

Investigation of novel approaches to improving nursery pig health

by

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ABSTRACT

Investigation of novel approaches to improving nursery pig health

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This thesis investigates two approaches for potentially improving the health of weaned pigs. In one approach, two trials were conducted to examine the relationship of antibody response to *Mycoplasma hyopneumoniae* and growth with iron status. Based on hemoglobin levels at weaning, no difference was found among anemic, iron deficient, and normal pigs ($P>0.05$). In addition, an experimental-challenge study was used to determine if additional iron supplementation could impact a pig's response to infection of enterotoxigenic *Escherichia coli* (ETEC). Iron supplementation preventing anemia and iron deficiency was not associated with reduced clinical signs ($P>0.05$). Additionally, a plant-based variant of the FaeG protein, rFaeG_{ntd/dsc} was evaluated as a treatment of ETEC infection using a similar experimental-challenge model. Pigs fed the highest level of FaeG experienced less severe diarrhea than controls ($P<0.05$), however, there was no difference among the treatment groups based on ETEC fecal shedding or histological evidence of colonization ($P>0.05$).

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CONTRIBUTIONS

Victoria Seip coordinated field work, contributed to project planning, data collection, data management and analyses, interpretation of results and was the principal author on these three research chapters

Emily Arndt helped with all lab work including sample processing and analyses

Dr. Josepha Delay performed histological analysis on post-mortem samples

Dr. Rima Menassa and lab created, grew the tobacco FaeG plant protein and tested the product at AAFC London research and Development

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LIST OF ABBREVIATIONS

AHL- Animal Health Laboratory

BHI- brain heart infusion

bp-base pairs

CBA- Columbia blood agar

CFU- colony forming units

CPHAZ- Centre for Public Health and Zoonoses

ELISA- enzyme-linked immunosorbent assay

ETEC- enterotoxigenic *E. coli*

GIT-gastrointestinal tract

HCT- hematocrit

Hgb- hemoglobin

I.M.- intramuscular injection

MAC- MacConkey agar

MCH-mean corpuscular hemoglobin

MCHC- mean corpuscular hemoglobin concentration

MCV-mean corpuscular volume

OVC- Ontario Veterinary College

PCR- polymerase chain reaction

PMN- polymorphonuclear leucocytes

PWD- post-weaning diarrhea

RFLP-PCR- restriction fragment length polymorphism – polymerase chain reaction

RBC- red blood cell

SBA- serum bactericidal antibody

SDPP- spray dried porcine plasma

S/P- sample-to-positive ratio

TIBC- total iron binding capacity

WBC- white blood cell

ZnO- zinc oxide

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The nursery stage is possibly the most stressful period in the pig's life (1). Therefore, it is important that the weaned pig be as strong and robust as possible to thrive during the nursery stage. One health concern that has been recently highlighted is the fact that the biggest and fastest growing pigs at weaning are commonly iron deficient. This raises the concern that pigs with anemia due to iron deficiency may be less able to combat disease challenges in the nursery as pigs with adequate iron status. At three to four weeks of age when piglets are typically weaned and moved from the farrowing room to the nursery, many changes occur all at once. At this time circulating antibodies gained from ingestion of colostrum are limited and the localized protection from milk antibodies suddenly cease so that the pig must rely on creating an active immune response to pathogens, however the immune system of these young pigs is still immature and therefore they are vulnerable to disease (2).

Another significant change that occurs at weaning is the sudden diet change from milk to a grain-based feed. This change in diet predisposes the pig to intestinal inflammation and possible disease. Enteric disease caused by enterotoxigenic *Escherichia coli* (ETEC) is possibly the most common cause of illness in newly weaned pigs (3–7). Despite major efforts to control ETEC, post-weaning diarrhea (PWD) continues to be an economically important disease in the swine industry because of the morbidity, mortality, and effect on the growth rate (7,8). Novel approaches at controlling ETEC are being investigated.

This review will discuss common management techniques that are used to provide supplemental iron and discuss the importance of why investigating the standard commercial dosage of iron is critical for production improvements. It will look at what research has been done

to determine the effect of iron status at weaning on the growth rate of pigs throughout the nursery. The focus will then move towards how ETEC bacteria develops in newly weaned pigs. It will identify the pathogenesis and fimbrial types involved with the bacteria and identify receptor attachment and enterotoxin production. Following an overview of the bacterial disease, treatment and preventative measures will be discussed. The final topic will be the potential use of transgenic tobacco plants containing a recombinant variant of the FaeG protein subunit that might be used in swine diets to reduce PWD through competitive inhibition.

1.2 Iron

1.2.1 Iron Deficiency and Anemia

Pigs are are an animal that are most prone to iron deficiency (9). Piglets are born with very limited iron stores of only approximately 50 mg and almost all of this iron is already being used to form hemoglobin (10,11). Iron is an essential mineral required by the animal for proper growth, tissue and enzyme function (12). Although milk is a very nutritional food source, sows' milk is deficient in iron (13–15). Deficiency is a major concern in commercial pig husbandry because piglets are not able to obtain iron from their environment unless they are raised outdoors (9,16). In order to keep up with the daily requirement of 7-10 mg of iron, iron supplementation is required within the first few days of life in order to ensure optimum growth and health (17). One major consequence of iron deficieny is a decrease in hemoglobin (hgb) levels and a decrease in red blood cells (anemia) (18). This limits the amount of oxygen that can be transported throughout the tissues of the body (10).

1.2.2 Hemoglobin

Hemoglobin is a protein that is found within red blood cells (RBCs) and is one of the most commonly used indicators of iron status in piglets. Approximately two-thirds of iron is found within hemoglobin (19). Hemoglobin functions by transporting oxygen throughout the body to tissue's and organs for proper function (20). When pigs are anemic, they have fewer RBCs and a lower concentration of hgb in blood, resulting in less iron being transported and stored throughout the animal (21). The brain receives oxygen preference over other organs which compromises the ability of the other tissues and organs to properly function. This may affect the production of immune cells in the body (19).

The iron status of young pigs can be assessed on the basis of hgb. A study by Bhattacharai and Nielsen (15) categorized pigs with <90 g/L of hgb as “anemic”, 90-110 g/L as “iron deficient” and >110 g/L as “normal”. Although hgb is a convenient and useful method of indirectly measuring iron status there may be more accurate techniques such as measuring serum ferritin levels or even other red blood cell parameters (15).

1.2.3 Iron Supplementation

In order to prevent iron deficiency in the suckling period, the swine industry many years ago adopted routine iron supplementation. Typically, on most modern pig farms, piglets are given an intramuscular injection of 200 mg of iron dextran or gleptoferon during the pig's first week of life (17,21,22). This 200 mg dosage was derived by taking into account the animal's daily requirements and a three week weaning period (15,23,24). Supplementation is especially critical in the first few weeks of life when piglets are still not adapted to solid feed consumption (23).

When they are relying on sows' milk as their main food source, adequate iron levels for hemoglobin production and other uses cannot be sustained.

A study conducted by Peters and Maha (11) identified that piglets that are supplemented with iron in the first few days post-farrowing have improved growth performance and hgb levels compared to pigs that do not receive intramuscular iron supplementation. Their study also looked at the animal's performance based on additional iron injection at weaning. They found that the pigs that did not receive an iron injection in the neonatal period had a more rapid response to iron supplementation at weaning. Iron levels were also improved with a second injection at weaning in the piglets that received iron in the farrowing room (11).

As litter size continues to increase and milk production continues to increase to stimulate faster growing pigs in the farrowing room, it is likely that piglets will need more than the historical standard 200 mg dose of iron dextran during the suckling period. Piglets use up their iron reserves from one single injection too quickly, which results in pigs being iron deficient or anemic at three weeks of age (11,23). With a daily requirement of 7-10 mg of iron, the standard 200 mg iron dose was developed in order to provide piglets with enough iron reserves for a three week weaning period (25). However, with fast growing pigs, the amount of iron administered to piglets must be re-evaluated. On the other hand, when a dose of too high iron is administered to piglets by means of a single injection, iron toxicity can occur. Piglets experiencing iron toxicity can react acutely following iron injection, developing weakness, tremors and sudden death (26,27). In addition to the risk of toxicity, excess iron supplementation may result in slower growth rate, and therefore increasing the injection of more than 200 mg of iron during the first week of life has not been widely adapted by the swine industry.

1.2.3.1 Sow Feeding

In order to make up for the lack of iron in sows' milk, one alternative to iron supplementation for suckling piglets is increasing the iron content of sow feeds during gestation with the goal of increasing iron reserves in the newborn and higher iron content of milk. Peters and Mahan (11) concluded that although adding additional iron to sow diets may influence the amount of iron consumed by the piglet, additional piglet supplementation in the first few days of life is still necessary to achieve optimum growth rates and hemoglobin levels of suckling piglets (11).

1.2.3.2 Oral Supplementation

An alternative mode of iron supplementation is to provide iron orally. When pigs are reared outdoors, soil can be a good source of iron. The pig's natural behaviour is to root in the soil and this promotes ingestion of earth (9). The amount of iron consumed by a suckling pig raised outdoors probably varies dramatically from animal to animal and there are few studies that have evaluated the effectiveness of this approach to meet the iron requirements of rapidly growing modern pigs. In commercial production in Canada, almost all pigs are raised on slatted flooring made from plastic, metal or concrete (9,28). Ingestion of sow feces is another alternative source of iron, however with many farrowing crates use slatted floors that minimize the amount of sow feces available for the piglets to ingest (11).

Providing an oral form of iron has been proven to be an effective alternative to intramuscular injections of iron in order to reduce anemia. After approximately one day once it enters the small intestine, iron enters the epithelial cells by pinocytosis (27,29). It is crucial that when supplementing iron orally it be done as soon as possible post-farrowing in order to promote adequate iron absorption which can be reduced once the intestinal tract fully closes in order to prevent bacteria from entering the piglet's system (29). After approximately one day of supplementation, iron in the neonate is absorbed by its epithelial cells to be used and stored throughout the body (27). Common forms of oral iron that have been provided to young piglets include; iron paste on the teat, spraying the udder with an oral form and access to soil or peat moss for rooting in the farrowing crate. One issue with these techniques is that constant ingestion is crucial in order for piglets to meet their daily requirements (27).

Supplementing sources of iron that can be readily absorbed in both creep and starter feeds is an efficient way to improve piglet iron reserves and is a practical form of administration, but requires that producer's promote feed intake in order for the animal to improve their iron status (23). Generally, feed intake alone is not enough for the piglet to meet their daily requirement since creep feed intake before day 14 is often minimal (27). Iron sulphate and iron fumarate are the most commonly sources used in these palatable feeds (11,23,27).

1.2.4 Iron Deficiency in the Nursery

At 3 to 4 weeks of age when pigs routinely are weaned and enter the nursery, anemia is commonly seen (10). With pigs growing very rapidly in the farrowing room, by the end of the suckling period, piglets have often used up their iron reserves. As sow productivity continues to improve and more pigs are born per litter, the result is less iron reserves per piglet (11). The

heaviest pigs at weaning are often the most likely to be iron deficient because they have a greater surface area for blood flow and have a greater requirement for iron (22). At weaning, pigs are faced with many stressors. It is common for feed intake to drop substantially within the first week post-weaning and as a result, piglets do not receive iron, despite adequate levels provided in the starter feed (30–32). The nursery period tends to be the time when the most expensive feed ingredients are used in order to promote feed intake and to help overcome challenges such as disease stress by using highly digestible ingredients (32).

1.2.5 Iron Status and Growth Response

Adequate growth rate in nursery pigs is one of the primary goals for producers. Since many pigs enter the nursery anemic, it is important to investigate the relationship between iron status at weaning and growth response in the nursery. Several studies have indicated that the heaviest pigs entering the nursery are also anemic (10,15,20,33). A study by Perri et al. (10) looked at the effect of iron status at weaning on the growth rate of pigs in the first three weeks of the nursery. This study found that the heaviest pigs entering the nursery were also the most anemic. When they followed the pigs for three weeks, they found that anemic pigs had a poorer growth rate than pigs that had normal iron status (10). Jolliff and Mahan found similar results that the heaviest pigs at weaning were also the most anemic (20). Another study conducted by Manner et al. (34) looked at the effect of different modes of iron administration had on the growth rate of pigs in the nursery. They found that more iron did improve the hgb status of pigs, however this had no affect on growth rate (34). Bruininx et al. (33) found similar results that an additional iron injection in the suckling period improved the pigs' hemoglobin status. They also found that the additional iron supplementation had no affect on growth performance after weaning (33).

1.2.6 Iron Status and Antibody Response

At weaning, pigs are often vaccinated in order to develop their humoral immune response to common bacterial and viral pathogens they may face at later production stages (35,36). Antibodies can be found in blood plasma and are synthesized within the bone marrow (36). Very minimal research has been done to identify the effect iron status has on immune response in nursery pigs. However, some work has been done looking at the effect of iron deficiency and anemia in humans and its impact on antibody production and immune response. Chandra and Saraya found that the effect on immunoglobulin production and immune response in humans was similar amongst iron deficient and normal individuals (37). Laboratory work has also been conducted in rats to look at the effect of supplementing dietary iron on the production of antibody titres against influenza virus. Dhur et al. reported lower antibody titres in the rats that were not fed additional levels of iron compared to those that were supplemented (38).

One reason it is important that the immune system of the newly weaned pig is functioning at an optimum level is because at weaning pigs are often vaccinated for various important diseases. If immune response is compromised then pigs may not respond well to vaccination and disease outbreaks may occur despite immunization. A common vaccine given to most pigs around the time of weaning is for the control of *Mycoplasma hyopneumonae*. This vaccine is used to protect pigs against the respiratory disease enzootic pneumonia which results in reduced growth rate and is considered economically important (39,40). Piglets receive their first vaccination at weaning and then generally a booster vaccine at three weeks into the nursery period to ensure proper antibody production is established. Antibody response to *M. hyopneumoniae* vaccine can be tested using an enzyme-linked immunosorbent assay (ELISA) (40).

1.3 *Mycoplasma hyopneumoniae*

Mycoplasma hyopneumoniae is a common pathogen in the swine industry, and leads to a respiratory disease called enzootic pneumonia. Generally, enzootic pneumonia affects pigs in the grower-finisher stage of production (40). This bacterial disease is spread through direct animal contact. Morbidity impacts the growth and causes a loss of profits for pork producers (35). Clinical signs often include coughing, lethargy, weight loss, lack of appetite and labored breathing, but usually low levels of mortality (40,41). In order to reduce the clinical impact of enzootic pneumonia in swine operations, it is common for producers to vaccinate their pigs at weaning and commonly a booster is given 2 to 3 weeks later to strengthen protection (40).

1.4 Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *E. coli* are gram negative bacteria and a common cause of diarrhea in newly weaned pigs and a common reason for antimicrobials use swine nurseries.

1.4.1 Fimbrial Adhesins

Digesta continually flows through the lumen of the gastrointestinal tract (GIT). This movement may prevent *E. coli* attachment and colonization, however some *E. coli* are still able to attach themselves to surface receptors. In order for a pig to become ill from ETEC, the bacteria must have fimbrial adhesions that are specific to the appropriate receptor (4,7,42). The fimbrial types most commonly associated with ETEC infection include; F4(K88), F5(K99), F6(987P), F17 (Fy/Att25), F41 and F18 (4,42,43). These fimbriae are composed of intertwined or straight proteinaceous extensions that are formed from the outer membrane of the bacterial cells (43,44).

In many cases of ETEC, the F4 (previously known as K88) and F18 fimbrial strains are the most common fimbriae to cause both neonatal and PWD (7,8,45,46). Infection due to the F4 variant of ETEC is specifically a threat to swine and provides limited risk to other farm animal species (45).

1.4.1.1 F4 (K88) Fimbrial Types

When faced with PWD caused by ETEC, long proteinaceous extensions are found on the surface of the bacteria called F4 fimbriae (2). The F4 fimbrial type of *E. coli*, is divided into three variants. These variants are; F4ab, F4ac and F4ad, which are part of the flexible fimbriae (44,45,47–49). The “a” refers to the common epitope of the protein, and the following “b”, “c” or “d” represent the more specific epitope (44,45,47). These three different variants have different attachment capabilities and some animals may be susceptible to more than one type (45). The common fimbrial types found on commercial swine farms are composed of major and minor subunits. The several hundred identical proteins found forming the fimbriae are major sub-units known as the FaeG protein and the minor sub-units FaeC protein (2,3,44). The FaeG is known for its adhesive properties which allow binding of the bacteria to the receptors in the digestive tract of the pig (50).

1.4.1.2 F4 Bacterial Attachment

When ETEC bacteria have been ingested by the pig, the bacteria move throughout the gastrointestinal tract and colonize and replicate in the small intestine. The bacteria attach themselves to the receptors in the epithelium via the fimbrial adhesions (4,45). Once the bacteria

have adhered in the small intestine, the function of the epithelial cells change with fluid being secreted into the intestinal lumen resulting in diarrhea (3). Once ETEC bacteria have attached and the animal has become infected, bacteria replicate quickly and accumulate in the jejunum and ileum (45). The number of bacteria that attach to receptors by the fimbriae is largely what determines the severity of infection (3).

1.4.1.3 F4 Receptors

In order for F4 ETEC to colonize in the small intestine, the physiology of the GIT of the pig must have an adherence site for the bacteria. The binding site is known as the F4 receptor and enables the bacteria to attach to the brush border of the small intestine epithelium. This allows bacterial colonization which leads to the development of PWD (4,44,48,49,51). The F4 receptor adhesion site is a genetically inherited dominant trait. When the F4 ETEC bacteria are present, only susceptible animals will be affected (3,52). Pigs can be categorized based on their adhesion properties. “Adhesive” animals experience bacterial attachment in the brush border of the small intestine, whereas “non-adhesive” animals cannot become easily infected by this fimbrial type of bacteria (3,49,51,52). Once the bacteria attach and colonize in the small intestine, the passage of intestinal contents moves some of bacteria towards the lower tract, allowing further colonization and spread of bacteria (44). Testing can be done using an *in vitro* adhesion assay to determine receptor status, classifying an animal as positive or negative for the receptor (48,49,51).

Sellwood et al. (51) performed an experiment to determine a method that can be used to indicate the status of brush borders of the F4 strain of *E. coli*. This *in vitro* design looked at classifying pigs as “positive” (F4R+) or “negative” (F4R-) based on the characteristics of the brush

border in the epithelium. Their results determined that bacterial attachment was determined by the fimbrial type of *E. coli*, due to the specificity of the receptor. They were easily able to classify the pigs as being F4R+ or F4R- based on whether the F4 bacteria were able to attach and colonize. They also concluded that the presence of the F4 receptor is likely a genetic trait since the bacteria were not able to attach and colonize in every animal (51).

A study conducted by Blomberg and Conway (53) looked at identifying the effect on age and ETEC bacterial colonization in pigs. Animals were euthanized at 5, 26 and 47 days of age to observe the differences in the gastrointestinal tract. Samples were taken to evaluate the differences in receptor susceptibility and bacterial counts within the animal at the different ages. They concluded that pigs around 26 days of age had significantly more F4 receptors than the 5-day and 47-day-old pigs (53). In Canada, the average commercial swine farm weans piglets around three weeks of age. When comparing these results to commercial swine production, we can see that the animals that are most at risk of ETEC infection are those approximately five days after weaning. This should be an important consideration for producers when raising and feeding nursery pigs.

1.4.1.4 F4 Receptor Phenotypes

Although there are two different receptor types, F4R+ and F4R-, a further subdivision can be made to categorize multiple phenotypes related to F4 ETEC bacterial attachment. A brush border adhesion assay experiment conducted by Bijlsma et al. (49) took samples from 65 pigs that were used to test F4 ETEC strains with the variants F4ab, F4ac and F4ad along with a control strain. Their first observation identified a specific receptor status (F4R+/F4R-) for each strain of *E. coli* due to adherence to the intestinal brush border, similar to Sellwood et al. (51). The second

part of their experiment enabled them to identify a number of phenotypes associated with adhesive animals (49,51). The five phenotypes that were discovered were labelled from A-E.

The subdivision of phenotypes was categorized based on different combinations of attachment of the three F4 variants. The phenotype A receptor could attach all three variants (F4ab, F4ac and F4ad) to the receptor. Phenotypes B and C were able to adhere two variants; F4ab and F4ac or F4ab and F4ad. Phenotype D was only able to adhere to the F4ad variant and lastly, phenotype E wasn't able to adhere to any of the three variants (49). The phenotype E was identified in this study as being a homozygous recessive trait. Based on the genetic testing done by Gibbons et al.(54), animals that are homozygous recessive for the receptor were not able to recognize the F4 ETEC bacteria, and therefore did not produce antibodies to eliminate bacteria (54).

Since pigs are litter bearing species, it is possible for litters to be divided into three categories of susceptibility when looking at the F4 receptor. These include non-segregating susceptible (SS), where the entire litter may be F4R+, non-segregating resistant (ss), where the pigs in the litter do not have the receptor (F4R-) and lastly, the litter may be segregated (Ss) indicating that both receptor phenotypes are present (45,54). Therefore, the susceptibility of the litter is quite variable.

1.4.2 Pathogenesis of ETEC in Swine

E.coli is an anaerobic bacterium that colonizes and survives in the small intestinal tract of animals (55,56). Within the first few days post-weaning, pathogenic *E.coli* can cause severe watery diarrhea, body weight loss, dehydration and sometimes mortality in pigs that are infected with the bacteria (42,45,56). Many factors around this time of production assist in morbidity due to ETEC

infection such as: termination of passive antibody transfer, dietary changes and the stress of weaning (45).

Before the piglet is born, the body and GIT are sterile and immunity and microbiota post-farrowing build up through passive transfer through lactogenic immunity and contact with the environment (56). *E.coli* causing PWD enter the animals GIT via ingestion of contaminated feed, feces, fomites, air and other vectors that may act as a host site for the bacteria (3,45,56).

For the pig to become infected and show signs of morbidity when faced with ETEC and for PWD to develop, several processes must happen. The bacteria must be present in sufficient numbers to eventually proliferate throughout the length of the jejunum and ileum. Secondly, the proper receptors must be present in order for the bacteria to attach onto the mucosal layer or epithelium (4,45,56,57). Once attached, toxic proteins are released, altering the movement of water and electrolytes. This results in excessive fluid secretion resulting in PWD (56). During this time, when pigs are faced with additional disease challenges such as rotavirus, more damage occurs within the intestinal epithelium and morbidity becomes more severe (45,58).

1.4.3 Enterotoxins

When newly weaned pigs have been faced with the challenge of ETEC and the bacteria have attached to receptors and started to replicate, the last step involved with morbidity is the production and release of toxins within the GIT. The secretions of toxic proteins known as enterotoxins is the main cause of diarrhea in ETEC infected pigs (57,59). The secretion of these toxins alters the proper function of fluid homeostasis causing the lumen of the intestine to fill with fluid. When pigs are challenged with ETEC, these plasmid-regulated proteins are secreted and alter the environment of the intestinal epithelium (3,57).

The primary role of the small intestine is to regulate secretion and absorption of nutrients and fluids which are required by the body for proper maintenance and growth (56). When enterotoxins are released after bacterial attachment to the epithelium, the proteins are released into the lumen. This results in an altered secretion of electrolytes (Na^+ , Cl^-) and water, and a decreased absorption of fluids resulting in scouring diarrhea and the potential for acidosis (3,4,56).

Enterotoxins involved with ETEC PWD are classified into two categories based on their thermal stability. The two categories are heat-labile (LT) and heat-stable (ST) (3,57). LT toxins can be categorized into LT-I and LT-II (3,56,60). Heat-stable toxins can be further categorized into STa, STb and EAST1. STb is most commonly found in cases of ETEC PWD, where STa is more often associated with ETEC neonatal diarrhea (3,57,60). EAST1 is similar to the STa family of enterotoxins and is known for its low molecular weight (45).

1.4.4. Clinical Signs of ETEC

For decades, ETEC PWD has been an issue faced by swine producers around the globe. Generally post weaning diarrhea caused by ETEC occurs within the first 14 days post-weaning, however receptors are still present when the pigs move onto the grow-finish stage of production and so outbreaks can occur at a later age as well but it is rare (61). When high numbers of bacteria have colonized, pigs start to show clinical signs of illness associated with post-weaning diarrhea. When infection is severe, the pigs stool will be very loose, have a watery appearance and may be light in colour and diarrhea may last up until a week of being in the nursery (62,63). The alkalinity of the diarrhea may cause the pig to have hair loss around the anus, and appear to have scalded the skin (64). Pigs may appear dehydrated, depressed and have a lack of appetite (63,64). Purple discolouration around the snout and ears is also common in severe cases. If pigs survive, weight

gain can be reduced throughout the nursery period (45). It has been reported that some piglets die without showing any signs of diarrhea within the first week post-weaning, however intestinal contents contain watery fluid (45,64).

A study conducted by Krsnik et al. (65) looked at the ways common behaviours that are seen in pigs such as eating, drinking, activity and defecating may be impacted by a pathogen challenge such as ETEC. They felt that by identifying changes in these common behaviours, this could potentially help identify early signs of PWD, rather than waiting until clinical signs of infection occur. In this study, pigs that were infected with *E. coli* showed a reduced appetite, less water consumption, a reduced or increased duration of lying time, along with an increased amount of defecation when compared to a control group of pigs not infected with the pathogen. They concluded that the normal behaviours seen among newly weaned pig's changes when they are faced with a disease challenge. By observing the frequency and behaviour once piglets enter the nursery, these changes could be an early indication of infection due to ETEC (65).

Economic losses due to the ETEC pathogen can be high. Producers faced with this pathogen challenge are more likely to have on-farm use of antibiotics, and/or use in-feed antimicrobials and other treatments to aid in the elimination of disease (66).

1.5 Preventative Measures for Dealing with ETEC

One of the rising issues with food animal production around the world is the appropriate use of antimicrobials. For the past 60 years, antimicrobials have served various purposes in food-animal production which contribute to their health and performance over the growth period (67). Improper use of antimicrobials is a concern for the general public due to emergence of resistant bacteria, which can be passed on to the human food supply chain (68,69). There is a constant

pressure for producers to reduce their use of antimicrobials in food production. In the nursery, antibiotics are used to help prevent ETEC infection, and resistance of this bacterial pathogen has arisen due to the use of antibiotics such as neomycin which has been used for decades by swine producers. In order for antibiotics to properly eliminate infection when present, a therapeutic concentration of the drug in the small intestine must be reached (64).

1.5.1 Antimicrobials Used for Post-Weaning Diarrhea

The administration of antimicrobials in order to reduce PWD has the added benefits of reducing morbidity, mortality and improving pig growth in swine production. The main reasons for mass medication of antimicrobials in feed or water are as follows: act as a prophylactic measure to reduce the risk of disease such as PWD; to provide metaphylactic benefits by treating large numbers of animals when disease is present in the herd (70,71) . On most farms, these additives are included in the nursery stage of production when pigs are faced with the most challenges such as PWD and tend to be eliminated when the pigs are close to market to avoid antimicrobial residues in the meat (67,71).

1.5.1.1 Heavy Metals for Preventing Post-Weaning Diarrhea

For the past several decades, therapeutic levels of heavy metals have been added to nursery diets to help both reduce PWD and also to increase growth rate (72). The two most common heavy metals used in this way are zinc and copper (73). Based on the animals requirements, the normal level of dietary zinc in swine diets is 125 mg/kg (74) and the inclusion of dietary copper is approximately 6 mg/kg (73). In order to prevent disease and improve growth, these heavy metals are often added to feed at 3000 mg/kg for zinc oxide and 125 to 200 mg/kg for copper sulfate.

The inclusion of dietary zinc has benefitted newly weaned pigs by helping to prevent and minimize post-weaning diarrhea (72–74). Zinc has many uses in the growing pig. It is essential for proper homeostasis in the body and improves weight gain and daily feed intake when fed at levels to meet nutritional needs (74). At very high levels, zinc can act as an antimicrobial agent reducing bacterial build-up and adhesion in the gastrointestinal tract and potentially resulting in less scouring (75). Dietary inclusion of zinc oxide ranging from 1500-3000 ppm is commonly used in early starter diets for the first few weeks after weaning to prevent diarrhea caused by ETEC (46,73). Studies have shown that by including this dietary level of zinc oxide, pigs showed more vigour, improved growth performance, better fecal scores and better overall health and gut development in the nursery (46,73,74,76,77).

Although dietary zinc has proven to be beneficial in the nursery stage there are some concerns about the use of these extremely high levels. One of the main concerns involves waste management. When pigs are fed higher levels than what their body can utilize and absorb, the excess nutrient is excreted in the feces and this can cause a concern for soil health and the environment over the long term (72,78).

Copper sulfate is another heavy metal that is commonly used in nursery diets to improve growth and health status in the nursery phase of production. Levels of 125-250 ppm have resulted in improved the health and growth of pigs (74,79). In the nursery phase of production, copper sulphate appears to have antimicrobial properties and results in enhanced growth rate of weaned pigs. However, the effect of copper on the pig's gut microflora and the mechanism by which it reduces disease is not fully understood (79).

1.5.1.2 Antibiotics for Preventing Post-Weaning Diarrhea

Antibiotic therapy use is one of the most common ways swine producers have treated animals to reduce and eliminate post-weaning diarrhea in the nursery. Several field trials have shown that on commercial farms, the most common antibiotics used are: gentamicin, apramycin, neomycin, tiamulin, tylosin, and cefitofur (66,80,81). A combination of these drugs such as neomycin and oxytetracycline can be used (82). Farms with a higher incidence of ETEC post-weaning diarrhea tend to use more antibiotics which increases the cost of production per pig (66). The most common forms of administration at pen level are through feed or water intake and through I.M. injection for individual treatment (66,81). Depending on the severity of disease, animals that are showing clinical signs of illness may need parenteral treatment if their water and feed intake is low (62). Because of the often acute nature of ETEC infection, treatment must be started quickly before the pig becomes severely dehydrated. An advantage of treating the entire pen or room of pigs at once through feed or water is that it does not require the herdsman to identify the very early signs of infection. It is important to consider that by eliminating the use of antimicrobials without an adequate substitute, there is the potential for higher amounts of morbidity and mortality, along with reduced feed efficiency on commercial swine farms (71). The herd veterinarian is often the one who makes the decision on what antibiotic should be used to treat the herd (64).

1.5.2 Herd Management

Weaning is one of the most stressful times in a pig's life due to the drastic changes that occur with the movement to the production stage. Piglets no longer have the company of the sow, they are in a new living environment and are fairly abruptly changed from a liquid milk to a solid, grain-based diet (30–32). This is often a time when piglets go off feed and rely on their body energy stores for survival for the first few days post-weaning. However, it's important to consider

that this is the time when they are also faced with disease challenges such as ETEC PWD. With bacterial proliferation and colonization and lack of feed intake, this often leads to high morbidity within the first week of weaning.

Herd management in the farrowing and nursery stages of production can help to reduce and eliminate post-weaning diseases that occur within the first 3-4 weeks of a piglet's life. Weaning age is one factor that can help reduce the colonization of bacteria in the digestive tract of the pig. On commercial swine farms, the age at which pigs are weaned is dependent on how many sows are going to farrow, the number of farrowing crates and the turn-around time from washing to sow entry (83). Many studies have shown that increasing weaning age from the standard commercial time frame of approximately three weeks can help reduce the incidence of disease. These studies showed that older pigs had lower *E.coli* fecal counts (31,84–86). Pigs that are weaned around four weeks of age have a more developed digestive system and may be more familiar with solid creep feed than younger animals. As a result, older pigs tend to have a less drastic growth lag, consume feed more quickly upon entering the nursery and have a stronger ability to overcome bacterial infections (84,86,87).

Following an all-in all-out pig flow system can also help to reduce the build-up of ETEC bacteria and improve piglet health. This form of pig flow reduces bacteria accumulation and proliferation with thorough washing and disinfection between groups (83). Moving entire groups of pigs allows less disease to be passed between different age groups of animals and therefore, pathogen challenge minimized.

1.5.3 Vaccination

Vaccines are one of the most common approaches to disease prevention in commercial swine settings. Producers decide to vaccinate their herd after taking into account vaccine efficacy, the cost of vaccination versus the cost of disease, and identifying the health status of the herd. Most neonatal pigs are protected against *E.coli* infections as a result of passive immunity through maternal vaccination (88–90). Prior to farrowing, sows are vaccinated and protection from maternal antibodies build the immune system in the neonate (91). It's important for producers to consider that passive protection declines with increasing piglet age and once the piglets are weaned, they are at risk of ETEC infection (88,91).

When dealing with disease challenges that are faced by the newly weaned pig it is crucial that their mucosal immune system is stimulated (88,89). Effective vaccines reduce bacteria colonization and antibodies that inhibit the attachment of the *E.coli* bacteria within the intestine (92). Live oral vaccines have proven to compete with bacterial attachment, however, mucosal immunity is not stimulated immediately upon administration resulting in a time period where the piglets are vulnerable to disease (91). Therefore, it is not always the most efficient choice to vaccinate pigs against ETEC upon arrival in the nursery, due to the risk of early infection.

1.5.4 Feeding Management

One of the most common ways swine producers try to minimize ETEC PWD is by using strategic feeding management to reduce the build-up of bacteria in the environment and gastrointestinal tract. Many research trials have analyzed the ways these techniques can control and limit post-weaning disease outbreak.

1.5.4.1 Spray Dried Porcine Plasma

Spray dried porcine plasma (SDPP) is a common feed ingredient that has been included in many creep feeds and phase one starter diets to try and limit ETEC disease shedding and outbreak (93). This supplement not only limits *E.coli* colonization and outbreaks but also provides many other benefits for the challenged animal by improving feed intake and average daily gain (94). The benefits that come from feeding SDPP are associated with the immunoglobulins found in the plasma, more specifically IgG which can help stimulate immunity in the piglet (94–96). When included in rations, this can be a more expensive but easily accessible protein source that stimulates feed intake in the newly weaned animal (94). Recommended inclusion rates in starter rations are typically around 4-8% of the diet (96). Studies have shown that pigs challenged with ETEC had improved performance and gain and less severe diarrhea when fed diets supplemented with SDPP (93–96).

1.5.4.2 Acidifiers

When piglets are transitioned from a milk to a solid diet, it is very common for them, in the first few days, to have a reduced feed intake due to the many changes and stressors being faced at this stage of production. One of the reasons piglets tend to go off or have a reduced feed intake when they are weaned is a result of the lack of hydrochloric acid secretion in the stomach which lowers the stomach pH (97–99). When a piglet is young and just weaned off milk, the pH of their stomach tends to be similar to their diet. This makes it harder for them to properly digest plant proteins such as soybean meal and allows bacteria to pass on through the digestive tract (97).

When acid secretion in the stomach is limited at weaning as a result of the immature digestive system of the pig, growing conditions are more ideal for bacterial colonization (99). Low amounts of hydrochloric acid also limit the breakdown of feedstuffs resulting in less nutrient

absorption in the GIT and higher levels of scouring (99). One of the ways to overcome high stomach pH and bacterial growth is to supplement starter diets with pure and mixtures of organic acids to make the contents of the stomach more acidic (97). As the pig gets older, stomach pH drops to below 3.5 which reduces bacterial growth within the GIT of the animal (97–99).

1.5.4.3 Creep Feeding

To create a healthy transitioning period, creep feeding is an effective way to introduce piglets to solid feed. When piglets are introduced to solid feed earlier, they are less likely to have as long of a post-weaning growth lag with a reduced feed intake once weaned (32). Creep feeds normally contain high quality ingredients such as lactose and SDPP which help to stimulate piglet feed intake. Providing creep feed during the suckling phase, has been credited with improving gut development and reducing the build-up of bacteria in the GIT (30).

1.5.4.4 Probiotics

When probiotics are fed to young weaned pigs, they serve a variety of purposes. They are fed to: inhibit harmful bacteria build up and colonization, improve the capacity of the GIT and lower stomach pH, stimulate the growth of good bacteria in the gut, and to help build up mucosal immunity for the animal (100). The idea behind feeding probiotics to newly weaned pigs is that these live bacteria will help mature their digestive system with very minimal nutrient requirements. They may even compete with the harmful bacteria such as *E. coli* to limit bacteria build up in the gut. Bacteria such as *Lactobacillus* sp. have proven to reduce gastric pH and compete and inhibit

the growth of pathogens such as *E.coli* by providing less adequate colonizing conditions and competing for adhesion sites (32,101). However, there is a high amount of inconsistency with probiotic use in starter rations as a result of product handling, feed processing and feed storage (100).

1.6 Transgenic Tobacco

1.6.1 Genetically Modified Crops

Genetically modified crops have been developed to improve the productivity of plant species along with providing them with more use in other industries. Scientists have worked to modify the genetic composition of plants to: provide protection against disease and to produce plants that are less susceptible to insect destruction and infection along with developing herbicide resistance (102). It wasn't until later in the 20th century that tobacco was identified as a potential crop that could be genetically modified. By transforming the nuclear genome, tobacco has proven to have uses outside the cigarette industry (102,103).

Plant products are used to make vaccines and therapeutics for several beneficial reasons. First, they are easy to produce and can be grown all year round in a variety of growing conditions (such as greenhouse production). The plants' development require minimal capital investment due to their ease in production and reproducibility. Lastly, plants are a very safe host because bacteria and viruses that are a risk to humans or animals cannot survive in their growing environment or host (50,104,105). When delivering the edible plant vaccine to the animal, there are many

advantages with its ease of administration including consumption through feed, avoids stressful procedures for the pig (106).

1.6.2 Genetic Modification

Chloroplast transformation using tobacco plants has been used to manipulate plant proteins to create vaccines, enzymes and antibodies that defend the host against pathogens and diseases (107). Chloroplast manipulation has been tested in tobacco plant leaves and deemed successful in a number of studies (2,6,75,103,107,108). It has been proven to be advantageous by having a high level of expression, little threat to the native species of tobacco, and minimal gene silencing (6). The foreign protein can be inserted into the chloroplast with minimal effect on the cells maturation and growth (50). Several studies have been successful in incorporating the FaeG protein into the tobacco plant chloroplast to stimulate an antibody response and mucosal immunity once received by the animal (2,6,108,109). This response may compete with the challenged bacteria to avoid morbidity and mortality, reducing ETEC PWD in newly weaned pigs.

1.6.3 Comparison of Research Studies

In the past several decades, several studies have looked at whether modification of crop plants, more specifically their chloroplasts, can reduce the incidence of post-weaning *E. coli* in newly weaned pigs. This bacterial challenge is one commonly faced by pork producers and finding new alterations to antimicrobial and antibiotic use are a priority.

Several studies have looked at the modification of tobacco plant chloroplasts with the FaeG protein that provides the adhesive properties of the bacteria. The main approach behind these

studies has been to use the plant product as a vaccine to stimulate the animal to produce immunity against bacteria (2,6,75,108,109).

Studies conducted by Shen et al. (6) and Huang et al. (108) and looked at immunizing mice to see if they built up a mucosal immune response from an FaeG based immunization. For weeks after injection, the mice were continuously tested to see if they had built up an immunity. To test whether there was an inflammatory response associated with the modified protein, a rabbit ileal loop analysis was performed to determine whether there was inflammation in the gut and whether the FaeG protein could neutralize the ETEC bacteria and prevent diarrhea. These experiments were deemed successful, indicating that this modified plant protein may be a potential additive to reduce post-weaning diarrhea in young pigs (6,108).

Kolotilin et al. (2) and Joensuu et al.(109) produced studies looking at whether an oral feeding of the modified protein could potentially survive in conditions similar to the gastrointestinal tract of the pig. This study produced solutions with a similar pH to the pig's stomach (approximately 3.5) and added intestinal contents to see whether a mucosal immune response occurred. In this study, researchers showed that the protein can bind to the epithelial F4 receptors, competing with bacterial adhesion, and reduce the incidence of disease (2,109).

Other studies have been conducted to determine whether other crop species can achieve a similar result to reduce bacterial attachment in the epithelial cells of the pig. Joensuu et al. (50) identified the ease in producing alfalfa plants with chloroplast modification including the FaeG protein and tested this modified plant within the animal. They determined that this product could stimulate a mucosal immune response and reduce *E. coli* excretion, but this was not as effective as the tobacco plant protein (50). Garg et al. (110) tested whether modifying the chloroplast in

soybeans could be a potentially more realistic alternative than feeding tobacco to pigs. Soybean meal is a common feed ingredient in swine rations. This study concluded that there was not enough protein in the plant chloroplasts to effectively compete with the *E. coli* bacteria (110). Rossi et al. (106) looked at feeding pigs tobacco seeds containing the FedA protein associated with the F18 fimbrial type of *E. coli* to see if the bacterial attachment could be reduced with the ingestion of the seeds. Tobacco seeds were identified as a good potential protein source due to the lower nicotine content than what is found in the leaves and their natural protection to avoid degradation in the stomach of the pig. They concluded that oral administration of ground transgenic tobacco seeds may protect the pig against post-weaning diarrhea with its ability to compete with bacterial attachment (106).

Therefore, tobacco chloroplast modification has been identified as one of the best potential protein sources to reduce ETEC in newly weaned pigs. Based on these research trials, it has been identified that oral, administered FaeG protein could successfully compete with bacteria to reduce colonization and growth.

1.7 Research Objectives

The nursery phase is a critical time in the life of a pig. Weaning is a very stressful time with many changes occurring all at once. This thesis will investigate methods to improve the health of pigs in the nursery by examining techniques to improve the pig's immune function and investigate a novel approach to reduce the impact of post-weaning *E. coli* diarrhea.

Chapter 2 of this thesis will look at whether iron status at weaning is related to growth performance and antibody response of pigs in the nursery. Chapter 3 will look at identifying whether a second injection of iron dextran at 14 days of age can ensure pigs at weaning have

adequate hemoglobin levels and whether preventing anemia can improve the piglet's ability to defend itself from post-weaning *E. coli* diarrhea. Therefore the overall objective of these two studies is to determine if pigs with normal iron status at weaning are immunologically stronger and healthier than pigs with anemia or iron deficiency. Chapter 4 will look at a unique alternative to the use of antibiotics to control post-weaning *E. coli* diarrhea. Newly weaned pigs will be fed lyophilized tobacco plant leaves containing a recombinant variant of the FaeG protein, rFaeG_{ntd/dsc}. The objective of this study is to determine if this plant-based product can protect susceptible pigs against enterotoxigenic *E. coli* by competing with the pathogenic *E. coli* for epithelial receptor sites.

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CHAPTER 2: THE RELATIONSHIP BETWEEN HEMOGLOBIN LEVELS AND GROWTH PERFORMANCE AND ANTIBODY RESPONSE IN NURSERY PIGS

2.1 Abstract

Piglets are born with low iron stores and sow's milk does not supply sufficient iron to meet their needs. It is routine practice for piglets to be given iron supplementation. However, pigs at weaning are commonly deficient in iron and possibly anemic, which might cause them to be less robust in the early nursery phase. The objective of this study was to determine whether the hemoglobin (iron status) of pigs at weaning can impact growth rate and antibody response to vaccination. In order to create pigs with varying levels of hemoglobin at weaning, 254 piglets at 3 days of age from 22 litters were randomly assigned to 1 of 3 iron treatments including; 100 mg of iron dextran on day 3 (Treatment 1), 200 mg of iron dextran on day 3 (Treatment 2) or 200 mg of iron dextran on day 3 and 14 (Treatment 3). At 3 weeks of age, 145 pigs were followed through a six-week nursery phase. Blood samples were collected, and individual weights were taken at 3, 6 and 9 weeks of age in order to determine the hemoglobin level and serum antibody response to *Mycoplasma hyopneumoniae* vaccine and the impact on growth rate respectively of the pigs

throughout the nursery phase. Pigs were categorized as; anemic (<90 g/L of hgb), iron deficient (90-110 g/L hgb) or normal (>110 g/L hgb) based on their hemoglobin level at weaning. Overall, 134 (92%) of pigs had a normal hgb status (>110 g/L of hgb) by the end of the nursery. Hemoglobin status at weaning did not have an impact on nursery growth rate or the pig's antibody response to vaccine ($P>0.05$).

2.2 Introduction

Iron deficiency is a concern for piglets during the first 3 weeks of life while they are nursing and consuming only small amounts of creep feed. Pigs are born with limited body iron reserves of approximately 50 mg and most of this is tied up in hemoglobin [1]. Milk is a poor source of iron and doesn't provide the daily iron requirement of 7-10 mg per day [2,3]. An intramuscular (IM) injection of 200 mg of iron (gleptoferron or dextran) during the first week of life is standard practice on most swine farms [4–6]. However, pigs have been constantly changing with intense genetic selection for faster growing and lean animals, and recent studies indicate that the fastest growing and largest piglets at weaning may be iron deficient [7].

Iron deficiency at weaning is a concern because the first few weeks in the nursery are a critical time in the pig's life. This is a period of transition and can be stressful. For example, this is a period when pigs encounter new pathogens and it coincides with the loss of passive immunity from the sow's milk. Therefore, it is important that the pig's immune system is as robust as possible to meet these disease challenges [8–10]. At weaning, pigs are also often vaccinated in order to develop immunity against common viruses and bacteria that they may face later in production [11,12]. For example, the *Mycoplasma hyopneumoniae* vaccine is commonly given at the time of weaning in order to protect pigs from clinical respiratory disease in the grower-finisher stage [13].

However, the impact of iron status at weaning on immune response to microbial pathogens and growth performance in nursery pigs is not well studied.

The objectives of this study were 1) to examine the relationship between iron status (hemoglobin) at weaning and immunity measured by evaluating response to vaccination of pigs against *M. hyopneumoniae* and 2) to determine whether iron status at weaning has an effect on the growth rate of pigs in the nursery stage of production.

2.3 Materials and Methods

The study was approved by the Animal Care Committee at the University of Guelph and conducted at the Arkell Swine Research Station.

Pig selection & iron administration

In order to create pigs with varying levels of hemoglobin at weaning 3 different iron supplementation regimens were applied to piglets at 3 days of age. Twenty-two litters of pigs (11 litters in each trial) were selected for this study. On the day after farrowing, all piglets were identified by an individual ear tag and assigned to one of 3 iron dose treatments through systematic random sampling. The treatment groups were: Low dose (treatment 1) 100 mg iron dextran (Uniferon®200, Pharmacosmos Inc., Watchung, NJ, USA) on day 3, Medium dose (treatment 2): 200 mg iron dextran on day 3, and High dose (treatment 3): 200 mg iron dextran on Day 3 and Day 14. Each iron dosage was given individually by IM injection. Creep feed was not provided in the farrowing room. Two replicates of this study were performed. In total, 145 pigs (Trial 1: n=70, Trial 2: n=75) were enrolled in the study. The parity of each sow was recorded and included in data analysis.

Nursery housing and phase feeding

At 3 weeks of age all piglets were weaned into the nursery. Pigs were housed in one nursery room and were split by gender according to pen, placing gilts and barrows in specific nursery pens. On average, 10 pigs were housed in each pen. Pigs were provided access to feed ad-libitum. Throughout the nursery phase, a total of four starter rations were fed over the course of 6 weeks (Table 2.1). Each starter phase was fed according to pig weight guidelines. Individual weights were taken at 3 (weaning), 6 and 9 weeks of age. At 9 weeks of age, piglets were taken off trial and moved into the grower unit.

Sample collection

At 3, 6 and 9 weeks of age, two individual blood samples were collected from each pig via the orbital sinus. To analyze the hgb level, one blood sample was collected into a tube (BD Vacutainer®, BD, Franklin Lakes, NJ, USA) containing an anticoagulant (EDTA). Whole blood samples were put on ice and stored in an insulated container before being submitted to the Animal Health Laboratory (AHL), University of Guelph within 3 hours after collection. The second blood sample was collected into a plastic serum tube (BD Vacutainer®, BD, Franklin Lakes, NJ, USA). The blood samples were centrifuged at 2500 RPM for 20 minutes and sera were collected into to 2mL tubes and stored at -20⁰C for further analysis of antibody response to *M. hyopneumoniae* vaccine.

Hemoglobin measurement

Whole blood samples were submitted to AHL. Samples were analyzed for hemoglobin determination using an ADVIA 2120/2120i Hematology system (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

M. hyopneumoniae vaccination and antibody detection

All pigs were vaccinated (2 mL IM) against *M. hyopneumoniae* (Suvaxyn® MP/HPS, Zoetis, Kirkland, QC) at weaning and 3 weeks post weaning (6 weeks of age). Frozen blood serum samples from pigs at 3, 6 and 9 weeks of age were submitted to AHL to test for antibody to *M. hyopneumoniae* (IDEXX M. Hyo Ab Test; IDEXX Laboratories, Inc., Maine, USA). The continuous values of S/P (sample-to-positive) ratio provided by the manufacturer were used to assess antibody response to the vaccine. Pigs were categorized as seronegative (S/P ratio <0.3), seropositive (S/P ratio >0.4), or suspected (S/P ratio between 0.3 and 0.4).

Hemoglobin (iron status) groups

Piglet iron status was categorized based on piglet hgb levels at 3 weeks (weaning), 6 weeks and 9 weeks (nursery exit) of age using the cut-off values described by Bhattacharai and Nielsen [6]. Pigs were categorized as “anemic” if hgb level was <90 g/L of hgb, “iron deficient” if hgb was within 90-110 g/L, and “normal” if hgb was >110 g/L [6].

Data analysis

Data were entered into Microsoft Excel 2016 (Microsoft, Redmond, Washington, USA) Version 15.28, and were imported into STATA 14 (STATA for Mac; StataCorp, College Station Texas, USA). The mean, median, proportion and standard deviation of each outcome including

S/P ratio and seropositivity to *M. hyopneumoniae* vaccine, hgb levels (iron status), and growth rates were described at each collection period.

A mixed effects multi-level linear regression method with sow (clustering) as a random effect was used to determine the relationship between seropositivity to *M. hyopneumoniae* vaccine and total average daily gain. A mixed-effects multi-level linear regression method with sow (clustering) and test (repeated measure) as random effects were also used to determine the association of hemoglobin with weight within the 6-week time period pigs were in the nursery.

The collinearity between independent variables was evaluated using a Pearson correlation coefficient. The outcomes were assessed for normality using a histogram visual aid. Linearity between the outcome and continuous independent variables was assessed using a lowess smoother curve and by generating a quadratic term. Independent variables including iron supplementation, parity, and trial were considered for inclusion into multivariable analysis if *P*-value <0.2 in

univariable analysis. The statistical significance of variables was included in the final model if $P<0.05$.

2.4 Results

The growth performance parameters and immune response parameters at three different points in time in each trial are shown in Table 2.2. In Trial 1, the average hgb level at weaning was 77.0, 100.2 and 122.1 g/L and in Trial 2 it was 81.8, 98.8, and 127.0 g/L for pigs that were “anemic”, “iron deficient or “normal”, respectively. The seropositivity to *M. hyopneumoniae* in all pigs at weaning were below negative cut-off values (S/P ratio <0.3) as pigs were raised in a *M. hyopneumoniae* free research farm. In Trial 1, the mean weaning weight for pigs that were “anemic”, “iron deficient” or “normal” levels were 7.8 ± 0.9 , 7.6 ± 0.9 and 6.3 ± 1.4 kg and in Trial 2, 7.2 ± 1.0 , 6.2 ± 1.3 and 6.2 ± 1.0 kg, respectively. In both trials, there was no significant difference in the average daily gain between sample collections or the entire 6-weeks spent in the nursery ($P>0.05$).

In Trial 1, pigs exiting the nursery at 9 weeks of age had a mean hgb level of 128.8 ± 5.8 g/L and all pigs were classified as “normal”; the mean S/P ratio was 0.42 ± 0.2 . The average exit weight of pigs in Trial 1 was 26.6 ± 3.5 kg with total average daily gain of 0.56 ± 0.08 kg/day from 3 to 9 weeks of age. In Trial 2, there were no anemic pigs leaving the nursery, but n=11 pigs were iron deficient (Appendix I Table 2). The mean hgb level of the iron deficient pigs and the normal pigs was 108.1 ± 2.1 g/L and 123.5 ± 5.1 g/L, respectively. The mean S/P ratios of the iron deficient and normal pigs was 0.81 ± 0.5 and 0.97 ± 0.5 , respectively. Based on the iron status of pigs at 9 weeks of age, there was no difference on the S/P ratio between the pigs that had a normal iron status and those that remained iron deficient ($P>0.05$). In Trial 2, the average exit weight of iron

deficient pigs was 22.9 ± 2.8 kg with a total average daily gain from 3 weeks of age to 9 weeks of 0.40 ± 0.06 kg/d. However, in “normal” pigs the mean exit weight was 25.8 ± 3.8 kg with a total average daily gain of 0.46 ± 0.07 kg/d. The individual weight at 9 weeks of age in the iron deficient pigs was significantly less than that in normal pigs ($P < 0.05$). In Trial 2, average daily gain was 0.10 kg/d less than in Trial 1 ($P < 0.05$). At the end of the nursery, combined final weights indicated that pigs with a “normal” iron status were 2.62 kg heavier than pigs that were “iron deficient” ($P < 0.05$).

The results of multivariable analysis for the factors associated with antibody response (S/P ratio) to *M. hyopneumoniae* measured at end of nursery in Trial 2 are shown in Table 2.3. The pigs with heavier weaning weights and pigs with a higher S/P ratio at 6 weeks of age had a higher S/P ratio at 9 weeks of age. However, piglets from older sows had a lower S/P ratio at 9 weeks of age ($P < 0.05$).

In both trials, the hgb (iron status) at weaning was associated with the weight of pigs at 3 weeks of age (Table 2.4). In Trial 1, “normal” pigs had a lower weaning weight than “iron deficient” pigs ($P < 0.05$). In Trial 2, “anemic” pigs at weaning had heavier weaning weights than “iron deficient” pigs ($P < 0.05$). In Trial 2, the total average daily gain was associated with the pig’s hgb level (iron status) at 9 weeks of age. Pigs with a “normal” status had 4 g/d higher average daily gain than “iron deficient pigs” during 9-weeks nursery stage ($P < 0.05$). In combined trials, pigs that had an iron status that was greater than 110 g/L at 9-weeks had a total ADG that was on average 6 g/d heavier than pigs that were iron deficient ($P < 0.05$).

2.5 Discussion

In order to investigate the relationship of iron status (hgb) with immune function and growth rates, three different of hemoglobin levels (iron status) was successfully induced in pigs by supplementation of three dosages of iron to suckling piglets. The objective of this study was to determine if iron status based on hgb levels at weaning influenced the growth rate and antibody response to *M. hyopneumoniae* in nursery pigs. This study found that there was no association between the iron status of pigs at 3 weeks of age and their antibody response to *M. hyopneumoniae* at 9 weeks of age. The antibody response was not detectable until 9 weeks, i.e. 3 weeks after pigs received the *M. hyopneumoniae* vaccine booster. Similarly, iron status at 3 weeks of age did not affect the total overall weight gain from 3 to 9 weeks of age. However, it is important to note that the majority of pigs were able to improve their iron status and have “normal” levels upon nursery exit. Of n=11 (8%) pigs that remained iron deficient throughout the nursery growth performance was reduced compared to n=134 (92%) of pigs that had normal iron levels at end of nine weeks.

Improving piglet weight at weaning is a primary goal for swine producers. When pigs are heavier at weaning, they have greater body and energy reserves to help them overcome the lag period many pigs experience in the first week post-weaning leading to fewer days to market. A trend has been seen throughout the literature indicating that heavier pigs at weaning are often more anemic. Studies have found contradicting results related to anemia in pigs entering the nursery and its impact on growth rate. Perri et al. (14) found that pigs that were “anemic” had heavier weaning weights than pigs with improved iron status. This study also found that anemic pigs at three weeks of age entering the nursery had a lower growth rate from 3 to 6 weeks of age than pigs with normal iron levels at weaning [14]. Similarly, Bhattacharai and Nielsen found that pigs that were both larger and heavier had reduced serum iron concentration at weaning [6]. Pigs that are larger often have a

greater iron requirement, especially in the last few days before switching to highly palatable starter feeds that include dietary iron. They require a greater blood volume and have an increased hemoglobin requirement to provide proper nutrients to tissues throughout the body [15]. They are limited by the 200 mg injection which is conventionally given in the first few days post-farrowing. On the other hand, the smaller pigs have a reduced iron requirement and may sufficiently thrive with the 200 mg administered in the first few days post-partum in order to meet their hgb and iron requirement in the first 21 days. In both trials in the current study, the heaviest pigs at weaning were anemic. This indicates that the standard 200 mg injection of iron dextran may not be enough for all piglets in the farrowing room, and that pigs that are larger with rapid growth may need additional supplementation at some point prior to weaning.

Because the majority of pigs were able to improve their iron status throughout these 6 weeks, it is not surprising that when controlling for starting weight, the highest ADG was seen in the pigs with a normal iron status. These results are similar to the work of Bruininx et al. (16) who found that an additional iron injection within the neonatal period positively influenced the iron status of pigs at weaning but showed no effect on overall average daily gain [16]. Contradicting results were found in a study conducted by Schrama et al. who found that an additional 100 mg of iron given at day 3 through I.M. injection positively influenced the growth rate of the animal [17].

The current study found that piglets that were anemic at weaning were able to improve their iron status throughout the 6-week nursery period to achieve normal levels upon nursery exit. However; in commercial production at weaning, when piglets significantly reduce their feed intake and the inclusion rate of antimicrobials in starter rations is high, pigs often remain or become more anemic in the first few weeks post-weaning [14,18]. This is one of the essential reasons that iron

is provided in starter feeds in a form such as ferrous sulphate or fumarate that is easily utilized by the gastrointestinal tract (GIT) of the pig in order to make up for this deficiency.

A study conducted by Perri et al. found that pigs that were anemic at weaning (3 weeks of age) became more anemic by 6 weeks of age [14]. These authors speculated that the inclusion of high levels of zinc oxide (ZnO) which is commonly used to reduce post-weaning diarrhea (PWD) in the early stage of the nursery interfered with iron absorption leading to the increased levels of anemia post-weaning. Dietary inclusions of ZnO is typically 2500 ppm or higher in starter diets for newly weaned pigs [19]. The use of high levels of ZnO is often widespread in the Canadian swine industry. However, since in this study was conducted on a research farm where pigs fed a diet with 150 ppm of ZnO the results may not be representative of what happens on the majority of commercial nursery farms. It is possible that if pigs had been provided with a starter diet containing a higher ZnO, then anemia might have persisted much longer in the nursery.

When looking at the relationship between iron status at 9 weeks of age and the growth performance of pigs leaving the nursery, the 11 pigs that were iron deficient had reduced final weights, poorer overall weight gain and a lower total average daily gain throughout the nursery period. The study by Perri et al. [14] found that pigs with poorer iron status had reduced growth rates in the nursery. One difference between these two studies was that those pigs were only followed until 6 weeks of age, not throughout the entire nursery period [14]. One advantage of the current study design was that pigs could be followed for 9 weeks from birth until they are entering the grower barn which allowed to explore the association between iron deficiency and overall growth rate and weight gain over entire nursery period.

In order to assess the impact of hemoglobin (iron status) on immune function, pigs were vaccinated against *M. hyopneumoniae* two times (at weaning and at 2 weeks post-weaning) and the antibody response was measured by ELISA. As this study was conducted on a *M. hyopneumoniae* free research farms, pigs had no maternal antibody nor been exposed to *M. hyopneumoniae* before. A positive response to the *M. hyopneumoniae* vaccine was not detectable until 9 weeks of age (3 weeks after the second vaccination). However, at end of nursery (9 weeks of age) the majority of pigs had antibody level interpreted as seropositive. There was no difference in S/P ratio (antibody titer) based on the pigs' iron status at 3, 6 or 9 weeks of age. This indicates that when pigs are able to improve their iron status throughout the nursery period, antibody production is not limited. The antibody response is usually detectable between 3 to 6 weeks after exposure to the vaccine [20,21]. Although the IgM antibody has been shown to appear earlier than IgG after the first vaccination, the ELISA that was used in the current study could detect the IgG antibodies. Testing IgM antibodies may have shown an earlier antibody response by our 6-week collection period. Further studies need to determine whether iron status is associated with IgM production after a single vaccination.

In the current study, piglets received their initial vaccine at weaning (3 weeks of age). The vaccine booster was received by the pigs at 6 weeks of age when the majority of the animals had overcome anemia. At both 3 and 6 weeks of age, all pigs were classified as being seronegative to *M. hyopneumoniae* vaccine with no significant differences were based on hgb status at weaning. A similar immune response was found in human based on their antibody response and immunoglobulin concentration [22]. A study conducted in rats showed that the iron deficient rats had less influenza antibody titers than rats that were supplemented with recommended levels of dietary iron [23]. Overall, little consistency has been found in the literature in both humans and

animals to determine how anemia may impact the antibody response and immune function in newly weaned pigs [5,12].

In summary, piglets that were anemic and iron deficient at weaning were able to improve their iron status by 9 weeks of age. Hgb status at 3 weeks of age was not associated with the pig's total average daily gain nor with the pig's antibody response to *M. hyopneumoniae*. However, a few pigs that did remain iron deficient at the end of the nursery period had a lower weight at 9 weeks of age and a lower total average daily gain throughout the nursery. Future studies should examine whether anemia at weaning persists for the first 3 weeks of the nursery when therapeutic levels of ZnO are included in starter rations.

2.6 Acknowledgements

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Table 2.1: Reference for analysis of the four starter rations that were fed the pigs in the nursery over the course of 6 weeks. Diet phases were fed according to body weight of the pigs

Component	Diet Phase			
	Starter 1 (7.2-11.9 kg)*	Starter 2 (11.9-16.2 kg)	Starter 3 (16.2-20.1 kg)	Starter 4 (20.1-28.1 kg)
Crude Protein (min) %	20	19.5	19	18
Crude Fat (min) %	3.0	3.0	2.5	2.0
Crude Fibre (max) %	2.5	3.5	3.0	3.5
Calcium (actual) %	0.83	0.8	0.69	0.66
Phosphorus (actual) %	0.67	0.66	0.57	0.55
Sodium (actual) %	0.3	0.25	0.2	0.2
Copper (actual) mg/kg	125	125	125	125
Zinc (actual) mg/kg	150	150	150	150
Vitamin A (min) IU/kg	10,000	10,000	10,000	10,000
Vitamin D (min) IU/kg	1,500	1,500	1,500	1,500
Vitamin E (min) IU/kg	70	70	65	65
Selenium mg/kg	0.5	0.5	0.5	0.5
Iron mg/kg	142	142	175	175
Lincomycin mg/kg	44	44	44	44
Kg Feed/Pig**	6	6	8	14

*weights refer to the average individual pig weight that was fed when pigs were transitioned onto each dietary phase

**kg of feed/pig is based on the average recommendation of feed intake per animal

Table 2.2: Mean (\pm Standard Deviation) of outcomes at 3, 6 and 9 weeks of age based on iron status at weaning

		Iron Status at 3 Weeks of Age					
<u>Pig Age</u>	<u>Parameter</u>	Trial 1 ^a			Trial 2 ^b		
		<i>Anemic</i> ^c n=23	<i>Deficient</i> ^d n=16	<i>Normal</i> ^e n=31	<i>Anemic</i> n=10	<i>Deficient</i> n=20	<i>Normal</i> n=45
3 Weeks (weaning)	Hgb ^f (g/L)	77.0 (6.34)	100.2 (5.16)	122.1 (5.83)	81.8 (6.03)	98.8 (4.88)	127.0 (9.87)
	Weight (kg)	7.8 (0.94)	7.6 (0.93)	6.3 (1.37) **	7.2 (0.96)*	6.2 (1.27)	6.2 (0.99)
6 Weeks	Hgb (g/L)	105.8 (13.14)	109.6 (7.46)	118.3 (12.53)	96.5 (8.70)	103.8 (7.98)	107.6 (8.12)
	Weight (kg)	14.0 (1.93)	14.0 (1.90)	12.4 (2.39)	13.5 (1.67)	13.28 (3.04)	13.1 (2.18)
	ADG Week 3-6 (kg/day)	0.29 (0.078)	0.31 (0.056)	0.29 (0.067)	0.26 (0.061)	0.30 (0.086)	0.29 (0.065)
9 Weeks	Hgb (g/L)	130.5 (6.02)	127.9 (5.78)	128.0 (5.59)	120.9 (7.65)	120.9 (8.31)	121.4 (6.81)
	S/P Ratio ^g for <i>M. hyopneumoniae</i> ELISA	0.45 (0.20)	0.37 (0.16)	0.43 (0.27)	1.18 (0.54)	0.99 (0.50)	0.88 (0.43)
	Weight (kg)	27.46 (2.90)	27.96 (3.07)	25.37 (3.83)	25.98 (3.46)	25.57 (4.59)	25.13 (3.58)
	ADG Week 3-9 (kg/day)	0.56 (0.080)	0.58 (0.073)	0.55 (0.090)	0.45 (0.073)	0.46 (0.839)	0.45 (0.712)

*represents parameters where iron status at weaning represented a significantly increased outcome ($P<0.05$)

** represents parameters where iron status at weaning represented a significantly decreased outcome ($P<0.05$)

^an=70 in Trial 1

^bn=75 in Trial 2

^cAnemic=<90 g/L hgb

^d Deficient= 90-110 g/L hgb (referent)

^e Normal=>110 g/L hgb

^fHgb= hemoglobin

^g S/P ratio= sample-to positive ratio representing the antibody titer

Table 2.3: Mixed effects multi-level analysis of factors associated with antibody response (S/P ratio) to *M. hyopneumoniae* vaccine measured at end of nursery in pigs in Trial 2

Variable	Coefficient ^a	Standard Error	95% Confidence Interval	P-Value
Weight at Weaning (Kg)	0.101	0.0427	0.0171, 0.184	0.018
S/P Ratio at 6 Weeks	1.924	0.936	0.091, 3.758	0.04
Parity ^b	-0.74	0.265	-1.26, -0.222	0.005

*Mixed effect linear regression model including sow id as a random effect.

^a The coefficient represents the change in each outcome if the variable is increased by one unit or compared to the referent category (ie. for every increase in kilograms of weaning weight, the S/P ratio of the pig will increase by 0.10).

^bThe parity of the sow was recorded at farrowing for each litter. Parity ranged from parity 1 to parity 5 sows.

SE= standard error

CI=confidence interval

S/P ratio- sample-to-positive ratio

Table 2.4: Mixed effects multi-level analysis of association between piglet hemoglobin (iron status) and piglet weight at weaning (kg)

Variable	Coefficient ^a	Standard Error	95% Confidence Interval	P-Value
<i>Trial 1</i>				
Hgb Status at 3 Weeks				
Anemic ^b	0.589	0.386	-0.383, 1.562	0.235
Deficient ^c	Ref			
Normal ^d	-1.976	0.386	-2.732, -1.220	<0.001
<i>Trial 2</i>				
Hgb Status at 3 Weeks				
Anemic	0.905	0.392	0.136, .674	0.021
Deficient	Ref			
Normal	0.072	0.265	-0.446, 0.590	0.786

* Mixed effect linear regression model including sow id as a random effect

^aCoefficient represents the change in each outcome if the variable is increased by one unit or compared to the referent category (ie. anemic pigs have an average weaning weight that is 0.589 kg heavier than pigs that are iron deficient at weaning)

^b Anemic=<90 g/L hgb

^c Iron deficient= 90-110 g/L

^d Normal=>110 g/L hgb

SE= standard error

CI=confidence interval

CHAPTER 3: THE ASSOCIATION BETWEEN PRE-WEANING IRON SUPPLEMENTATION AND POST-WEANING DIARRHEA IN PIGS CHALLENGED WITH ENTEROTOXIGENIC *E. COLI*

3.1 Abstract

Rapidly growing piglets are often iron deficient at the time of weaning and iron is thought to play a role in immunity. The primary objective of this study was to determine if pigs receiving additional iron supplementation prior to weaning would be less susceptible to enteric disease compared to pigs given the standard or less than standard iron supplementation. The secondary objective identified whether other blood parameters are associated with an additional iron injection in the neonatal period. Piglets were randomly assigned to 1 of 3 iron treatments including; 100 mg at 3 days of age, 200 mg at 3 days of age (standard), or 200 mg of iron dextran I.M. at 3 and 14 days of age. Shortly after weaning 60 pigs with susceptibility to F4 *E. coli* were challenged orally with 2 mL of 10^9 CFU/mL F4 enterotoxigenic *E. coli*. Pigs were observed for clinical signs of diarrhea and rectal swabs were taken and tested for presence of ETEC. There were no differences in clinical signs and severity of disease among pigs from different iron treatments ($P>0.05$). Additional iron supplementation in the neonatal period increase hemoglobin level at weaning along other hematological parameters ($P<0.05$).

3.2 Introduction

In late fetal development and the neonatal period, iron is critical to ensure proper neurological function and growth (1). Pigs are more prone to iron deficiency than other livestock due to their lack of iron stores at birth and fast growth rates during the first few weeks of life while nursing (2). Sows' milk is a poor source of iron, providing piglets with only about 1 mg of iron per day, which is only approximately 14% of their daily requirement (2–5). The most immediate result of iron deficiency is anemia because iron is essential for hemoglobin development. It is

common practice on commercial farms to inject piglets with 200 mg intramuscularly with iron dextran or gleptoferin in the first few days of life (4,6–8). However, recent studies have shown that this level of iron supplementation may not be adequate for large, fast growing suckling pigs and these pigs may become iron deficient or anemic by the time they are weaned (4,9,10). Hemoglobin (hgb) is typically measured as a common indicator of iron sufficiency, with anemia being diagnosed when levels of hgb fall below 90g/L (11). In addition, iron is a major component of hemoglobin and therefore, critical in oxygen transport. There is evidence that iron affects immune function and may impact the immune system's ability to properly produce immune cells (12).

At weaning, piglets are faced with many stressors including dietary and environmental changes and exposure to pathogens at a time when their immune system is immature (13–15). It may be that anemia causes pigs to be more susceptible to enteric pathogens such as enterotoxigenic *E. coli* (ETEC) which is one of the most common bacterial pathogens causing post-weaning diarrhea (PWD) (2).

The primary objective of this study was to determine whether iron supplementation, particularly a second injection of 200 mg of iron dextran before weaning to ensure adequate hgb levels in newly weaned pigs could influence the development and severity of post-weaning diarrhea in weaned pigs that were experimentally challenged with enterotoxigenic *E. coli*. A secondary objective was to examine the effect of 3 different iron supplementation protocols on blood parameters, including hgb at weaning in order to determine if a second injection of iron dextran could prevent iron deficiency at weaning.

3.3 Materials and Methods

This study was approved by the Animal Care Committee of the University of Guelph and was conducted in accordance with the guidelines of the Canadian Council on Animal Care. Two trials were performed.

Twenty-two litters of pigs (11 litters for each trial) at the Arkell Swine Research Unit, University of Guelph were selected for this study. On the day after farrowing, all piglets (n=254) were identified by an individual ear tag and assigned to 1 of 3 iron treatments through systematic random sampling. The treatments groups were: Low dose (Treatment 1, 87 pigs) 100 mg iron dextran (Uniferon®200, Pharmacosmos Inc., Watchung, NJ, USA) on Day 3, medium dose (Treatment 2, 85 pigs) 200 mg iron dextran on Day 3, and High dose (Treatment 3, 82 pigs) 200 mg iron dextran on Day 3 and Day 14. Piglets were not provided with creep feed for the purpose of this trial. Each iron dosage was given individually by I.M. injection.

Enterotoxigenic *E. coli*- F4 receptors

Whole blood samples were collected at 7 days of age and at weaning (21 days of age) from a subset of pigs (n=164) into vacutainer tubes containing an anticoagulant (EDTA) (BD Vacutainer®, BD, Franklin Lakes, NJ, USA). Pig DNA was extracted from blood samples taken at 7 days of age by the Omega ENZA Tissue DNA Kit® (Omega Bio-tek, Norcross, United States). An RFLP-PCR was used to determine the polymorphism (susceptible versus resistant pig genotype) in the porcine *Mucin 4* gene (16). In each reaction well, 10 µl of 2x Taq Froggammix, FroggaBio®, 0.8 µl of primer A (*muc1*), 0.8 µl of primer B(*muc2*), 1.0 µl of the template DNA and 7.4 µl for a total of 20 µl was mixed (FroggoBio, Toronto, Canada). The primers used were 5'GTGCCTTGGGTGAGAGGTTA/ 5'CACTCTGCCGTTCTCTTCC along with 0.25 units HotStarTaq (16). The GenepHow Gel/PCR Kit® (Geneaid Biotech Ltd., New Taipei City, Taiwan)

was used to further purify PCR product. Then, 10 µl of PCR product was subjected to digestion by *Xba*I enzyme (New England Biolabs, MA, USA) according to the manufacturer's instructions. The PCR product is 367 bp which is not digested by *Xba*I if the pig does not have the F4 receptor (resistant allele) but is digested into 151 bp and 216 bp segment if the pig has the F4 receptor (susceptible allele) (Figure 3.1).

Blood parameters

At 3 weeks of age, individual whole blood samples were collected from each individual susceptible pig. Blood tubes were stored on ice in an insulated container for approximately 2 h before and submitted to the Animal Health Laboratory (AHL), University of Guelph. Samples were examined for a full non-differential analysis using an ADVIA 2120/2120i Hematology System (Siemens Healthcare Diagnostic, Deerfield, IL, USA). The blood parameters measured included hemoglobin (hgb), white blood cell (WBC), red blood cell (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Pigs were classified as “anemic” if their hemoglobin levels were less than 90 g/L, “iron deficient” if their hemoglobin levels ranged from 90-110 g/L, and “normal” if they had a hemoglobin level greater than 110 g/L (10).

Challenge trial

For each of the 2 trials, 36 susceptible weaned pigs of approximately 21 days of age were transferred to a level-2 biosafety facility and housed in 6 rooms with 6 pigs per room (2 pigs from each iron treatment per room). On arrival (Day 0), pigs were provided with non-medicated starter feed *ad-libitum*. On Day 1, the pigs in all but one room (control) were challenged using 2 mL of 10^9 CFU/mL ETEC by oral gavage. In total, 60 pigs (30 pigs in each trial) were challenged. On

Day 3, all pigs were euthanized, and samples collected for bacteriology and histological examination.

Challenge inoculum

E. coli O149:K91:F4 (JG280) isolate recovered from a clinical case was used to prepare the challenge inoculum. The isolate was first cultured in 5 ml brain heart infusion (BHI) (Thermo Scientific, Lenexa, Kansas) and incubated at 37°C for approximately 24 h. Then, 75 µl of seed culture was transferred to one of three 15 mL BHI flasks and incubated at 37°C on shaker (200 rpm) overnight. In order to determine the CFU/mL, the serial dilution of the overnight culture was prepared and plated onto Columbia blood agar (CBA) and MacConkey agar (MAC) and incubated at 37°C overnight.

Clinical observations

Prior to challenge and every 12 h post-challenge, pigs were observed and scored based on the general condition, diarrhea, depression, dehydration, and appetite. Rectal temperatures were also taken at each recording. Fecal scores were classified as 0 (normal), 1 (mild, pasty or loose stool), 2 (moderate, stool was quite liquid but coloured), and 3 (severe watery, clear diarrhea with scalding on the skin).

Sample collection, bacterial culture, and histology

A fecal swab was taken from each animal prior challenge and every day after challenge. The swabs were cultured on CBA and incubated at 37°C for 24 h. A pig was defined as being ETEC positive if a hemolytic colony was observed on CBA plate on at least one sampling.

On Day 3, all pigs were euthanized, and tissue samples taken from the jejunum, ileum and colon and were placed in jars containing formalin and submitted to AHL, University of Guelph for histological examination. The adherence of bacilli, presence of significant lesions from each section of the GIT, and the state of the intestinal villi were examined. Animals were scored as having histological lesions if one or more lesions were present. In addition, a sample from the colon was collected in a plastic Whirl Pak® bag (Nasco, Fort Atkinson, Wisconsin). The colon samples were then plated on CBA and incubated at 37°C overnight. Each pig was defined as having a positive culture if a hemolytic bacterial colony was observed on CBA plate.

Statistical analysis

Data were entered into Microsoft Excel 2016 (Microsoft, Redmond, Washington, USA) Version 15.28 and after cleaning imported into STATA 14 (STATA for Mac; StataCorp, College Station Texas, USA).

A mixed-effects multi-level logistic regression method with sow as random effect to account for clustering was used to determine the association of both iron treatment and hemoglobin with diarrhea, the presence of ETEC cultured from rectal swabs, bacterial colonization, bacteria culturing from post-mortem intestinal samples and histology lesions. A pig was classified as having diarrhea if at any time over the four observation periods they had a fecal score of 2 or greater. A pig was classified as ETEC positive if F4 *E. coli* was cultured from at least 1 fecal sample. Histological lesions were classified as present if a pig had at least one lesion in the jejunum, ileum or colon. In addition, a mixed-effects multi-level linear regression method was applied to assess the impact of three iron supplementation protocols on hemoglobin levels at weaning. A mixed-effects multi-level linear regression method with sow (clustering) and pig

(repeated measurements) as random effects was also used to determine the difference in rectal temperature in pigs with different levels of hemoglobin or iron treatment. A Pearson correlation coefficient was used to determine whether hgb was correlated with RBC, HCT, MCV, MCH, MCHC.

3.4 Results

A total of 60 pigs were challenged with ETEC while 12 pigs were not challenged (controls). One control pig in Trial 1 and two control pigs in Trial 2 were classified as having diarrhea. However, all 12 control pigs remained free of ETEC at all sampling points. In Trial 2, one pig from the Low treatment died acutely as a result of ETEC infection. Descriptive statistics looking at various parameters associated with clinical signs of infection are summarized in Table 3.1.

Overall, from the 20 pigs that were challenged and received the Low Treatment, 70% of the pigs were classified as “anemic” (<90 g/L hgb) and 30% of the pigs were “iron deficient” (90-110 g/L hgb). In the Medium Treatment, 15%, 30%, and 55%, of pigs were classified as anemic, iron deficient and normal. In the High Treatment 10% of the pigs were iron deficient and 90% of pigs were classified as normal (Appendix II Table 1).

The mean blood parameters are presented in Table 3.2. Pigs that received the Low Treatment had significantly lower hgb, RBC, HCT, MCV, MCH than pigs that received the Medium treatment ($P<0.05$). For pigs that received the High Treatment, hgb, HCT, MCV, MCH and MCHC parameters were significantly higher than pigs that received only 200 mg (medium treatment) ($P<0.05$). The Pearson correlation coefficient identified that RBC, HCT, MCV, MCH and MCHC blood parameters values were highly correlated with hgb (cut-off >0.6) (Appendix II Table 2).

Overall, no differences among treatment groups were noted regarding diarrhea score, fecal and colon ETEC culture, presence of ETEC on intestinal villi based on histological examination, or rectal temperature ($P>0.05$). However, in Trial 2, a trend was seen since diarrhea was more likely in pigs received High dose of iron (Treatment 3) deemed to be more diarrheic compared to pigs received the Medium dose (Treatment 2) ($OR= 1.43$; 95% CI=0.023-1.23; $P= 0.079$). In Trial 2, pigs that received the Low dose had a higher mean temperature than pigs that received the Medium Treatment ($OR= 0.270$; 95% CI= 0.075-0.465; $P<0.05$).

3.5 Discussion

Neonatal piglets are at risk of iron deficiency and anemia due to their high demand of iron because of fast growth in the first few weeks of life and due to the limited amount of iron reserves within the piglet (2,17,18). The main goal of this study was to determine whether increased iron supplementation during the suckling period to correct an iron deficiency would improve the pig's ability to resist ETEC infection. The results of this experimental challenge study showed that although a second injection of iron dextran was effective in correcting iron deficiency, iron supplementation did not affect the development of clinical signs of ETEC infection. However, providing an additional 200 mg of iron dextran at day 14 could significantly reduce the number of anemic and iron deficient pigs at weaning which is consistent with previous studies (7,19).

A small proportion of the control animals were classified as having diarrhea and with further examination, also had histological lesions. These pigs however were not shedding *E. coli* bacteria, nor did they have bacteria colonized on the intestinal villi or have bacteria cultured from their colon. This indicates that there may have been another form of infection causing these pigs to have diarrhea. Over 60% of pigs that were challenged with *E. coli* were also found to have histological lesions, however lesions are not always associated with an ETEC infection. It is more

common to see a high abundance of bacteria colonized on the intestinal villi after they have attached to receptor surfaces (20). Therefore, it is possible that prior to arrival, pigs may have had another underlying infection causing them to scour and have histological lesions.

Blood hemoglobin levels are one of the most common ways to identify iron deficiency in piglets (10,11). Hemoglobin is required to transport oxygen within the blood throughout the body and is required for proper cell function (9,18,19). Over two-thirds of iron is found within hgb which is one of the reasons why hgb is often used as an indicator of iron deficiency (21). Providing an extra 200 mg of iron dextran at day 14 can positively influence blood parameters that are associated with hgb and RBCs in the animal. However, the observation that RBC, HCT, MCV, MCH and MCHC were highly correlated with hgb may have several implications. First, hemoglobin was an appropriate choice to evaluate iron levels within the blood to identify how iron treatment impacted hgb level and to look at how hgb may influence a newly weaned piglets' ability to cope with an ETEC infection. Secondly, this analysis also indicates that any of those parameters could have been used as potential indicator of iron level.

A study conducted by Bhattari and Nielson (10) looked at other ways to identify iron deficiency in order to determine if hgb should be the most common indicator used. Their results found that other parameters including serum iron, total iron-binding capacity (TIBC), mean corpuscular hemoglobin (MCH) and mean corpuscular hgb concentration (MCHC) were a more accurate representation of iron deficiency in large versus small pigs at weaning. In fact, hgb was not different among the size of various piglets and it was determined to be less accurate than other blood measurements as iron deficiency indicator (10). Both MCH and MCHC can also be evaluated using a full non-differential analysis. However, in order to determine serum iron and

TIBC, an additional blood sample must be tested. TIBC refers to the amount of blood plasma proteins that are able to bind to iron (22).

An issue that can be problematic by administering high levels of iron is the risk of aiding in bacterial growth. Bacteria have been known to have a high requirement for iron. When iron is found in excess amounts within the bloodstream bacteria can more readily replicate (4,23,24). It was thought that pigs that had a normal iron status at weaning, induced by two injections of 200 mg 11 days apart, may have an improved ability to cope with an ETEC infection. It is possible that this higher inclusion of iron by two injections of 200 mg may have promoted bacterial growth if iron was provided in excess amounts of what the piglets could properly use and store. However, when looking at the results of combined trials, there was no difference in bacterial presence observed among the treatment groups in each trial. Further, based on the reference interval stated in the samples non-differential analysis, none of the pigs in this study were classified as having an excessive level of hgb over 158 g/L.

A study conducted by Masawe et al. found that children who were iron deficient may have been protected against bacterial infection (25). Dallman (23) reported that in humans, people that were iron deficient were more likely to become clinically ill due to respiratory or enteric infection. However, in human populations, it can be hard to determine if iron deficiency was the primary cause of illness (23). Because iron plays such a critical role in proper bodily function and health, anemia in the early growth stage can severely impact growth and performance later on in swine production (1).

When iron is supplied in excess of the animal's requirement, iron toxicity can occur. This is most common when there is a deficiency of the vitamins and antioxidants such as vitamin E

(24,27). Toxicity occurs within the first few hours after iron administration, which results in, lethargy, weakness, tremor and often sudden death of piglets (5,24,26). In this study, iron toxicity was not seen following iron administration. In the High Treatment, the 400 mg of iron dextran that was supplied to piglets on day 3 and 14 post-farrowing was split into two 200 mg injections. As a result, toxic levels were not achieved, and no piglets were found dead following iron administration.

In order to achieve a consistent number of pigs with a normal hemoglobin status (>110 g/L hgb) at weaning, based on the findings in this study and the results of Bruininx et al. , it is beneficial to provide more than one 200 mg iron injection within the neonatal period (7). Although this provides an advantage of an improved iron status at weaning, implementing this second injection requires additional pig handling and may increase the cost and time of labour. One of the easiest ways to provide this injection would be administering it at weaning when the pigs are being vaccinated and handled prior to entering the nursery. This however may not provide an immediate response in blood iron levels, although this is essential within the first few days post-weaning.

A second alternative could be to administer the second iron dose at the time of processing. Piglets tend to be handled at around one week of age in the farrowing room when castration takes place. This may be a time when a second injection could be administered to avoid the time and cost it would take to handle the pigs a third time. It is critical that iron injections are administered properly (5). Creep feed is another alternative way to supplement iron in the farrowing room. Iron sulphate is one of the most common palatable iron sources found in creep feed. It has more availability than substances such as iron carbonate and ferric oxide (5). It is crucial that creep feed is consumed by the pig in order to help provide extra iron to the piglet's dietary needs. If pigs do consume creep feed during the suckling phase, these animals overcome anemia more quickly (2).

Sanitation is one of the key contributors to F4 ETEC in commercial production. If pig nurseries are not cleaned properly and the bacteria stays within the environment, this causes pigs to ingest the bacteria and potentially infect pigs that are susceptible. In commercial settings where disease pressure is natural, additional iron supplementation may help improve the pig's ability to respond to bacterial pathogen load. However, in this experimental challenge study, the bacterial challenge was more concentrated than what would be seen in a commercial environment since the pigs were administered a more concentrated form of inoculum (10^9 CFU). As a result, this study makes it more challenging to identify if improved hgb status at weaning may improve the pig's ability to deal with a bacterial pathogen load in commercial production.

In summary, iron treatment and hemoglobin level did not affect the piglet's ability to resist against ETEC infection under experimental conditions. Iron treatment did influence the hemoglobin level of the pig along with other hematological parameters. Future research could determine the optimal time of a second iron injection during the neonatal period. This could be implemented by looking at whether iron administration was more successful when administered at processing, at 14 days of age, or at weaning. Another direction could focus on identifying the impact of iron treatment on post-weaning diarrhea in pigs in a commercial setting, which would be a useful way to account for bacterial load. Determining the impact of iron supplementation on a different disease of interest such as Salmonella may also be an effective way to determine how iron treatment in the suckling phase may contribute to the overall health status of newly weaned pigs.

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Table 3.1: Clinical observations, histology, and bacteriology results in pigs receiving different dosage of pre-weaning iron supplementation and challenged with ETEC

Analyte	Outcome	Score	Iron Treatment					
			Trial 1			Trial 2		
			Low ^a	Medium ^b	High ^c	Low	Medium	High
Diarrhea Score ^d	18h post-challenge	0	70	100	80	50	50	40
		1	10	0	10	10	10	20
		2	10	0	10	30	30	20
		3	10	0	0	10	10	20
	24h post-challenge	0	40	80	80	40	50	30
		1	30	10	0	20	10	30
		2	10	0	20	30	10	10
		3	20	10	0	10	30	30
	36h post-challenge	0	40	30	60	67	30	30
		1	10	20	10	11	0	20
		2	20	20	10	11	30	30
		3	30	30	20	11	40	20
Bacterial Culture	Fecal Swab ^e 18h post-challenge	0	20	30	20	20	20	20
		1	10	0	10	10	10	0
		2	0	10	10	10	10	0
		3	30	20	10	20	30	50
		4	40	40	50	40	30	30
	Fecal Swab 36h post-challenge	0	0	0	0	0	0	0
		1	10	10	20	0	0	0
		2	10	10	0	20	10	10
		3	20	40	10	10	30	10
		4	60	40	70	70	60	80
	Colon ^f	0	10	0	0	0	0	0
		1	10	0	0	0	0	0
		2	0	0	0	0	0	0
		3	20	20	10	40	30	20
		4	60	80	90	60	70	80
Colonization	Adherent Bacilli ^g	--	60	60	50	40	40	70
Histology Lesions ^h	Jejunum	--	70	70	70	30	10	20
	Ileum	--	60	40	50	20	0	0
	Colon	--	10	10	10	60	40	20
Temperature (°C)	18h post-challenge	--	39.2 (0.28)	39.2 (0.42)	39.4 (0.36)	39.6 (0.35)	39.4 (0.50)	39.7 (0.39)
	24h post-challenge	--	39.0 (0.41)	38.9 (0.44)	39.1 (0.36)	39.1 (0.45)	39.3 (0.38)	39.5 (0.32)
	36h post-challenge	--	39.2 (0.53)	39.4 (0.31)	39.3 (0.54)	39.3 (0.62)	39.2 (0.66)	39.6 (0.44)
Weaning Weight (kg)		--	6.94 (1.30)	7.92 (1.74)	6.49 (1.64)	6.38 (0.94)	5.82 (0.76)	6.00 (1.10)
Hemoglobin (g/L)		--	80.3 (6.62)	102.3 (13.65)	125.4 (10.20)	93.6 (11.91)	119.8 (8.34)	131 (10.71)

*At weaning blood samples were submitted to the Animal Health Laboratory for a CBC non-differential analyses to determine hemoglobin levels in each pig

^a Low Treatment= 100 mg of iron dextran on day 3

^b Medium Treatment= 200 mg of iron dextran on day 3

^c High Treatment= 200 mg of iron dextran on day 3 and 14

^d Diarrhea score; 0=none (stool maintains form), 1=mild (pasty or loose stool), 2=moderate (stool is liquid but coloured), 3=severe (watery and clear)

^e Fecal swabs were cultured and scored; 0=non-pathogenic, 1=non-pathogenic with slight discolouration, 2=pathogenic with slight hemolysis, 3= pathogenic with moderate hemolysis around and 4= pathogenic with severe hemolysis

^f Colon samples were cultured and scored; 0=non-pathogenic, 1=non-pathogenic with slight discolouration, 2=pathogenic with slight hemolysis, 3= pathogenic with moderate hemolysis around and 4= pathogenic with severe hemolysis

^g Intestinal samples were classified as positive if they had adherent bacilli

^h Histology lesions were identified as being present/not in sections of the jejunum, ileum and colon

Table 3.2: Mean (Standard Deviation) of blood parameters based in pigs among three different pre-weaning iron supplementations

Blood Parameter	Iron Dose		
	Low ^a	Medium ^b	High ^c
Hgb ^d (g/L)	90.54** (13.60)	112.13 (13.38)	127.13* (10.00)
WBC ^e ($\times 10^9$ /L)	8.70 (2.40)	10.02 (3.63)	9.72 (2.28)
RBC ^f ($\times 10^{12}$ /L)	5.38** (0.55)	5.93 (0.43)	5.93 (0.53)
HCT ^g (L/L)	0.32** (0.040)	0.38 (0.031)	0.42* (0.029)
MCV ^h (fL)	59.58** (3.80)	64.83 (5.09)	72.67* (4.69)
MCH ⁱ (pg)	16.83** (1.61)	19.13 (2.07)	21.67* (1.74)
MCHC ^j (L)	281.96 (13.05)	294.17 (14.46)	297.67* (11.02)

*Represents a blood parameter that was significantly greater than in the Medium treatment ($P<0.05$)

**Represents a blood parameter that was significantly less than in the Medium treatment ($P<0.05$)

^aLow Treatment= 100 mg of iron dextran on day 3

^bMedium Treatment= 200 mg of iron dextran on day 3

^cHigh Treatment= 200mg of iron dextran on day 3 and 14

^d Hgb= hemoglobin

^e WBC= white blood cell

^fRBC= red blood cell

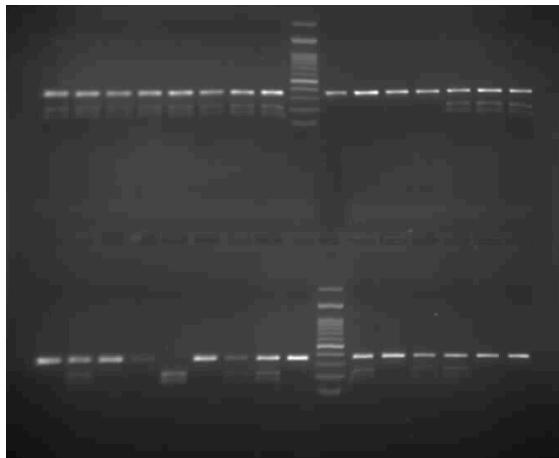
^gHCT= hematocrit

^h MCV= mean corpuscular volume

ⁱMCH= mean corpuscular hemoglobin

^jMCHC= mean corpuscular hemoglobin concentration

Figure 3.1: RFLP-PCR gel image representing pigs that are susceptible to F4 ETEC. Pigs with the susceptible allele is represented by bands at 151 bp and 216 bp segments



CHAPTER 4: USE OF PLANT-BASED FAEG FED TO NEWLY WEANED PIGS TO COMPETE WITH F4+ *E. COLI* FOR BINDING SITES AND TO PREVENT DIARRHEA

4.1 Abstract

Enterotoxigenic *E. coli* (ETEC) is one of the most common bacterial disease challenges facing newly weaned pigs. It causes morbidity and mortality in the nursery along with reduced growth rate in the later stages of production. The objective of this control-challenged study was to identify if feeding oral plant-based protein FaeG could reduce the clinical signs of illness associated with ETEC. Weanling pigs were challenged with *E. coli* O149:K91: F4 after receiving five dosages of plant-based FaeG protein for three days. In total, 72 pigs were assigned to one of four treatment groups; Control (fed wild-type), Wildtype (challenged and fed wild-type), Low (challenged and fed low dose of protein) and High (challenged and fed a high dose of protein). Feeding a high level (5g/day) of this product reduced the incidence of diarrhea ($P<0.05$). However, it did not affect the bacterial colonization, fecal culture or histology lesions of these piglets ($P>0.05$). Future studies could identify a more effective way to administer the plant-protein and if a greater dosage would be more effective at stimulating a greater mucosal response for piglets challenged by ETEC.

4.2 Introduction

At the time of weaning, pigs are faced with many stressors and challenges involved with moving from the neonatal to the nursery stage of production. Generally, at about 3 weeks of age, piglets are weaned, and mixed with other piglets during the nursery stage while suddenly being switched from milk to plant-based solid feed. In addition, weaned pigs no longer receive lactogenic immunity, which until this stage helped protect them from infectious enteric disease (1,2).

Diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is one of the major bacterial challenges facing newly weaned pigs on modern swine farms worldwide (3–6). Clinical signs include weight loss, dehydration, loss of appetite, severe watery diarrhea and possibly sudden death (1,4,7). Economic loss associated with *E. coli* diarrhea includes a negative impact on production parameters as well as the costs of treatment and prevention, which commonly involves group medication with antibiotics. Oral live vaccines have been proven to be somewhat effective, however, piglets are faced with the disease challenge before immunity is stimulated (8,9).

ETEC have F4 fimbrial appendages, which allow the bacterium to colonize and attach to the F4 receptor in the mucosal layer of the gastrointestinal tract (GIT) (7,10–12). The most common variant found on commercial swine operations is the F4ac which is composed of the major sub-unit FaeG (5,7,13,14). FaeG allows the bacteria to become adhesive and bind to specific receptors in the GIT of the pig (15). After ETEC attachment to the small intestine mucosa, the movement of intestinal contents allow further colonization and spreading towards the hind-gut of the pig (6,16). Once the fimbria attach to the receptor sites, heat-stable enterotoxins (STa and STb) along with heat-labile enterotoxins (LT) are released affecting homeostasis and fluid absorption in the GIT of the pig (5,17). This causes diarrhea and potential acidosis as a result of altered secretion of electrolytes and water in the GIT lumen, along with reduced absorption of fluids (4,6,18).

A recombinant variant of the FaeG protein, rFaeG_{ntd/dsc} has previously been produced in plant chloroplasts, and accumulation levels of 2.0 g rFaeG_{ntd/dsc} per 1 kg fresh leaf tissue have been obtained (10). We hypothesized that the plant FaeG protein administered orally to post-weaning pigs may compete for receptors along with the GIT and therefore block ETEC from attachment and colonizing in the GIT of the pig to reduce disease caused by ETEC.

The objective of this study was to determine whether a plant-based product containing the *FaeG* protein can protect newly weaned pigs from developing diarrhea when challenged by F4+ ETEC.

4.3 Materials and Methods

This study was approved by the Animal Care Committee of the University of Guelph and was conducted in accordance with the guidelines of the Canadian Council on Animal Care. Three replicates of this trial were performed. For each replicate, five litters from the Arkell Swine Research Unit, University of Guelph were selected for the study. On average, two gilts and two barrows were selected from each litter using systematic randomization and identified using an ear tag. For each trial, 24 susceptible pigs at three weeks of age, were transported to a level 2 biosafety facility, University of Guelph.

On arrival to the OVC Isolation Facility (Day 0), pigs were placed in four rooms (6 pigs per room) by blocking gender and littermates. In optimal conditions, three gilts and three barrows were placed in each treatment group. Each room was then assigned to a different treatment: Treatment 1 “Control” (2.5g/feeding of wildtype of tobacco, not challenged with ETEC); Treatment 2 “Wildtype” (2.5g/feeding of wild type of tobacco and were challenged with ETEC); Treatment 3 “Low” (1.25g/feeding of *FaeG* protein and were challenged with ETEC); Treatment 4 “High” (2.5g/feeding of *FaeG* plant protein and were challenged with ETEC). Over the course of the trial, piglets were provided with a non-medicated starter feed ad-libitum. Pigs were given two days to become acclimatized before receiving treatment. Each of the four treatment groups received five feedings of freeze dried, finely ground tobacco leaves mixed with 100 ml of chocolate milk. Pigs received individual allocated treatment twice per day (in the morning and afternoon) on Days 2 and 3, and once on Day 4 prior to challenge.

Enterotoxigenic E. coli- F4 receptors

At one week of age a whole blood sample was taken from a subset of 65 piglets through systematic randomization for each trial. DNA was extracted using the Omega ENZA Tissue DNA Kit® (Omega Bio-tek, Norcross, United States). In order to detect the polymorphism of the porcine *Mucin 4* gene, an RFLP-PCR was conducted (19). The PCR protocol and primers were described by Jensen *et al.*, (19). In each reaction well, 10 µl of 2x Taq Froggammix, FroggaBio®, 0.8 µl of primer A (*muc1*), 0.8 µl of primer B(*muc2*), 1.0 µl of the template DNA and 7.4 µl distilled water for a total of 20 µl was mixed (FroggaBio, Toronto, Canada) (19). The primers used for the polymerase chain reaction were primer A: 5'GTGCCTTGGGTGAGAGGTTA/ primer B: 5'CACTCTGCCGTTCTCTTCC along with 0.25 units HotStarTaq. The GenepHow Gel/PCR Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) was used to further purify the PCR product. Then, 10 µl of PCR product was used for *Xba*I (New England Biolabs, MA, USA) enzyme digestion according to manufacturer. The PCR product is 367 bp which is not digested by *Xba*I if the pig does not have the F4 receptor (resistant allele) but is digested into 151 bp and 216 bp segment if pig has the F4 receptor (susceptible allele) (19). Areas where the enzyme had been digested into 151 and 216 bp were considered susceptible. This image can be seen in Figure 4.1.

Preparation of plant-based FaeG

A transplastomic approach in tobacco was used to produce a recombinant variant of the FaeG protein, rFaeg_{ntd/dsc}, following the procedure outlined by Kolotilin *et al.* (10). The *E. coli* that the FaeG_{ntd/dsc} is derived from is the naturally-occurring ETEC strain C1360-79 (Serotype F4ad; Protein Data Bank entry 3GEA) (20). Kolotin *et al.* (10) determined that the properties of FaeG_{ntd/dsc} are very similar to the characteristics seen in oral vaccines, allowing the plant protein to bind to F4 ETEC receptor sites. The plant material was tested *in vitro* and shown to survive

gastric digestion (10). To scale up production of FaeG_{ntd/dsc}, 60 transplastomic tobacco plants were grown in a research greenhouse on a bench area of 20 m² at Agriculture and Agri-Food Canada. Sixty wild type, untransformed tobacco plants were grown in the same greenhouse compartment on a bench area of 20 m². Tobacco plants were grown in 8" pots and fertilized automatically with 20:8:20 NPK fertilizer at 0.5 g/L. The plants were grown for 11 months, harvested every 6-8 weeks, cut back to 15 cm above soil level at harvest and allowed to regrow. At each harvest, leaves were frozen at -80°C and then freeze dried (Labconco Corp. Kansas City, MO). Dried leaves were then pulverized in a Thomas-Wiley Laboratory Mill Model 4 (Thomas Scientific, Swedesboro, NJ) through a 2 mm screen and stored at room temperature in airtight containers. The amount of FaeG_{ntd/dsc}/dry weight was determined in the final product by quantitative SDS-PAGE versus known amounts of previously purified FaeG_{ntd/dsc}.

Challenge inoculum

An *E. coli* O149:K91:F4 (JG280) strain was first cultured in 5 ml Brain Heart Infusion (BHI) (Thermo Scientific, Lenexa, Kansas) and incubated at 37°C for approximately 24 h. Seventy five µl of seed culture was then transferred to one of three flasks containing 15 mL of BHI broth and shaken at 37°C (200 rpm) overnight. Serial dilution of the overnight culture (10⁻¹-10⁻⁸) was prepared and plated onto Columbia blood agar (CBA) and MacConkey agar (MAC) and incubated at 37°C overnight to determine the CFU/mL.

Challenge trial

For each trial, 24 susceptible, 3-week-old weaned pigs used. On arrival (Day 0), pigs were placed in 1 of 4 rooms. Pigs were provided with non-medicated starter feed *ad-libitum*. Each room contained one of four treatment groups with six pigs receiving each treatment. On Day 1 until the morning of Day 4, pigs were administered their feeding treatment. Room 1 contained Treatment 1

which were classified as “control” pigs. On Day 4, pigs receiving Treatments 2, 3 and 4 were challenged using 2 mL of *E. coli* O149:K91: F4 (JG280) strain by oral gavage. On Day 6, all pigs were euthanized, and post-mortems were conducted on each pig.

Clinical observations

Prior to and post-challenge, each pig was observed and scored based on their general condition, diarrhea score, depression, dehydration and appetite. Rectal temperatures were also taken at each recording. Fecal scores were accounted as 0-normal to 3- severe watery diarrhea. If at any time pigs had a fecal score 2 or greater, individual animals were classified as having diarrhea. General condition was observed as 0- normal to 3-severely abnormal. Depression was observed as 0-normal to 3- severely depressed. Dehydration and appetite were scored based on 0-good appetite or 1- off feed.

Sample collection, bacterial culture and histology

A fecal swab was taken from each animal prior to challenge and every day after challenge. The swabs were cultured on Columbia Blood Agar (CBA) and incubated at 37°C for 24 h. A pig was defined as being ETEC positive if at least at one sampling time a hemolytic colony was observed on a CBA plate. On Day 6, all pigs were euthanized, and tissue samples taken from the jejunum, ileum and colon and were placed in jars containing formalin and submitted to the Animal Health Laboratory (AHL), University of Guelph for histological examination. Samples were classified as positive or negative for adherent bacilli and comments were made indicating whether significant lesions from each section of the GIT were present and the state of the intestinal villi. Animals were scored as having histological lesions if one or more lesions were present.

A sample from the colon was placed in a plastic Whirl Pak® bag (Nasco, Fort Atkinson, Wisconsin) and plated on CBA and incubated at 37°C overnight. Each pig was defined as having a positive culture if a hemolytic colony was observed on CBA plate.

Statistical analysis

Data were entered into Microsoft Excel 2016 Version 15.28 and further imported into STATA 14. In each treatment group, 18 pigs in total were used over the course of three trials. In order to determine the effects of feeding treatment on pigs that were challenged with ETEC, Treatments 2, 3 and 4 were used for statistical analysis. Treatment 2 was used as the referent to determine the effectiveness of the feeding treatment. For analytic purposes in our mixed-effect logistic regression model, outcomes were made dichotomous. Pigs were classified as having diarrhea if at any time over the four observation periods they had a fecal score of 2 or greater. Hemolytic fecal swabs were classified as positive if the total culture score of the swabs was greater than one. Bacteria colonization was recorded as being present or not. Culturing results from the bacteria level of post-mortem colon samples were classified as hemolytic if they had a score of three or greater. Histological lesions were classified as present if a pig had at least one lesion in the jejunum, ileum or colon.

A mixed-effects logistic regression model was used with sow (to account for clustering) as random effect in order to look at the impact that feeding treatment had in the challenged pigs on dependent variables of interest (diarrhea score, fecal culturing, post-mortem culturing, post-mortem colonization and histological lesions). A mixed-effects linear regression model was used with sow (for clustering) and test (repeated measures) as random effects to look at the significance of temperature at each time point based on the feeding treatment that was fed to each group of pigs. Descriptive statistics were used to describe bacteria level, *E. coli* on fecal swabs,

colonization, histology lesions and diarrhea score for the different treatment groups for each trial.

Both individual trial and combined results for the three trials were analyzed.

4.4 Results

Plant growth in 40 m² greenhouse

Over the course of 11 months, 200.16 kg of leaves were harvested from wild type plants and 213.65 kg of leaves expressing FaeG_{ntd/dsc} (Table 4.1), generating about 20 kg of freeze dried leaves from each group. The average accumulation levels of FaeG_{ntd/dsc} in those plants was 7% of total soluble proteins (TSP), 1.45 g/kg fresh leaf weight and of 5.8 g/kg dry leaf weight (Table 4.1). Considering that the piglets were each administered a maximum of 5 doses of 2.5 g dry weight, our yield in a small greenhouse compartment of 20 m² would allow us to treat about 1,600 animals at this regimen.

Presence of ETEC in colon and fecal contents

In Trial 1, the average colony count in the challenge inoculum was 3.3×10^9 CFU/mL and in Trials 2 and 3, 2.7×10^9 CFU/ mL. The results for the bacterial colonization are shown in Table 4.2. A small subset (n=3) of the control pigs did have bacteria colonized in their GIT. For the remaining groups, approximately half of the pigs that were challenged with ETEC had bacteria colonized in their GIT. Overall, there was no significant difference in bacterial colonization in pigs among the treatment groups ($P > 0.05$).

The results for the bacterial culturing identified through post-mortem samples of the colon are shown in Table 4.2. Only 3 of the Control pigs had bacteria cultured from the colon. However, *E. coli* could be recovered from colonic contents of all pigs which were challenged with F4+

ETEC. Therefore, no difference in the culturing results was seen between pigs challenged with F4+ ETEC ($P>0.05$).

The results for ETEC shedding in each treatment group are shown in Table 4.2. Pigs were classified as shedding *E. coli* bacteria if the cultured fecal swabs on CBA showed a hemolytic bacterial colony. In our Control group, all of the pigs were classified as negative for ETEC. However, over 85% of pigs in the remaining three treatments were found shedding ETEC bacteria. Therefore, no difference in fecal shedding was seen between Treatments 2, 3 and 4 ($P>0.05$).

Histology lesions

The results for the histological examination identified through post-mortem samples of the jejunum, ileum and colon are shown in Table 4.2. Pigs were classified as having histology lesions if there was at least one lesion found in the jejunum, ileum or colon. There was no difference in the presence of histology lesions based on feeding treatment since at least one lesion was seen in the majority of challenged pigs ($P>0.05$)

Diarrhea score

Pigs that were classified as having diarrhea in each of the three trials are shown in Table 4.2. Pigs were classified as having diarrhea if at any time they had a fecal score that was 2 or greater. In Wildtype and Low treatments, over half of the pigs were scored as having diarrhea. The pigs in High treatment were less likely ($OR=0.19$, 95% CI= 0.04-0.94) to have diarrhea than pigs in the Wildtype treatment ($P<0.05$) (Appendix III Table 1).

4.5 Discussion

The objective of this study was to determine if a high or low feeding level of a plant-made FaeG fed to newly weaned pigs could protect them from infection with F4+ ETEC. In each of the three trials, only one pig in Control had diarrhea. This indicates that these pigs successfully remained as control group because they remained free of clinical signs of illness. Diarrhea is one of the most common clinical signs associated with *E. coli* infection. This is generally one of the first symptoms found when pigs are infected by the bacteria. When looking at clinical signs of disease associated with ETEC, improved fecal scores of pigs that received the high treatment indicates that 2.5g of protein may have been able to reduce PWD.

Bacterial colonization results are a good indicator of whether *E. coli* have been able to replicate and attach in the epithelial cell of small intestine of the pig. In severe cases of infection, intestinal villi are often found covered in bacteria (1). However, since there was no difference in bacterial colonization based on feeding treatment, the plant-based FaeG was not able to successfully compete with the bacteria fimbriae in order to block receptor surfaces. Whether or not bacteria are able to be cultured from colon samples is an indicator of the severity of infection associated with ETEC. The receptor surfaces are found only in the small intestine, which means that after bacterial attachment and proliferation, bacteria may have moved further down the GIT into the hind gut of the pig.

Improved fecal scores of pigs fed a high dose of the plant- made FaeG may be a result of some competitive inhibition between plant protein and bacterial attachment to the F4+ ETEC receptor sites. This indicates that the protein may have acted in a way to reduce electrolyte imbalance and reduce the number of enterotoxins released by bacteria. When enterotoxins are released, this is what alters fluid homeostasis in the GIT (1). Higher concentrations of water are

secreted in the gut causing a fluid imbalance leading to diarrhea (21). It is possible that improved fecal scores may have been a result of some FaeG protein attachment on the epithelial layer of the small intestine, resulting in less diarrhea.

For plants to be used for oral administration, the edible part of the plant must be modified to incorporate certain antigen proteins that are related to pathogenesis of the microbial agent (22–24). Kolotilin et al. (10) genetically modified tobacco plants to incorporate the rFaeG_{ntd/dsc} protein into the chloroplast genome for the production of an oral ETEC supplement in the leaf tissue. Their study concluded that tobacco plants have the ability to synthesize high levels of the FaeG protein, needed to bind to F4 ETEC receptors in the piglets GIT (10). It is, however, unknown how much plant leaf needs to be consumed by each piglet to effectively compete with *E. coli* binding to F4 receptors for competitive inhibition (24). In this study, feeding dosage was determined by observing the weight of the finely ground, dried plant product and taking into account the limitation and palatability in accordance with gut fill of the piglet (25,26). Based on various weights, 5 g per day as the high dose and 2.5 g per day as the low dose seemed appropriate amounts for piglet consumption. It is possible that this did not have enough FaeG antigens to compete with ETEC to attach F4 receptors on GIT epithelial cells. If it is feasible to increase FaeG protein consumption to more than 5 g per day, this may increase the likelihood of the protein attaching to receptor binding sites to reduce bacterial attachment more substantially.

When FaeG antigens bind to the epithelial layer of the lining in the small intestine, this inhibits bacteria from attaching to specific binding sites (23,27–30). However, the plant protein must be able to survive pig gastrointestinal conditions (10) and sufficient amounts of antigens need to remain intact (23,30,31). It should be noted that the cell wall provides a protective barrier to inhibit or reduce protein breakdown and allow the antigen to reach the epithelial lining of the

small intestine (27,32). It has been shown that the leaf tissue was more resilient than the purified protein (10). Therefore, feeding the leaf tissue containing the FaeG protein rather than providing the purified protein as an oral drench has a greater likelihood of preventing ETEC infection.

In this study, pigs were orally challenged with 2 mL of 2.9×10^9 CFU/mL of ETEC. However, the bacterial load in the environment of farrowing rooms and nurseries in commercial production might be lower than this inoculum. Once a pig ingests the ETEC bacteria, it may take several hours for the bacteria to attach to the mucosal layer with its fimbria, replicate, and release enterotoxins and reach this high concentration (1). ETEC often remain in the nursery environment and must be ingested by the pig to cause infection. To look at the feasibility of administering FaeG orally in a commercial environment, future studies are needed to identify the effect of feeding this plant-based protein to newly weaned pigs in a commercial setting where post-weaning *E. coli* diarrhea is an ongoing problem. In many cases, ETEC outbreaks can be related to management, sanitation, and housing environment. When nursery rooms are not properly sanitized, or the temperature is cool and has a draft, this can increase the severity of infection (1,33). Since general management in the nursery plays a big part of infection severity, introducing this study in a commercial setting may be beneficial to see if the protein can reduce morbidity more substantially.

As the livestock industry continues to move towards a decrease in antibiotic and antimicrobial use, alternatives must be found in order to maintain the health and welfare of nursery and grower pigs (23,31,34). With advances in plant-based production methods, deriving subunit vaccines from plants may soon become more affordable than other current vaccine production methods, particularly due to their low cost, use earlier in life, ease in storage, production and transportation (22,35,36). Studies have also been conducted looking at ETEC oral supplements using other more palatable plants such as alfalfa and soybeans which have been modified for oral

delivery (15,37). Use of a more palatable plant source with adequate levels of FaeG might be an effective way to increase protein intake in order to implement the protein into starter rations. Further, feeding the plant product as a top-dress may be a more effective way to promote protein attachment to the receptor binding sites if the animal continually ingests the protein during the two-weeks that it is most susceptible to ETEC after weaning. This would also be a more feasible approach in a commercial environment with regards to protection of the entire nursery population. However, a potential limitation with this feeding approach is that within the first few days post-weaning, piglets often reduce their feed intake (26). It may also be beneficial to allow piglets access to the plant protein while they are still nursing in the farrowing rooms, thereby allowing them to become used to the taste and texture of the feed additive. This would allow piglets intestinal mucosa more time for FaeG protein attachment to increase protection.

In summary, this controlled challenge study which successfully infected pigs with F4+ ETEC found that feeding a high level (2.5g /feeding) of freeze dried tobacco leaves containing 14.5 mg of FaeG protein was able to improve the fecal score of newly weaned pigs. Diarrhea is one of the most prominent clinical signs of illness associated with ETEC infection. Further research is needed to identify a feasible way to administer this protein on farm in order to make it a potential aid in dealing with post-weaning *E. coli* diarrhea in the two weeks post-weaning.

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Table 4.1: Yield of tobacco leaves and FaeG_{ntd/dsc} content in 20 m² greenhouse over 11 months

Harvest	Fresh leaf Yield		Average rFaeG levels (% of TSP ^a)	Average rFaeG levels (g/kg FW ^b)	Average rFaeG levels (g/kg DW ^c)
	Wild Type	FaeG _{ntd/dsc}			
1 st	19.7 kg	18.9 kg	5.28	0.83	
2 nd	25.6 kg	37.8 kg	5.79	0.95	
3 rd	41.9 kg	-			
4 th	30.9 kg	49.2 kg	6.67	2.18	
5 th	35.71 kg	52.75 kg	7.87	1.33	
6 th	46.35 kg	55.00 kg	9.8	2	
Total	200.16 kg	213.65 kg	7.08	1.45	5.8

^aTSP= total soluble protein

^bFW= fresh weight

^cDW= dry weight

Table 4.2: ETEC shedding and colonization, histological lesions, diarrhea score, and rectal temperature in pigs in each of the four Treatment groups

<i>Outcome</i>	Trial 1				Trial 2				Trial 3				Combined Trials^e			
	<i>1^a</i>	<i>2^b</i>	<i>3^c</i>	<i>4^d</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>
Bacteria Colonized in GIT	1	3	4	2	1	3	3	3	1	3	4	4	3	9	11	9
Bacteria Cultured in Colon	2	6	6	6	0	6	6	6	0	4	6	6	2	16	18	18
Histology Lesion	5	6	5	4	2	3	5	5	2	6	5	5	9	15	15	14
Fecal Shedding	0	6	6	5	0	5	5	6	0	5	6	6	0	16	17	17
Diarrhea Score	0	5	5	1	0	3	4	2	1	5	3	3	1	13	12	6
Temperature (°C)	39.9	39.2	39.4	39.7	39.6	39.7	39.9	39.5	39.6	39.5	39.4	39.3	39.7	39.4	39.5	39.4

* Three replicates were done for each treatment group, each composed of 6 pigs per trial. The number of pigs in each treatment were those classified as being positive for ETEC. Colonization was classified as positive based on a histology examination of intestinal tissue. Pigs were classified as having diarrhea if they had a fecal score of 2 or greater. Pigs were classified as shedding hemolytic *E. coli* bacteria and testing positive for culturing if hemolysis was seen on Columbia Blood agar.

^aTreatment 1: n=6 pigs that were fed 2.5g/feeding of a wild type of tobacco and were not challenged with ETEC

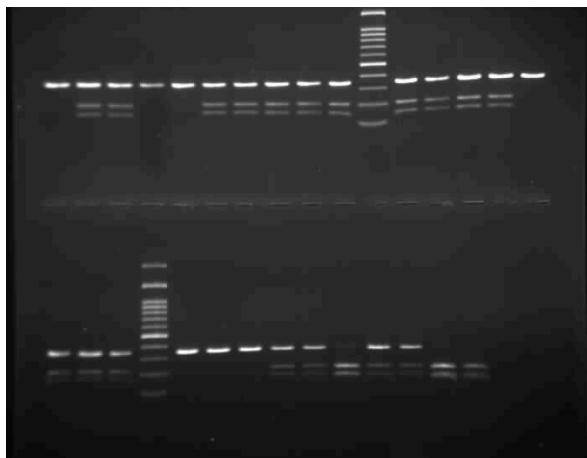
^bTreatment 2: n=6 pigs that were fed 1.25 g/feeding of a wild type of tobacco and were challenged with ETEC

^cTreatment 3: n=6 pigs that were fed 2.5g/feeding of FaeG product and challenged with ETEC

^dTreatment 4: n=6 pigs that were fed 2.5 g/feeding of FaeG product and were challenged with ETEC.

^eCombined Trials represents a total of 18 pigs per treatment group.

Figure 4.1: Gel image of an RFLP-PCR showing pigs susceptible to F4 ETEC tested by RFLP-PCR. The presence of a 151 bp and 216 bp segments indicates that the pig does have the susceptible allele.



CHAPTER 5: CONCLUSIONS

5.1 Research Summary and Conclusions

Typically newly weaned pigs suffer a lag in growth during the first 2 weeks in the nursery as they acclimatize to their new surroundings, feed changes, loss of maternal antibodies and other stressors associated with weaning (1,2). In addition, this is a time when pigs are vulnerable to disease challenges. Over the past 30 years the swine industry has adapted many practices to improve weanling pig performance. The use of high quality, palatable feed ingredients in starter rations that are easily digested and result in minimal gut inflammation are now used as well as the inclusion of antimicrobials in the feed. Disease challenge has been greatly reduced with the use of all-in/all-out management and specialized housing that maximizes cleanliness and pig comfort (3). Unfortunately, proper management cannot always prevent enteric disease and other health challenges. A further issue that has arisen is the public pressure to reduce the use of antimicrobials in livestock production so that new approaches to control post-weaning disease problems need to be found. One approach to improving pig performance in the nursery and minimizing the problem of disease is to ensure pigs are healthy and robust when they are weaned. Recent work has shown that in modern pig production growth rate during the suckling period has improved to the point where traditional levels of iron supplementation are sometimes inadequate, and pigs are entering the nursery in an iron deficient state.

The second chapter of this thesis looked at the impact of iron status at weaning on both average daily gain and total weight gain throughout the six weeks spent in the nursery. In addition, the role of iron in immunity was investigated by comparing the antibody response of anemic and iron deficient pigs with normal levels of hemoglobin. This was done by vaccinating pigs at 3 and 6 weeks of age against *Mycoplasma hyopneumoniae* and using an ELISA test to measure the

antibody response. In this study neonatal pigs were assigned one of three iron treatments (low, medium and high) in order to induce pigs of varying iron status. By categorizing piglets based on their hemoglobin status at weaning, it was determined that there was no difference in growth response or antibody production based on the animal's iron status (anemic, iron deficient or normal) at 3 weeks of age. It was noted that pigs in the nursery quickly recovered from iron deficiency and there were no anemic pigs at 9 weeks of age. This is most likely the reason no relationship was found between iron status at weaning and overall nursery performance or antibody production at 9 weeks of age. There were a small number of pigs ($n=11$) in the study that were still iron deficient at 9 weeks of age and interestingly these pigs did have poorer growth rate and lower weights at 9 weeks of age than the other pigs. Previous studies have found that nursery pigs on some farms remain anemic at least for the first 3 weeks in the nursery (4). It has been suggested that the common practice of using therapeutic zinc oxide at levels of around 3000 ppm might result in poor iron absorption and been the underlying cause of this prolonged anemia in the nursery. In the current study zinc was fed at about 150 ppm to meet nutritional requirements but not at a therapeutic dosage. Future research could look at inclusion of high levels of ZnO and copper sulfate and if this practice results in prolonged iron deficiency. Pigs that remain anemic for the first 3 weeks of the nursery may produce different results with regard to antibody production and growth compared to pigs in this study that quickly recovered once they began to eat iron in the starter feed.

There are several different methods to determine if the immune system is strong or to test if iron deficiency is somehow weakening the pig's ability to fight off disease. In Chapter 2 we evaluated the pig's ability to produce antibodies, and in the next chapter of the thesis we examined whether there was a difference between anemic pigs and normal pigs in their ability to defend

against infection using an experimental challenge of enterotoxigenic *E. coli*. We chose to use ETEC in this disease challenge model because it continues to be one of the most common and economically important diseases of post-weaned pigs (5,6). It is also one of the most common reasons for pork producers to use antibiotics in feed or water. If it could be shown that by improving iron status and making pigs stronger at weaning, it might be possible to reduce the need for antibiotic use in the nursery phase of pork production.

Chapter 3 investigated whether a second injection of 200 mg of iron dextran at 2 weeks of age would result in adequate hemoglobin levels and whether these pigs with good iron status were more robust and better able to defend against infection from an ETEC challenge compared to anemic pigs. This study found that additional iron treatment at 14 days of age resulted in adequate hemoglobin levels, but when challenged with there was no significant difference in the pig's ability to deal with infection. All animals were found to have diarrhea, shed bacteria and have bacteria colonized in their gastrointestinal tract (GIT). It is however very important to note that this was an experimental challenge study. The pathogen load that was administered to the 3-week-old pigs was possibly more concentrated than what would be seen in a commercial setting where ETEC is an ongoing environmental and sanitation issue. It might be worth performing a similar study under field conditions to determine whether a natural infection would produce similar results

It is important to consider that iron is required for many metabolic functions in the animal. In both Chapters 2 and 3, the additional injection of 200 mg on day 14 was an effective way to improve the iron status of the animal at weaning. In both of these studies the iron treatments that piglets received consistently produced categories of pigs that were anemic, iron deficient or had normal hemoglobin levels. These studies confirmed that large, fast growing suckling pigs require more than, a single 200 mg injection of iron dextran at 3 days of age (7).

The final research chapter of this thesis continued to examine the problem of post-weaning diarrhea caused by ETEC and again using an experimental *E. coli* challenge model to evaluate efficacy. In this chapter the approach was altered from trying to strengthen the pig's immune system by correcting a nutrient deficiency to examining a novel product that might be able to compete with pathogenic *E. coli* for the intestinal receptors and therefore prevent *E. coli* colonization. Enterotoxigenic *E. coli* can only cause disease if they can attach themselves to intestinal villi and not be swept away, by the flow of digestion. In order to achieve this adhesion *E. coli* use fimbriae. The most common strain of *E. coli* associated with post-weaning pig diarrhea possess F4 fimbriae which are composed of a protein FaeG. By using a plant-made FaeG it was hypothesized that the plant product would compete with *E. coli* for receptor sites and if sufficient sites were occupied by the plant FaeG product the *E. coli* would be unable to colonize. In this study 2.5g of plant product per feeding appeared to improve fecal scores of pigs, but treated pigs were seen to have very large numbers of bacteria adhering to villi at post-mortem examination in the treated pigs as well as controls and all challenged pigs shed the enterotoxigenic *E. coli*.

The protein is incorporated into the chloroplast of the tobacco plant, and leaves are harvested and freeze-dried in order to be fed to the animal. Several harvests allow high levels of production, however for this study, plants were grown in a small-scale greenhouse production system. Part of the issue in determining the feasibility of this protein in commercial production is knowing the right level to feed to the animal. In this pilot study, pigs were individually bottle fed their required feeding dosage, however this cannot be done on commercial operations. Another limitation is determining how to feed the protein. It cannot be included in pelleted starter rations because it is at risk of protein denaturation. Including this product as a top-dress may be the most feasible way to incorporate this into the industry, however, piglets often refuse to eat in the first

few days post-weaning. It may be beneficial to mix the protein with creep feed or peat moss in the farrowing crate to give them earlier access to receptor attachment. In this experimental challenge study, the concentration of the ETEC was possibly higher than what pigs would face in a commercial environment and doing field trials could be useful in further evaluating this product. There are many considerations and applications that need to be tested to determine whether growing this plant protein for commercial sale can show additional benefits before incorporating large-scale production.

Overall, these trials were able to incorporate a successful challenge model into new approaches to overcome ETEC infection. Although they were not successful at reducing the incidence of disease, we were able to determine that providing an extra 200 mg of iron dextran in the suckling period was able to iron levels in the pig along with other hematological parameters. In the nursery study, 92% of the pigs had a normal iron status by the time they left the nursery at 9 weeks of age. These results were rather unique to what is generally found in the literature and should be further studied to determine effective alternatives for overcoming anemia in the nursery. Feeding an oral FaeG protein to newly weaned pigs was able to improve the fecal scores of pigs challenged with ETEC, however it was not an effective treatment to prevent bacteria from colonizing in the GIT and reduce bacterial shedding in newly weaned pigs.

5.2 References

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APPENDIX I

Appendix I Table 1: Percentage of pigs in each treatment group classified by the pig's individual hemoglobin status at 3 weeks of age (weaning)

Hemoglobin Status at Three Weeks of Age						
Treatment Group^a	<u>Trial 1</u>			<u>Trial 2</u>		
	<i>Anemic^b</i> n=23	<i>Deficient^b</i> n=16	<i>Normal^d</i> n=31	<i>Anemic</i> n=10	<i>Deficient</i> n=20	<i>Normal</i> n=45
Low	88	8	4	36	57	7
Medium	8	54	38	0	17	83
High	0	5	95	0	0	100

^a Iron treatment received in the farrowing crate (low=100mg of iron dextran on d 3; medium=200mg of iron dextran on d 3; high=200mg of iron dextran on d 3 and 14)

^b Anemic=<90 g/L hgb

^c Deficient= 90-110g/L hgb

^d Normal=>110 g/L hgb

Appendix I Table 2: Percentage of pigs in each treatment group classified by the pig's individual hemoglobin status at nine weeks of age (nursery exit)

Hemoglobin Status at Nine Weeks of Age

Treatment Group^a	<u>Trial 1</u>			<u>Trial 2</u>		
	<i>Anemic^b</i> n=0	<i>Deficient</i> n=0	<i>Normal^d</i> n=70	<i>Anemic</i> n=0	<i>Deficient</i> n=11	<i>Normal</i> n=64
Low	0	0	100	0	29	71
Medium	0	0	100	0	8	92
High	0	0	100	0	4	96

^a Iron treatment received in the farrowing crate (low=100mg of iron dextran on d 3; medium=200mg of iron dextran on d 3; high=200mg of iron dextran on d 3 and 14) ^b

Anemic=<90 g/L hgb

^c Deficient= 90-110g/L hgb

^d Normal=>110 g/L hgb

APPENDIX II

Appendix II Table 1: Percentage of pigs from combined trials challenged with ETEC from each iron treatment with anemic, iron deficient or normal hemoglobin levels at weaning

Iron Treatment**	Iron Status*		
	Anemic ^a n=17	Deficient ^b n=14	Normal ^c n=29
Low n=20	70	30	0
Medium n=20	15	30	55
High n=20	0	10	90

*Iron status was categorized into; anemic, iron deficient or normal based on hemoglobin levels (g/L) at weaning

** Iron treatment defined by dosage of iron dextran administered by intramuscular injection: Low; 100 mg on day 3, medium; 200 mg on day 3, high; 200 mg on day 3 and 14

^a Anemic pigs = hgb <90g/L

^b Deficient pigs = hgb 90-110g/L

^c Normal pigs = hgb >110g/L

Appendix II Table 2: Pearson correlation coefficients between hemoglobin and other hematological indices

	HGB	WBC	RBC	HCT	MCV	MCH	MCHC
HGB	1						
WBC	0.1791	1					
RBC	0.658	0.2764	1				
HCT	0.9673	0.1425	0.7165	1			
MCV	0.7606	-0.0425	0.0824	0.748	1		
MCH	0.8381	0.0515	0.1754	0.7762	0.9487	1	
MCHC	0.7316	0.2666	0.3175	0.5811	0.5367	0.7537	1

HGB=hemoglobin; WBC= white blood cell; RBC=red blood cell; HCT= hematocrit; MCV=mean corpuscular volume; MCH=mean corpuscular hemoglobin; MCHC= mean corpuscular hemoglobin concentration

Appendix II Table 3: The effects of hemoglobin on diarrhea score, rectal temperature, ETEC shedding and colonization, and histological lesions in newly weaned pigs challenged with enterotoxigenic *E. coli*

Outcome	Variable	Trial 1				Trial 2				
		OR ^a	SE	95% CI	P-Value	OR	SE	95% CI	P-Value	
Diarrhea	Hemoglobin ^e	0.966	0.020	0.927, 1.007	0.105	1.040	0.024	0.995, 1.087	0.083	
Fecal Culture ^c	Hemoglobin	0.987	0.022	0.945, 1.031	0.559	1.061	0.063	0.945, 1.192	0.313	
Bacterial Culture ^d	Hemoglobin	1.326	0.508	0.626, 2.808	0.461	N/A				
Bacterial Colonized	Hemoglobin	1.009	0.019	0.973, 1.047	0.623	1.065	0.035	0.998, 1.137	0.058	
Histology Lesion	Hemoglobin	1.005	0.025	0.957, 1.056	0.838	0.984	0.023	0.939, 1.03	0.484	
Temperature (°C)	Hemoglobin	<u>Coef^b</u>	<u>0.001</u>	<u>0.002</u>	<u>-0.002, 0.004</u>	<u>0.451</u>	<u><0.001</u>	<u>0.002</u>	<u>-0.004, 0.005</u>	<u>0.681</u>

*Mixed logistic regression model was used for dichotomous outcomes including sow id as a random effect. A mixed linear regression model was used for the continuous outcome temperature including both sow id and test as random effects

^aOR (odds ratio) represents the odds of an outcome based on every g/L increase of hemoglobin (ie. the odds of a positive bacterial culture is 1.326 times greater for every increase in g/L of hemoglobin)

^bCoef (coefficient) represents the change in each outcome if the variable is increased by one unit or compared to the referent category (ie. anemic pigs have a temperature 0.0347 degrees less than pigs that are iron deficient at weaning)

^c Fecal culture refers to fecal swab that was cultured on Columbia Blood Agar

^d Bacterial culture refers to post-mortem colon sample that was cultured on Columbia Blood Agar

^e Hemoglobin was measured in g/L using non-differential CBC Advia analysis

SE= standard error

CI=confidence interval

Appendix II Table 4: The effects of iron treatment on diarrhea score, rectal temperature, ETEC shedding and colonization, and histology lesions in newly weaned pigs challenged with enterotoxigenic *E. coli*

Model	Variable	Trial 1				Trial 2			
		OR ^a	SE	95% CI	P-Value	OR	SE	95% CI	P-Value
Diarrhea	Iron Treatment ^c								
	Low ^d	0.624	10.637	0.085, 4.614	0.644	0.375	0.383	0.051, 2.773	0.337
Fecal Culture ^f	Iron Treatment								
	Low	1.000	1.118	0.112, 8.947	1.000	1.000			
Bacterial Culture ^g	Iron Treatment								
	Low	N/A				N/A			
Bacterial Colonized	Iron Treatment								
	Low	0.639	0.617	0.097, 4.236	0.643	5.217	6.607	0.436, 62.433	0.192
Histology Lesion	Iron Treatment								
	Low	0.549	0.719	0.042, 7.154	0.647	1.000	0.913	0.167, 5.385	1.000
Temperature	Iron Treatment	<u>Coef^b</u>							
	Low	0.113	0.883	-0.060, 0.286	0.202	0.270	0.099	0.075, 0.465	0.007
	High	-0.004	0.082	-0.164, 0.156	0.964	0.015	0.099	-0.179, 0.209	0.087

^aMixed logistic regression model was used for dichotomous outcomes including sow id as a random effect. A mixed linear regression model was used for the continuous outcome temperature including both sow id and test as random effects

^bOR (odds ratio) represents the odds of an outcome for pigs with different iron treatments compared to the referent (medium treatment) (ie. the odds of having diarrhea is 0.624 times less in pigs that received the low treatment of iron compared to pigs that received the medium treatment)

^cCoef (coefficient) represents the change in each outcome if the variable is increased by one unit or compared to the referent category (ie. pigs that received the low iron treatment have a temperature 0.113 degrees higher than pigs that received the medium treatment)

^dIron treatment= iron dose that pigs were administered on day 3 and 14 (ref= medium treatment level (200 mg on day 3))

^eLow Treatment= 100 mg of iron dextran on day 3

^fHigh treatment=200 mg of iron dextran on day 3 and 15

^gFecal culture refers to fecal swab that was cultured on Columbia Blood Agar

^hBacterial culture refers to post-mortem colon sample that was cultured on Columbia Blood Agar *note all pigs were classified as having bacteria cultured

SE= standard error

Appendix II Table 5: The effects of iron status on diarrhea score, rectal temperature, ETEC shedding and colonization, and histology lesions in newly weaned pigs challenged with enterotoxigenic *E. coli*

Model	Variable	Trial 1				Trial 2			
		OR ^a	SE	95% CI	P-Value	OR	SE	95% CI	P-Value
Diarrhea	Iron Status ^c								
	Anemic ^d	0.770	0.884	0.081, 7.304	0.820	0.334	0.452	0.0235, 4.738	0.417
	Normal ^e	0.132	0.183	0.009, 1.995	0.144	2.600	2.292	0.462, 14.629	0.278
Fecal Culture ^f	Iron Status								
	Anemic	2.747	3.180	0.284, 26.559	0.383	0.429	0.674	0.019, 9.364	0.590
	Normal	2.249	2.623	0.229, 22.123	0.487	1			
Bacterial Culture ^g	Iron Status								
	Anemic	1.000				N/A			
	Normal	1.000				N/A			
Bacterial Colonized	Iron Status								
	Anemic	0.072	0.124	0.002, 2.128	0.128	0.769	1.265	0.031, 19.306	0.873
	Normal	0.120	0.203	0.004, 3.290	0.210	3.412	3.710	0.405, 28.736	0.259
Histology Lesion	Iron Status								
	Anemic	0.257	0.486	0.006, 10.450	0.473	1.000	1.225	0.091, 11.028	1.000
	Normal	0.212	0.424	0.004, 10.725	0.438	0.636	0.545	0.119, 3.411	0.598
Temperature (°C)	Coef ^b								
	Iron Status								
	Anemic	-0.035	0.087	-0.204, 0.135	0.689	0.100	0.143	-0.180, 0.381	0.481
	Normal	0.095	0.074	-0.240	0.201	0.075	0.128	-0.176, 0.325	0.560

^aMixed logistic regression model was used for dichotomous outcomes including sow id as a random effect. A mixed linear regression model was used for the continuous outcome temperature including both sow id and test as random effects

^bOR (odds ratio) represents the odds of an outcome in pigs that are anemic or have a normal iron level compared to the referent (deficient pigs) (ie. the odds of having diarrhea is 0.132 times less in normal level pigs than deficient pigs)

^cCoef (coefficient) represents the change in each outcome if the variable is increased by one unit or compared to the referent category (ie. anemic pigs have a temperature 0.0347 degrees less than pigs that are iron deficient at weaning)

^dReferent was iron deficient hemoglobin level (90-110g/L)

^eAnemic (<90g/L)

^fNormal (90-110g/L)

^gFecal culture refers to fecal swab that was cultured on Columbia Blood Agar

^hBacterial culture refers to post-mortem colon sample that was cultured on Columbia Blood Agar

SE= standard error

APPENDIX III

Appendix III Table 1: Results of mixed-effect logistic regression models comparing pigs fed different tobacco treatments to evaluate the effect of treatment type on bacterial colonization, fecal shedding, histology lesions and diarrhea score

Parameter	OR ^a	SE	95% CI	P-Value
<u>Colonization</u>				
<i>Wildtype</i>	Ref			
<i>Low</i>	1.58	1.11	0.42, 6.22	0.512
<i>High</i>	1.03	0.72	0.26, 4.08	0.97
<u>Fecal Shedding</u>				
<i>Wildtype</i>	Ref			
<i>Low</i>	3.01	5.63	0.08, 117.08	0.555
<i>High</i>	1.76	3.04	0.06, 51.67	0.743
<u>Histology Lesions</u>				
<i>Wildtype</i>	Ref			
<i>Low</i>	1.08	1.11	0.14, 8.15	0.943
<i>High</i>	0.73	0.73	0.10, 5.17	0.755
<u>Diarrhea Score</u>				
<i>Wildtype</i>	Ref			
<i>Low</i>	0.77	1.53	0.18, 3.21	0.718
<i>High</i>	0.19	0.16	0.04, 0.94	0.042

*The three treatment groups analyzed were all challenged with F4 ETEC. The wildtype treatment was used as the referent to account for treatment efficacy.

** Wildtype: n=18 pigs that were fed 2.5g/feeding of a wild type of tobacco. Low: n=18 pigs that were fed 1.25g/feeding of FaeG product. High: n= 18 pigs that were fed 2.5g/feeding of FaeG product.

^aOR (Odds Ratio) represents the odds of an outcome in Treatment 2 or 3 when comparing to the referent ie. the odds of having bacterial colonization in Treatment 4 is 1.58 times greater than in Treatment 2.

SE= Standard Error

CI=Confidence Interval

Appendix III Figure 1: A recombinant variant of the *FaeG* protein, rFaeG_{ntd/dsc} in tobacco plants with accumulation levels of 2.0 g rFaeG_{ntd/dsc} per 1 kg fresh leaf tissue



Appendix III Figure 2: Histological examination of enterotoxigenic *E. coli* bacteria colonized to receptor surfaces on jejunum villi

