonization factors. These factors are divided into four different morphological types: fimbrial also known as pilus, fibrillar, helical, and afimbrial (Madhavan and Sakellaris, 2015). CFA/1 and CS1 fimbriae are frequently found in human ETEC. The structure of these two factors is made up of multiple copies of an identical structural subunit protein which is responsible for immunological characteristics of the factor (Gaastra and Svennerholm, 1996). The factors play an important role in the epidemiology of the strain, as strains that lack the fimbriae adhesions have lost the ability to induce diarrhea in the host (Satterwhite et al, 1978).

A host will begin to experience symptoms of ETEC with in one to three days after exposure to the bacteria. Typically, contact with ETEC will occur via contaminated food or water with the ultimate source being human or animal feces. Patients who become infected will exhibit watery diarrhea and abdominal cramping for approximately three to four days. Other symptoms that may occur but are less likely are: fever, nausea with or without vomiting, and headaches. Recovery is normally seen without the use of antibiotics or hospitalization (CDC, 2014). However, ETEC can have a severe impact on the immunocompromised, especially children under the age of five.

Transmission of ETEC can occur through several pathways of fecal-oral contamination. ETEC has been discovered in drinking and environmental water in developing countries. The contamination of water can occur through defecation of livestock and wildlife into irrigation water, rivers, and other water sources. Though the immersion of the pathogen in water halts the growth and division stages, the bacteria can still remain viable for long periods of time which is a threat for the spread of disease (Gonzales-Siles and Sjöling, 2016). Lothigius et al. (2010) discovered at the end of a three month incubation period, clinical ETEC strains in water samples had bacterial cells remaining. The genes responsible in the metabolic pathways were still intact and toxin transcription was detected in all of the strains after the three month period. Black et al. (1982) showed a correlation between increased temperatures and frequency of *E. coli* infection in the water. Thus, water doubles as a reservoir and transmission route for ETEC. Another route of transmission is through contaminated food. Historically, 70% of ETEC outbreaks in the United States that have been reported to the Centers for Disease Control (CDC), were foodborne cases (Mead et al, 1999). In 2006, 55% of ETEC episodes in the United States were travel-associated (Scallan et al, 2011). The high incidence of ETEC in young children living in developing countries could be attributed to contamination of weaning foods. In a study of rural Bangledesh, 41% of weaned food samples were infected with *E.coli* and 49% of the milk samples were marked positive for *E.coli* (Black et al, 1982). Barrell and Rowland, 1979 found that the contamination of weaning foods in The Gambia significantly increased with the time elapsed after preparation. As 34.9% of food samples contained *E.coli* one hour after preparation whereas eight hours after preparation, 84.6% of samples contained *E.coli*.

Background and Pathogenesis of Environmental Enteropathy

Environmental enteropathy (EE) was first discovered in the 1960s when various studies reported similar morphological changes in the jejunum of adults living in tropical locations. Thus, the disease adopted the name of tropical enteropathy (Cook et al, 1969, Korpe and Petri 2012). Further research revealed that the etiology of tropical enteropathy is not based upon geographical location but rather environmental conditions as the condition was absent in tropical areas with high socio-economic status. Therefore, the disease has been renamed to environmental enteropathy (Menzies et al, 1999, Prendergast et al, 2012).

The onset of EE is thought to occur in people who live in poor sanitary conditions and are chronically exposed to fecal-oral contamination. This chronic exposure leads to dramatic morphological changes in the gut barrier, typically in the duodenum and jejunum of the small intestine. In a normal intestine, finger-like villi line the intestinal wall and are protected from luminal contents by a layer of mucus (Figure A2). The changes that occur to the intestinal barrier include: blunting of the villi, increased crypt depth, and lymphocytic infiltration of the lamina propria. Invasion of the lamina propria begins with the attack of the tight junctions which are formed between epithelial cells, creating a protective barrier. Once this barrier is broken down, the luminal contents can leak into the lamina propria (Su et al, 2009). These transformations decrease the surface area available for nutrient absorption thus leading to malabsorption. Along with malabsorption, the impaired function allows for bacterial or lipopolysaccharide translocation across the gut barrier and into the bloodstream. Unlike other gastrointestinal disorders, affected individuals remain fairly asymptomatic. (Korpe at al, 2012, Trehan et al, 2016, Watanabe and Petri, 2016). This led researchers to believe that EE was minute in terms of global health issues especially since it could be found in assumedly healthy

individuals. It was not until recently that EE has been recognized as a key player in the stunting syndrome as well as playing a role in impaired cognitive function.

Biomarkers of Environmental Enteropathy

A current disadvantage in the diagnosis of EE is the lack of a definitive biomarker that indicates early intestinal dysfunction. Though small bowel biopsy is a possibility, the invasiveness and expense of this procedure make it unlikely to become an accepted practice for testing children who do not appear to be clinically ill. In order to prevent damage from EE, it is imperative to be able to identify it early in life. Therefore, a biomarker or series of biomarkers need to establish the absorptive function, mucosal permeability, inflammation, and immune activation of an individual's gastrointestinal tract (Table A1, Keusch et al, 2014).

Fecal markers for inflammation and gastrointestinal permeability include neopterin, myeloperoxidase, and calprotectin. Neopterin is a marker for the TH1 immune activation and has been used in the diagnosis of celiac disease, which resembles EE in a clinical and histopathologic manner (Fuchs et al, 1983, Kosek et al, 2013). Myeloperoxidase is a marker for neutrophil activity and has been associated with inflammatory bowel disease. There is an argument that it is a more suitable biomarker than calprotectin because it is not elevated in the fecal samples of breastfed children or neonates who are less than four years of age (Dorosko et al, 2008, Kosek et al, 2013, Syed et al, 2016). Guerrant et al, 2016 found a positive correlation between increased myeloperoxidase levels and decreased subsequent growth. In addition, combined observation of myeloperoxidase and neopterin levels were correlated with the poorest growth outcome. In a study designed to link geophagy, stunting, and EE, it was observed that increased EE scores based upon three biomarkers: alpha-1-antitrypsin, myeloperoxidase, and neopterin, were significantly associated with increased concentrations of myeloperoxidase and neopterin (George

et al, 2015). Like myeloperoxidase, calprotectin is associated with neutrophils as it is a calcium and zinc binding protein that is produced by neutrophils and monocytes. The secretion of calprotectin is done extracellularly from stimulated neutrophils and monocytes or is released by cell disruption/death (Syed et al, 2016). High concentrations of calprotectin and incidence of inflammatory bowel disease have been positively correlated; fecal calprotectin tests have shown to associate with clinical symtpoms of ulcerative colitis (D'Haens et al, 2012, Keusch et al, 2014). An advantage to using calprotectin as a biomarker is it remains stable in fecal samples that are kept at room temperature for up to seven days and is resistant to heat and enzymatic degradation in the gut (Prata et al, 2016). In the first animal model of EE, mice who were both malnourished and inoculated with BG (bacteroidales and *E. coli*) exhibited concentrations of calprotectin that were two and half times greater than the control and malnourished mice (Brown et al, 2015).

A common biomarker that is utilized in the field for testing EE is the lactulose:mannitol urinary test. This dual sugar absorption test is used to analyze gut barrier integrity and mucosal function. Lactulose is a non-digestible disaccharide that is too large to cross through a healthy mucosa. Mannitol is a small monosaccharide for which uptake is dependent upon the small bowel absorptive capacity. The excretion of the two sugars will result in the L:M ratio. This ratio is derived from the urinary excretion that occurs five hours post consumption of the test solution. Individuals who are experiencing intestinal permeability will have increased levels which occur from having high lactulose levels paired with low mannitol levels (Campbell et al, 2002, Keusch et al, 2014).

Stunting Syndrome and Environmental Enteropathy

Stunting is defined as falling two standard deviations below the World Health Organization (WHO) child growth standards (Weise, 2014). Globally, stunting affected 155 million children under the age of five with the highest prevalence of stunting in the WHO African region (WHO,2017) Long-term effects are associated with stunting such as: decreased cognitive function and physical development, reduction of reproductive abilities, and increased risk of degenerative diseases (WHO/Weise, 2014). The relationship of EE, the stunting syndrome and other outside factors results in a cyclic cycle (Figure A3).

Neurodevelopment is essential for infants during their first six months of life. During this time, infants will also experience maximal growth velocity. Meanwhile, the growth period from six to twenty-four months of age is one of the most critical periods for linear growth but consequently is the peak time for children to be stunted (Prendergast and Humphrey, 2014). In a cohort of data from Brazil, Guatemala, India, the Philippines and South Africa, stunting at twenty-four months of age was associated with a 16% likelihood of failing at least one grade in school and children had an estimated reduction of .9 years of school time (Martorell et al, 2010). In the past, researchers have labeled chronic diarrheal episodes as the underlying cause of growth stunting in children. Recent developments have suggested that environmental enteropathy may be a main causative agent in stunting syndrome. An observation of Gambian children indicated that growth faltering was not a result of diarrheal episodes but rather intestinal dysfunction as marked by the lactulose to mannitol ratio. The ratio accounted for 42.9% of linear growth failure and 38.8% of decreased weight gain. The prevalence of diarrhea throughout the study was only 7.3% but growth-depressing permeability values were 76% of the time. Therefore, suggesting that stunting in children is attributed more to intestinal damage rather than incidence of diarrhea (Lunn et al, 1991).

Enterotoxigenic Escherichia coli and Its Role in Post Weaning Diarrhea

Post weaning diarrhea (PWD) has continually been an issue in the pork industry as it is responsible for economic losses due to decreased growth rate, mortality, and medication expenses. Piglets are most susceptible to PWD during the first two weeks after weaning as their immune system is functioning at a low capacity. This is a result of the piglet undergoing several stress factors such as removal from the sow, social and environmental changes, dietary changes as well as morphological changes of the gastrointestinal tract. The most common cause of PWD is ETEC (Rhouma et al, 2017).

ETEC strains that possess the fimbrial adhesins F4 (K88) or F18 have traditionally posed the greatest threats to weaning pigs. Of 219 isolates from four to eight week old piglets with PWD, Frydendahl (2002) discovered 44.7% to be of the F4 origin and 39.3% to belong to F18. The F4 fimbriae occur as F4ab, F4ac, or F4ad, with the F4ac being the most prevalent type. The adhesion of F4 strains is possible through F4 receptor on the intestinal epithelial cells. Adherence via F4 is species specific, being mainly in swine. PWD ETEC of the F4 type will typically contain the STb and LT enterotoxins (Taillon et al, 2008). Some pigs can be genetically resistant to colonization by F4-positive strains, as they do not have the genes capable of making the receptor. In pigs that do not lack the receptor, colonization will occur through the length of jejunum and ileum. The F18 fimbriae come in two different types, F18ab and F18ac. The F18 strains rarely will produce LT enterotoxins rather they produce STa or STb. F18 strains are only found in pigs older than three weeks and are not know to induce disease in neonatal pigs. As in the F4 strains, pigs can be resistant to the colonization of F18 ETEC (Fairbrother et al, 2005).

Pigs with PWD will have an episode of watery diarrhea that can last up to five days. In some cases, pigs will die suddenly without displaying signs of PWD. The pathways by which the

enterotoxins induce diarrhea in the pig are the same as described for human ETEC, as described above.

Advantages of a Swine Model

Murine models have been responsible for the advancement of human knowledge in many areas of research. There are numerous reasons why the mouse has been selected as a prime research subject including: the small stature, ease of handling, reproductive efficiency, and low requirement of food and housing space. However, mice lack several essential similarities to humans.

Pigs are similar to humans in the areas of anatomy, physiology, and genetics. The most rudimentary similarities between the two is the fact that both are monogastrics and true omnivores. However, swine differ from humans in the physical structure of their small intestine by having the ratio of 1:8:1 duodenum, jejunum, and ileum, respectively. Humans have shorter duodenums with iliea that tend to be slightly longer or be a similar length to the jejunum (Swindle et al, 2012). Additionally, the pig's colon has an arrangement of centrifugal and centripetal coils which distinguishes itself from the human colon (Meurens et al, 2012). Even though swine exhibit slight anatomical differences from the human gastrointestinal tract, their digestive physiology is highly similar. Pigs have been proven to be similar in metabolic functions of the gastrointestinal tract, intestinal transport time, ion transport as well as motility and neonatal development of the gastrointestinal tract. Studies have been conducted in each of these areas by using the pig as a research model (Swindle et al, 2000).

The close relationship that the pig and human immune systems share has allowed for the development of models for human infectious disease. Through analysis of the human, mouse, and pig immunological associated genes, it was discovered that the frequency of orthologous

genes between pigs and humans is 80%, much higher than the 6% between mice and humans (Dawson, 2011). Consequently, pigs are increasingly being utilized to conduct human infectious disease research.

Pigs have been used as human models for several gastrointestinal pathogens including *Helicobacter pylori*, Rotavirus, *Cryptosporidium parvum* and *Shigella dysenteriae*. Rotavirus, like ETEC, induces diarrhea in young children. A gnotobiotic pig model has been used to study the pathogenesis of the disease as well as study vaccine efficacy. Pigs are the only animal model that has been shown to be susceptible to human rotavirus diarrhea. Neonatal pigs not only experience the same symptoms as an infant but also are immunocompetent at birth but remain immunologically immature, like human neonates (Saif et al, 1996, Meurens et al, 2012). Jeong et al, 2010 developed a successful human model of Shigellosis. Pigs that were inoculated with the pathogen experienced comparable symptoms to humans such as watery diarrhea, dehydration and anorexia coupled with increased cytokine-producing cells expressing IL-8 and IL-12. Though using swine as a research subject can increase the cost of the project in terms of feed, housing, and cost of the animal, the undeniable similarities between humans and pigs makes pigs the superior choice in studying gastrointestinal physiology and disease.

CHAPTER III

DEVELOPMENT OF A WEANED PIG MODEL OF ENTEROTOXIGENIC *E.COLI*-INDUCED ENVIRONMENTAL ENTEROPATHY

OBJECTIVES

The objectives of this study were to develop a weaned pig model that best represents the environmental enteropathy condition seen in children of developing countries.

MATERIALS AND METHODS

Animals and Facilities

All procedures involving animals and their care were approved and monitored by the Animal Care and Use Committee of the Southern Plains Agricultural Research Center location of the United States Department of Agriculture, Agricultural Research Service. A group of 24 weaned barrows (Landrace X Large White;; initial BW 6.35 kg) were used in this study. The pigs were housed at Southern Plains Agricultural Research Center in College Station, TX. Pigs were randomly assigned to pens with 2 barrows per pen. Each pen had solid concrete flooring and was equipped with a nipple waterer, rubber mat, and feeder. Pens were 4.634 m^2. Pigs were provided ab libitum access to feed and water. The diet was a corn-soybean meal based pelleted diet (Table B1) formulated to meet or exceed NRC (2012) recommended requirements of nutrients. The pigs were held 2 d prior to the start of treatment in order to be prescreened for signs of entertoxigenic *Escherichia coli* (ETEC). The pigs were then randomly allotted to 1 of 3

treatments: Non-challenged control, acute challenge (single dose of ~10^9 CFU), and chronic challenge (daily dose of ~10^7 CFU). The bacterial challenge strain was a nalidixic acid resistant derivative of *E. coli* strain ATCC 23545 (O138:K81(B):H14, STII+, Stx2+) obtained from the American Type Culture Collection. Bacteria were cultured overnight (16-18 h) in trypticase soy broth medium (TSB, Difco) at 37 ºC with aeration. There were a total of 4 pens per treatment. All pigs were housed in the same barn; however, the control pigs were housed separate from the challenged pigs in order to prevent cross-contamination. The acute treatment received one dose on d 1 and the chronic treatment received a daily dosage starting on d 1 until the termination of the trial. Pigs along with feeders were weighed on d 0, 1, 3, and 6 for calculation of average daily gain (ADG), average daily feed intake (ADFI), and gain to feed (G:F). The d 1 collection of growth, fecal, and blood data was approximately 12 hrs after the initial dose was administered. All animals were necropsied on d 7 for collection of intestinal scrapings and samples. The intestinal scrapings were used for analysis of *E.coli* colonization as well as determination of villi blunting.

Virulence Gene Screen via PCR

A pre-screen was conducted prior to the commencement of the trial in order to determine any pre-existing *E.coli* colonization. On d -1, approximately 1g of feces was collected from each pen and mixed with 9 ml phosphate buffered saline (PBS, Hyclone). The samples were vortexed until homogenous then serially diluted and plated to MacConkey agar plates (Difco). After an overnight incubation, 3 colonies were selected from each pen and mixed with 150 µl TE buffer. Each sample was boiled for 10 minutes then centrifuged at 8,000 x g for 2 minutes. The supernatant was transferred into a new tube and used as a template for PCR. The PCR conditions were adapted from Fagan et al, 1999 and Osek, 2001 using the uspA, heat labile toxin, and Shiga

toxin 1 and 2 primers as described. The resulting PCR product was electrophoresed on a 1.5% agarose gel and visualized with gel red (Biotium). Polymerase chain reaction products resulting in appropriate sized bands were analyzed by PCR a second time with individual primer sets in order to confirm the presence of the virulence gene.

Fecal Collection and Determination of Enterotoxigenic Shedding in Feces

A representative fecal sample was taken from each pen on d 1, 3, and 6 in order to determine *E.coli* counts. The samples were collected in sterile 50 ml conical tubes (Falcon, BD) and transported on ice. The fecal samples collected on d 1, 3, and 6 were processed and diluted in the same manner as described above, with 20 ul spots plated to MacConkey agar amended with 50ug/ml nalidixic acid. The d 1, 3, and 6 spotted plates (20ul) were incubated for only 10-12hrs at 37°C to avoid colonies merging within the spots. The plates were taken out after the allotted time and assessed for determination of colony growth as well as count. Fecal calprotectin was determined by a commercially available porcine ELISA kit (MyBioSource, San Diego, CA). The minimum detection of the kit is 6.25 ng/ml with an intra-assay CV of less than 15%.

Blood Sampling and Serum Analysis

Blood samples were collected from 2 pigs per pen during 1 sampling period (d 6 of the study). Blood was collected from the cranial vena cava using a 38 mm 20 gauge needle and a 10 mL serum vacutainer tube (BD, with clot activator and gel for serum separation). After collection, blood samples were inverted and allowed a minimum of 30 minutes to clot. Samples were centrifuged at 1,600 × g for 10 min at 2 ˚C. The separated serum samples were stored at -80˚C in 5 mL polypropylene tubes until analysis was performed. Serum concentration of interleukin 6 (IL-6) and interleukin 8 (IL-8) were determined by commercially available porcine

ELISA kits (R&D Systems, Minneapolis, MN). The minimum detection for IL-6 is 18.8 pg/ml with an intra-assay CV of 4.0%. The minimum detection for IL-8 is 62.5 pg/ml with an intra-assay CV of 8.3%. All analyses were conducted as outlined in the protocols provided by the manufacturer.

Intestinal Sampling and Histology

Pigs were anesthetized with 2.5 ml dose of a mixture of ketamine, Rompum, and Telazole. This was injected into the ham via a syringe with a 1 inch 18 gauge needle. Pigs were then injected intracardially with 12ml of euthanasia solution (Euthasol at 86 mg/kg pentobarbital) via a syringe with a 1.5 inch 18 gauge needle.

The animals were necropsied to take samples for *E.coli* counts and histology in intestinal sections from the duodenum, jejunum, and ileum, cecum and colon. The small intestine was sectioned into the three respective sections via zip ties in order to prevent cross contamination between sections. The distal end of each section was utilized for bacterial counts histology. Cecum and colon contents were collected along with scrapings from the duodenum, jejunum, ileum, cecum and colon. The scraping samples were placed into sterile 15 ml conical tubes (Falcon, BD) tubes with 2mm beads, 8 ml of PBS was added for homogenization. Scraping samples were vortexed for 5 minutes at 3000 rpm. Content samples were placed in 50 ml concial tubes with 25 ml PBS. Each was vortexed for 15 seconds at 3000 rpm. After vortexing, a sample homogenates were serially diluted and spot-plated to MacConkey agar + 50 µg/ml nalidixic acid as described above.

For histology, the distal end of each section was used. Samples were positioned onto a 5.08 cm x 5.08 cm cardboard sections and secured with small clips to prevent tissue curling. Consecutive tissue samples were fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10%

glacial acetic acid) at a 20:1 ratio for 30-45 minutes and 10% neutral buffered formalin (VWR, catalog # 89370-094) at a 10:1 ratio for 24 hours, followed by storage in 70% EtOH until further processing. Tissues were trimmed into longitudinal sections of 5 mm width and embedded into paraffin using standard procedures. After processing, 4 µm sections were placed on slides and stained with hematoxylin and eosin. The slides were analyzed by a board certified pathologist for rod attachment. A total of 12 pigs, 4 from each treatment, were randomly selected for the evaluation of small intestine villi length.

Statistical Analysis

Growth performance along with cytokine and intestinal bacterial counts data were analyzed using the PROC MIXED procedure in SAS 9.3 (SAS Inst. Inc., Cary, NC). The model fixed effect was treatment with pen set as a random effect for growth performance, cytokine and intestinal bacterial count data. Fecal samples were collected on a pen basis, therefore, pen was not included as a random effect. Calprotectin levels and fecal colony counts were analyzed as a repeated measures using the PROC GLIMMIX procedure. Treatment, day, and treatment × day served as fixed effects. Day of collection also served as the repeated measure with pen as the subject. Results were considered significant at $P \leq 0.05$ and marginally significant between $P > 0.05$ and $P \leq 0.10$.

RESULTS

Growth Performance

Overall ADG, ADFI, and G:F of pigs challenged with ETEC were not different (P > 0.184; Table B2).

Serum Cytokine Expression

On d 6, pigs challenged with ETEC had increased ($P < 0.05$) concentrations of serum IL-6 compared to the control pigs (Table B3). However, there was no difference between the pigs given acute and chronic treatments. As for IL-8, there was a marginally significant overall treatment effect ($P = 0.089$). Pigs that received a chronic dose of ETEC had increased ($P < 0.05$) concentrations of IL-8 in the serum compared to control pigs, with those receiving the acute dose being intermediate.

Fecal Shedding of ETEC 23545 and Calprotectin Concentration

All pigs were determined to be healthy and uninfected by ETEC at the start of the trial through PCR quantification. There was no treatment × day interaction ($P > 0.575$) or day main effect ($P > 0.768$) for fecal colony counts (Table B4). The acute and chronic treatments had higher prevalence of ETEC in feces when compared to fecal samples from control pigs on all sampling days. However, there was no difference in fecal ETEC between fecal samples from pigs administered the acute and chronic treatments. There were no treatment x day interactions or an ETEC challenge or day main effect for fecal calprotectin concentration (Table B5).

Intestinal Scrapings and Content ETEC Colonization

The ability of the strain to colonize the GIT of inoculated piglets was determined by plate counts of the homogenized intestinal sample or intestinal content. The duodenum of pigs administered the chronic and acute treatments had increased ($P = 0.093$) colonization ($\log_{10}$ CFU per gram) ETEC compared to pigs from the control group. There were no differences in the jejunum and ileum ($P > 0.114$). The ingesta located in the cecum ($P = 0.004$) and colon ($P < 0.001$) in the dosed animals were heavily populated by ETEC when compared to the control group. However, the intestinal scrapings of both the cecum ($P = 0.114$) and colon ($P = 0.110$) displayed

that the attachment of ETEC rods was not different across the three treatments. (Table B6).

Villi Length

Inoculated pigs' villi did not differ in length from the control pigs for the duodenum (P=.7125), jejunum (P=.3719), and ileum (P=.7783) (Table B7).

DISCUSSION

Environmental enteropathy is a subclinical disorder marked by a chronic inflammatory state of the small intestine. This is denoted by histological changes such as reduced villi length with flattened mucosa and crypt hyperplasia (Trehan et al, 2016). Though histological abnormalities occur, EE rarely manifests symptoms in the host like diarrhea (Korpe et al, 2012). The etiology of EE is poorly defined, but is hypothesized to result from a chronic exposure to fecal pathogens due to an unhygienic environment (Watanabe and Petri, 2016). The increased global health interest in EE is derived from EE being a contributing factor to both cognitive and physical stunting, poor response to oral vaccines, and childhood mortality (Trehan et al, 2016).

It has been shown that ETEC infections can be manifested in a weanling pig by a single dose of approximately 10^9 CFU of pathogenic *E.coli* (Berberov et al, 2004, Wellock et al, 2008), though this treatment most likely does not accurately represent the EE infection seen in humans as it is typically due to chronic exposure to the pathogen.

Pigs administered the acute and chronic dose of the ATCC 23545 strain had a 54.68% and 14.93% reduction in ADG compared to the control pigs. The control group had the least ADFI among the three treatments with acute and chronic having an increase of feed intake by 17.27% and 29.95%. These findings are in agreement with previous data that showed a 24% decrease in

the control pigs compared to the pigs inoculated with ETEC 0149 on d 3 to d 6. (Wellock et al, 2008). Madec et al. (2000) had similar results with a decrease in weight of pigs inoculated with strains possessing K88 fimbriae, from day 0 to day 2 then recovered by day 9 of the trial. The acute group had the poorest mean gain: feed conversion with the control group having the highest mean feed efficiency. Growth faltering has been associated with persistent enteric infection even without the presence of diarrhea (Lunn et al, 1991). Children with EE are known to be stunted in both height and weight when compared to global averages (Korpe et al, 2012, Keusch et al, 2014). This stunting is product of impaired gut function as nutrients cannot be properly absorbed by the damaged villi and infiltrated lamina propria (Trehan et al, 2016)

Inflammation induced by infection is mediated by increased levels of pro-inflammatory cytokines. Interleukin-8 is known to be upregulated in patients with *Helicobacter pylori* induced chronic gastritis (Crabtree et al,1993) It possesses chemotactic factor and is expressed in the enterocytes and macrophages where it attracts other inflammatory factors (Stadnyk, 1994 and Jung et al, 1995). Interleukin-6 is produced by several cell types including macrophages, endothelial cells, β cells and mast cells. It plays a critical role in the host response to inflammation and infection (Akira et al, 1993). Elevated levels of serum IL-6 have been induced by Gram-negative sepsis along with being positively correlated with inflammatory bowel disease patients (Hack et al, 1989 and Stevens et al, 1992). It has been shown that IL-6 can be used as a serum marker for subclinical viral and bacterial infections in pigs (Fossum, 1998). The IL-6 levels were lower in our control pigs compared to the inoculated groups. This is similar to the response seen by Fossum et al. (1998), pigs inoculated with *Actinobacillus pleuropneumoniae* without antibiotic treatment; however, their peak response was on day 4 where we continued to see a response on day 6. There were numerical differences in the IL-8 levels with only the

control and chronic treatments being statistically different. In contrast to our findings, Bosi et al. (2004) and Daudelin et al. (2011) found higher intestinal IL-8 levels in pigs given a challenge of enterotoxigenic *E.coli* K88. Though, Lee et al. (2016) discovered that the serum IL-8 levels peaked between day 0 and 2 then declined between days 2 and 7. This could explain the lack of elevated levels of circulating IL-8 in our challenged groups as we only measured the levels in the day 6 serum. Jiang et al (2014) associated elevated serum IL-6 levels with poor motor scores in infants with febrile disease, from birth to 24 months, living in poverty in Bangladesh. This suggests that heightened serum IL-6 levels has opposing effects on neurodevelopment, thus making it a useful biomarker for EE as studies have linked EE to poor neurodevelopment and vaccine failure (Guerrant et al, 2013, Korpe and Petri, 2012).

The presence of fecal enteropathogens in non-diarrheal stool samples has been associated with EE. (Platts-Mills et al, 2015). Though there was no response to day or treatment × day, there was a treatment response to fecal shedding of ETEC. A numerical peak in ETEC fecal shedding was seen at d 1 for the acute group and at d 3 for the chronic group. The acute treatment is consistent with other studies which observed peak shedding between 24 hours to 2 days post-inoculation (Owusu-Asiedu et al, 2003 and Jones et al, 2001). The difference in the mean peak shedding points between the groups could be explained by the dose each group received, as the acute had a higher infectious dose at approximately 10^9 CFU/ml. We projected that the acute treatment pigs' fecal counts would decline through the progression of the project with the lowest shedding point to be on day 6. In spite of that, the acute group mean shedding decreased from day 1 to day 3, but had increased *E.coli* counts from day 3 to day 6. This is likely a reflection of enterotoxigenic *E.coli* ability to be shed and cause a reinfection of the host. An

altered fecal microbiome composition is an indicator of enteric disease which is hypothesized to cause EE (Keusch et al, 2014).

The assessment of endoscopic biopsies is the optimal choice to assess EE; however, it is an invasive measure and is not applicable for research in impoverished countries. Thus, there is a need to determine a fecal biomarker or biomarkers that positively correlate with EE (George et al, 2015). Increased concentrations of fecal calprotectin have been positively correlated with the histological activity of inflammatory bowel disease in humans (Roseth et al, 1997). As fecal calprotectin is an adequate marker of neutrophilic intestinal inflammation (Costa et al, 2003). Brown et al. (2015) correlated mice induced with a cocktail of commensal *Bacteroidales* species and *Escherichia coli* with heightened fecal calprotectin levels in an EE model. Past studies have investigated calprotectin in the swine plasma, luminal intestine, and jejunal mucosa. All of which were found to be correlated with bacterial infection (Splichal et al, 2005 and Xiao et al, 2014). Lalles and Fagerhol (2001) conducted a study in order to determine the reference values for swine calprotectin. They concluded that newborn pigs at d 0 had lower calprotectin levels compared to that of human newborns with a mean concentration of 24 mg/kg. Whereas unweaned, 28 d old pigs were determined to have a mean calprotectin concentration of 21 mg/kg. This is the first study to our knowledge to test fecal calprotectin in pigs inoculated with *E.coli*. Pigs administered acute or chronic doses of *E. coli* did not differ in fecal calprotectin concentrations compared to the control pigs. In an investigation of exposure to enteric pathogens via geophagy, EE, and stunting, care-giver geophagy was associated with children possessing higher EE scores, combination of fecal myeloperoxidase, alpha-1-antitrypsin, and neopterin, and fecal calprotectin concentrations (George et al, 2015). Prata et al (2016) discovered a strong correlation between fecal calprotectin, lactoferrin, and myeloperoxidase concentrations and their

ability to be linked to malnutrition in children. Kosek et al (2012) combined three biomarkers, fecal myeloperoxidase, alpha-1-antitrypsin, and neopterin, into an EE disease activity score. This score was able to account for greater growth deficit than any marker alone. It would be beneficial for future animal models to develop an EE disease score in order to correlate impaired growth with intestinal inflammation.

Animal models are imperative for the advancement of knowledge in human disease as well as the development of interventions. Rodent models have often been used to gather information on biological functions, however these models are at a disadvantage when it comes to accurately representing human diseases and syndromes (Walters et al, 2011). Swine more accurately resemble humans in regard to anatomy, genetics, and physiology, making them a more appropriate model for human biomedical research (Bendixen et al, 2010). As there are already existing swine models in the fields of heart physiology, reproductive function, skin physiology, transplantation, gut physiology and nutrition, brain function, biomechanical models, tissue engineering, respiratory function and infectious disease models (Lunney, 2007). We believed it would be advantageous to develop a swine model for EE since the pigs' gastrointestinal tract is not only similar in structure, but also comparable to humans when it comes to nutrient absorption, the intestinal microbiome, and intestinal digestive enzymes/secretory proteins (Patterson et al, 2008 and Zhang et al, 2013). These considerations make the pig a relevant model to gain knowledge on EE in humans.

CHAPTER IV

CONCLUSION

Based upon observations, the weaned piglets acutely challenged with an STII+, Stx2+ *E. coli* most closely mimicked environmental enteropathy in children. The acute treatment had the poorest mean growth performance paired with the highest fecal shedding of *E.coli* and elevated colonization of the jejunum, ileum, and colon, as well as elevated levels of serum IL6. However, fecal calprotectin measurements and histological examination of intestinal sections from challenged animals did not demonstrate characteristics previously associated with EE in humans. Further study is required do develop an infection model that reflects all aspects of human EE in swine.

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APPENDIX A

CHAPTER II FIGURES

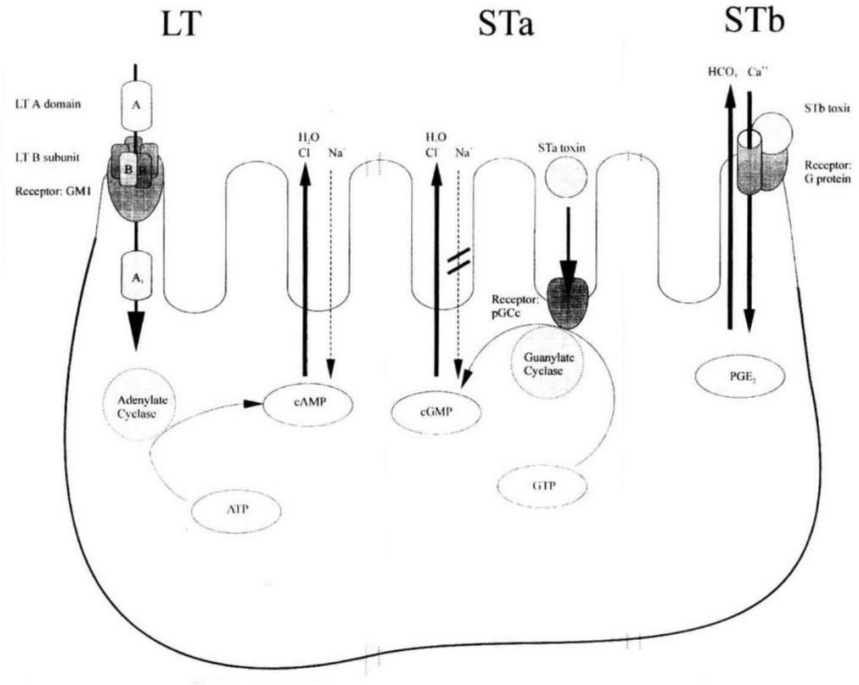

Figure A1. A schematic representation of mechanisms of action of heat-labile (LT) and heat-stable (STa and STb) enterotoxins of enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Figure was reprinted(Nagy and Fekete, 1999).

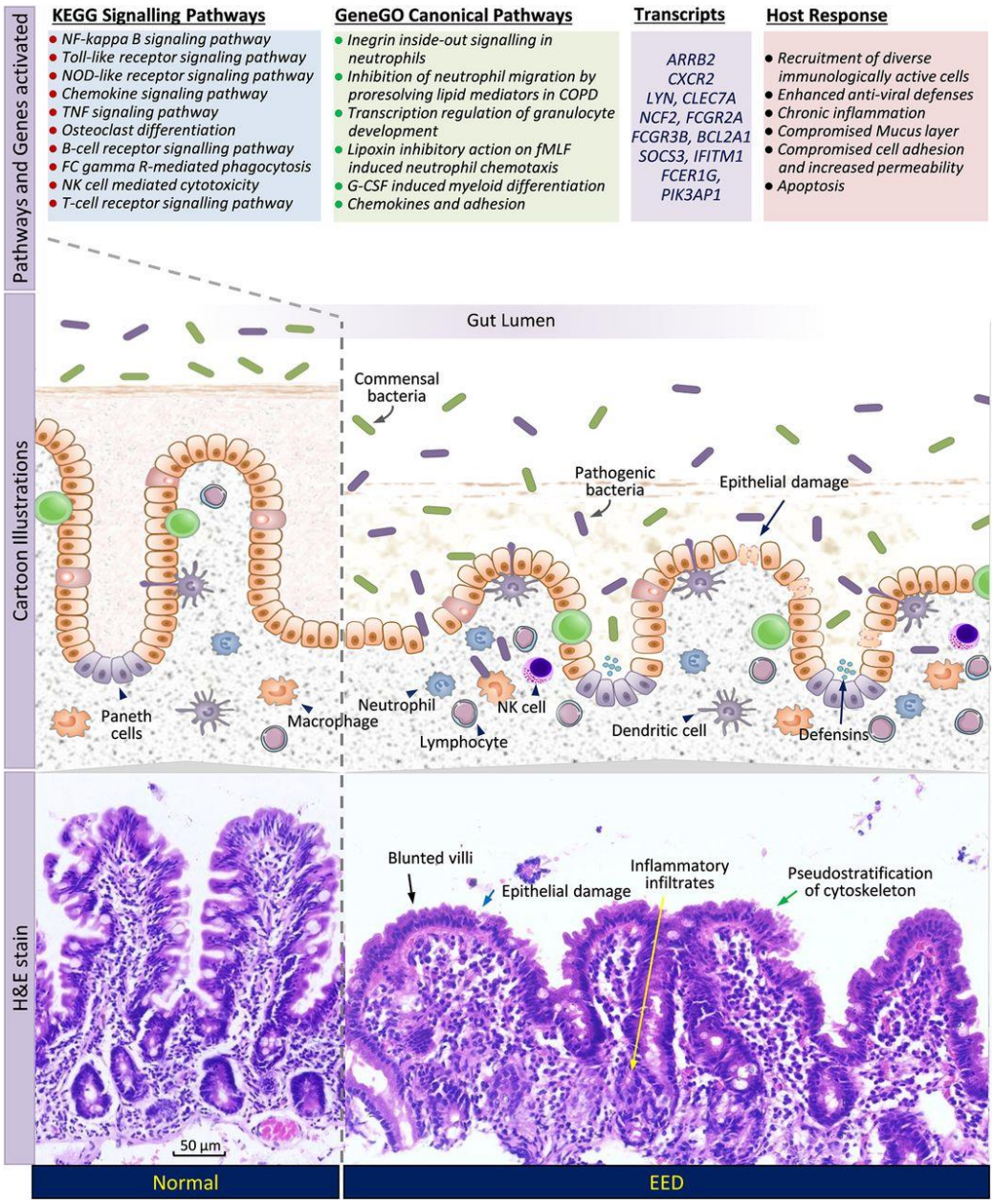

Figure A2. List of pathways, transcripts and host responses activated in environmental enteric dysfunction, juxtaposed with a schematic diagram of cellular interactions and histological photographs of damaged intestinal mucosa. Figure was reprinted (Trehan et al, 2016).

Biomarkers to Assess Environmental Enteric Dysfunction

Category	**Biomarkers**
1. Intestinal absorption and mucosal permeability	D-xylose, mannitol, or rhamnose absorption; lactulose paracellular uptake; α1-anti-trypsin leakage into gut lumen
2. Enterocyte mass and function	Plasma citrulline and/or conversion of alanyl-glutamine to citrulline, lactose tolerance test (as a marker of brush border damage)
3. Inflammation	Plasma cytokines, stool calprotectin, myeloperoxidase, or lactoferrin
4. Microbial translocation and immune activation	Stool neopterin, plasma LPS core antibody and/or LPS binding protein, circulating soluble CD14

Abbreviation: LPS, lipopolysaccharide.

Table A1. Biomarkers to assess environmental enteric dysfunction. Table was reprinted (Keusch et al, 2014).

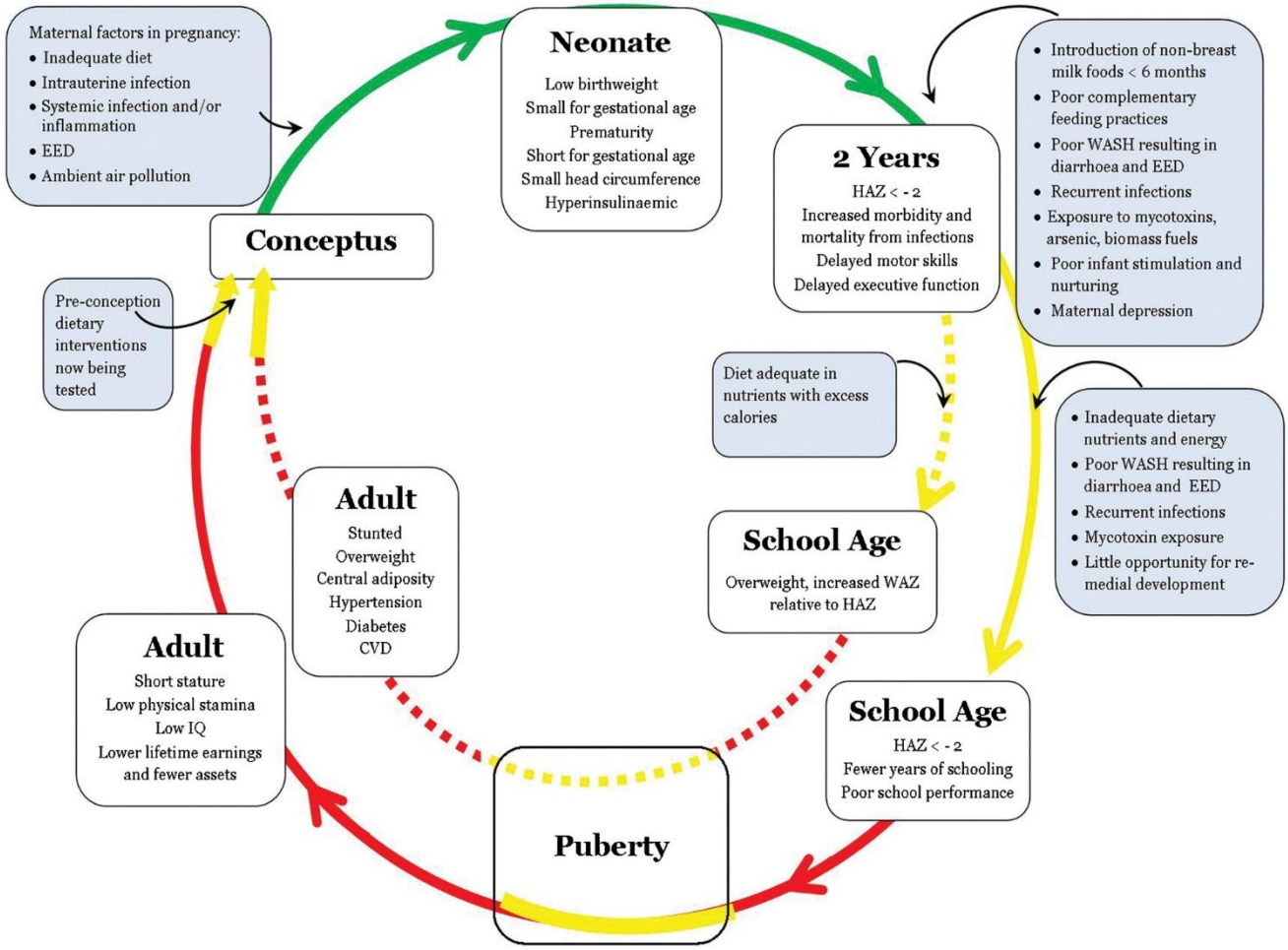

Figure A3. The stunting syndrome. Figure was reprinted (Prendergast and Humphrey, 2014).

APPENDIX B

CHAPTER III TABLES

Table B1. Diet composition (as-fed basis)

Ingredient	%
Corn	36.65
Soybean Meal (46.5.% CP)	10.6
Fermented soybean meal	10.0
Milk, Whey Powder	30.0
Fish Meal Combined	10.0
Soybean Oil	1.0
Limestone	0.5
Sodium chloride	0.3
L-Lys-HCL	0.265
DL-Met	0.175
L-Thr	0.105
Trace mineral premix[1]	0.15
Vitamin premix[2]	0.25
Phytase[3]	0.02
Total	100

[1]Provided per kilogram of diet; 110 mg of iron, 110 mg of zinc, 33 mg of manganese, 17 mg of copper, .3 mg of iodine, and .3 mg of selenium (Kansas State University)

[2] Provided per kilogram of diet; 1,102 IU of vitamin A, 1,378 IU of vitamin D_3, 44 IU of vitamin E, .04 mg of B_{12}, 4.4 mg of menadione, 8.3 mg of riboflavin, 28 mg of pantothenic acid, and 49 mg of Niacin (Kansas State University).

[3] Ronozyme HiPhos (DSM Nutritional Products, Parsippany, NJ) 540 FYT/kg feed, with a release of 0.13% available P.

Table B2. Effects of ETEC 23545 treatment on nursery pig performance[1]

	Treatment[2]				
Item	Control	Acute	Chronic	SEM	Probability, *P* <
d 0 to 6					
ADG, g	263	119	225	55.93	0.224
ADFI, g	179	210	232	43.05	0.688
G:F	2.02	.66	0.89	0.722	0.184

[1] A total of 24 weaned barrows (initial BW approximately 14 lb; Landrace × Large White) were used in a 7 d study. Pigs were housed 2 pigs per pen with a total of 4 pens per treatment.
[2]Control= Pigs administered a sham challenge of PBS each day, Acute = pigs administered approximately 10^9 CFU of 23545 on d 1 then sham challenged for the remaining time, Chronic = pigs administered approximately 10^7 CFU of 23545 from d 1 to d 6.

Table B3. Effects of ETEC 23545 treatment on serum cytokine levels.[1]

	Treatment[2]				
Item	Control	Acute	Chronic	SEM	Probability, $P <$
IL-6	4.11[a]	4.67[b]	5.15[b]	0.186	0.003
IL-8	5.04[a]	5.27[ab]	5.87[b]	0.368	0.089

[1] A total of 24 weaned barrows (initial BW approximately 14 lb; Landrace × Large White; PIC; Kansas) were used in a 7 d study. Pigs were housed two pigs per pen with a total of 4 pens per treatment. Blood was collected on d 6. Data was transformed using Log_{10}.
[2] Control= Pigs administered a sham challenge of PBS each day, Acute = pigs administered approximately 10^9 CFU of 23545 on d 1 then sham challenged for the remaining time, Chronic = pigs administered approximately 10^7 CFU of 23545 from d 1 to d 6.
[a,b,]Means with different superscripts within each row differ ($P < 0.05$).

Table B4. Effect of ETEC 23545 treatments on fecal colony counts.[1]

	Treatment[2b]				Probability, *P* <		
Item[3]	Control	Acute	Chronic	SEM	Treatment	Day	Treatment × Day
d 1	1.62[a]	6.87[b]	4.77[c]	.813	.0001	.7681	.5755
d 3	.72[a]	6.08[b]	5.47[b]				
d 6	.67[a]	6.61[b]	5.26[b]				

[1]A total of 24 weaned barrows (initial BW approximately 14 lb; Landrace × LargeWhite; PIC,Kansas) were used in a 7 d study. Pigs were housed two pigs per pen with a total of 4 pens per treatment. Values are Log_{10} of CFU/g.

[2]Control= Pigs administered a sham challenge of PBS each day, Acute = pigs administered approximately 10^9 CFU of 23545 on d 1 then sham challenged for the remaining time, Chronic = pigs administered approximately 10^7 CFU of 23545 from d 1 to d 6.

[3]All fecal samples were taken approximately 12hrs after dosing was completed for the respective day. The samples were taken by a per pen basis.

[a,b,c]Means with different superscripts within each row differ (P<0.05).

Table B5. Effect of ETEC treatment on fecal calprotectin concentrations.[1]

	Treatment[3]				Probability, *P* <		
Item[2]	Control	Acute	Chronic	SEM	Treatment	Day	Treatment × Day
d 0	122.93	106.87	117.46	15.124	0.954	0.485	0.955
d 1	105.83	92.82	98.04				
d 3	94.34	99.96	103.22				
d 6	94.71	109.82	103.85				

[1] A total of 24 weaned barrows (initial BW approximately 14 lb; Landrace × Large White; PIC, Kansas) were used in a 7 d study. Pigs were housed two pigs per pen with a total of 4 pens per treatment. Fecal samples were taken on a per pen basis. The average calprotectin concentration (ng/ml) was taken and values were converted to Log_{10}

[2] The d 0 fecal sample was collected before treatments were implemented. The d 6 fecal sample was taken ~12hrs prior to necropsy.

[3] Control= Pigs administered a sham challenge of PBS each day, Acute = pigs administered approximately 10^9 CFU of 23545 on d 1 then sham challenged for the remaining time, Chronic = pigs administered approximately 10^7 CFU of 23545 from d 1 to d 6.

Table B6. Effect of ETEC 23545 treatment on colony counts of intestinal scrapings and content.[1]

	Treatment				
Item[2]	Control	Acute	Chronic	SEM	Probability, *P* <
Intestinal Scrapings					
Duodenum	0[a]	1.30[ab]	2.32[b]	.711	0.093
Jejunum	0	2.35	.84	.770	0.117
Ileum	0	2.30	2.01	.805	0.114
Cecum	0	2.28	2.35	.860	0.114
Colon	0	2.32	2.29	.850	0.110
Intestinal Content					
Cecum	0[a]	3.83[b]	3.97[b]	.836	0.004
Colon	0[a]	5.43[b]	3.72[b]	.643	0.0001

[1]A total of 24 weaned barrows (initial BW approximately 14 lb; Landrace × Large White; PIC; Kansas) were used in a 7 d study. Pigs were housed two pigs per pen with a total of 4 pens per treatment. Pigs were necropsied on d 7 for the collection of intestinal samples. The values are the average of 8 pigs and have been converted to Log_{10}

[2] The proximal end of each section was used to determine colony counts. The cecum and colon contents were consisted of the remaining digesta in the respective section.

[a,b]Means with different superscripts within each row differ (P<0.05).

Table B7. The effect of ETEC 23545 treatment on small intestine villi length.[1]

	Treatment				
Item[2]	Control	Acute	Chronic	SEM	Probability, *P* <
Duodenum	735.10	753.24	712.58	48.55	.7125
Jejunum	540.38	566.22	618.98	53.86	.3719
Ileum	572.72	590.44	525.47	93.56	.7783

[1]A total of 24 weaned barrows (initial BW approximately 14 lb; Landrace × Large White; PIC; Kansas) were used in a 7 d study. Pigs were housed two pigs per pen with a total of 4 pens per treatment. Pigs were necropsied on d 7 for the collection of intestinal samples. The values are the mean of 4 pigs randomly selected from each treatment group.
[2] The distal end of each section was used for histology.