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Effect Of Feeding A Probiotic, Prebiotic Source, And Synbiotic Diet To Gestational And Lactational Sows On The Mucosal Immunity Of Their Weaned Piglets

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Effect of Feeding a Probiotic, Prebiotic Source, and Synbiotic Diet to Gestational and Lactational Sows on the Mucosal Immunity of Their Weaned Piglets

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Biographical Sketch

Lauren Kloc was born on May 29, 1988 in Belews Creek, North Carolina. She graduated from North Carolina State University in 2010 with Bachelor of Science degrees in Animal Science and Poultry Science. She is a candidate for the Master of Science degree in Animal Health Science.

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List of Symbols

β	Beta
BSA	Bovine Serum Albumin
CO_2	Carbon Dioxide
CON	Control
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent assay
HRP	Horseradish Peroxidase
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IgG	Immunoglobulin
IL	Interleukin
M	Molarity
µg	Microgram
µl	Microliter
ml	Milliliter
N	Normality
NCAT	North Carolina Agricultural and Technical State University
ng	Nanogram
nm	Nanometer
O	Oat
PBS	Phosphate Buffered Saline
ppm	Parts Per Million
PWD	Post-Weaning Diarrhea
rpm	Revolutions Per Minute

SD	Standard Deviation
TBS	Tris-Buffered Saline
TMB	Tetramethyl benzidine
TNF-α	Tumor Necrosis Factor- Alpha
YC	Yeast Culture

Abstract

A common practice, in the swine industry, is to administer antibiotics in piglet diets to reduce the incidence of post-weaning diarrhea (PWD). However, due to the link between the overuse of antibiotics in agriculture and the emergence of antibiotic-resistant bacteria, alternatives to using antibiotics that boost natural immunity are being sought. Diets rich in probiotic, prebiotic, or synbiotics enhance intestinal health. Since research has shown that a mother's diet during both gestation and lactation can play an important role in the development of their offspring, a study was designed to investigate whether feeding a probiotic, prebiotic source, or synbiotic diet to sows during lactation or gestation and lactation would enhance the intestinal immune system of their piglets. IgA levels were measured in the milk on the day of farrowing and in the lavage fluid collected from the duodenum, jejunum, and ileum of piglets on day 0 (day of weaning) and days 7, 14, 21, and 28 post-weaning. In addition, levels of the pro- and anti-inflammatory cytokines, TNF-α, IL-6, and IL-10, were measured within the small intestine lavage fluid on the same days. No differences were observed in IgA levels present in the milk on the day of farrowing. Measurements taken on day of weaning suggest that the inclusion of yeast culture and oats may contribute to increased IgA levels in the small intestines of piglets on the day of weaning, but may not contribute to long-term IgA production. The levels of pro-inflammatory cytokine detected also suggest that yeast culture and oat may contribute to intestinal inflammation. Therefore, the inclusion of yeast culture and oats in the diet of sows may affect IgA production and inflammatory responses within the intestine on the day of weaning.

CHAPTER 1

Introduction

Currently, the United States is a major global contributor to the swine industry. The United States is the second largest pork producer behind China, accounting for 10% of the total world supply of pork ("Hog Farming," 2007). To maintain increased hog production, many strategies are employed by the swine industry. Some of these production strategies include increasing the litter size and cross fostering extra piglets that the sow cannot raise (Guthrie et al., 1978; Lecce and King, 1981). Most sows do not exhibit signs of estrus or ovulation when they are lactating; therefore, another common strategy used to increase the numbers of piglets per sow each year and ultimately increasing production, is to wean piglets at the early age of 21 days (Guthrie et al., 1978).

Although weaning piglets at early ages shortens the time period when the sows are out of production, the performance of their piglets is negatively affected, which can cause economic losses for producers. When piglets are weaned early, their immune systems are weak and immature. The stress of weaning coupled with immature immune systems makes piglets prone to developing post-weaning diarrhea (PWD). PWD is a common condition seen in weanling piglets that can cause impaired growth rates and an imbalance of gut microflora (Modesto et al., 2009). To avoid PWD, growth-promoting antibiotics (GPA) have been included in piglet diets. The problem is that GPAs depress beneficial organisms in the gut and are possibly linked to the emergence of antibiotic resistant bacteria (Modesto et al., 2009). In Europe, the use of antibiotics in livestock has been banned and in the future, more countries are likely to start banning the use of these drugs (Stuyven et al., 2009). Therefore, alternatives to GPAs that maintain piglet health and boost immunity are being investigated. Several possible alternatives are probiotics, prebiotics, and synbiotics. Probiotics are live microorganisms that can provide

health benefits to the host while prebiotics are nondigestible food ingredients that are known for stimulating the growth of beneficial bacteria (Modesto et al., 2009). When probiotics and prebiotics are fed together, they are referred to as synbiotics and can provide synergistic effects to the host (Modesto et al., 2011).

Incorporation of probiotics, prebiotics, and synbiotics into the diet are good potential alternatives to the use of GPAs in swine production (Vondruskova et al. 2010). Therefore, the goal of this research was to investigate the use of probiotics or prebiotics alone and in combination as alternatives to GPAs. Furthermore, maternal diets can play an important role in the health of their offspring and the development of their immune systems (Sanz, 2011). Therefore, the focus of this study was to investigate the effect that feeding a probiotic, prebiotic source, or synbiotic diet to gestational and lactational sows has on the intestinal mucosal immunity of their piglets at the time of weaning. The hypothesis was that yeast culture and oat would function to modulate intestinal immunity.

CHAPTER 2

Literature Review

The United States is a major global contributor to the swine industry ("Hog Farming," 2007). Therefore, to maintain increased production, a common strategy is to wean piglets at 21 days of age when their immune systems are naive and immature (Guthrie et al. 1978). However, the stress of weaning coupled with immature immune systems makes piglets prone to developing PWD, which can cause substantial economic burdens for the producer (Zhang 2007). In 2007, it was estimated that the swine industry lost approximately 90 million dollars due to piglets dying from PWD (Zhang 2007). To reduce the incidence of PWD, GPAs are typically administered in piglet diets; however, due to the increased concern of antibiotic resistant bacteria, alternatives to using GPAs such as probiotics, prebiotics, and synbiotics are being evaluated (Vondruskova 2010).

2.1 The Immune System

The word, immunity, refers to the body's ability to protect itself from foreign and environmental agents such as microorganisms, drugs, chemicals, dander, or pollen (Coico and Sunshine, 2009). An individual's immunity can be classified as either innate or acquired. Innate immunity is a type of immunity that is present in animals, from birth, regardless of whether they have been exposed to an infectious agent in the past (Tizard, 2009). This immunity is the first line of defense and responds immediately to the invasion of pathogens and foreign substances into the body (Tizard, 2009). Innate immune responses are quick, non-specific, and without memory (Coico and Sunshine, 2009). Unlike innate immunity, adaptive immunity is acquired after birth and usually occurs in response to exposure to specific antigens like microorganisms or environmental agents such as pollen (Coico and Sunshine, 2009). In contrast to innate immunity,

adaptive immunity is slow, specific, and has memory, in which it will recognize and respond to the same infectious agents during future exposures (Coico and Sunshine, 2009). Two important elements of the innate and adaptive responses are cytokines and immunoglobulins.

2.1.1 Cytokines. Cytokines are proteins, which when secreted by cells, are responsible for mediating cellular interactions along with regulating the growth of cells and their secretions (Coico and Sunshine, 2009; Tizard, 2009). These proteins play an important role in facilitating innate immune responses and regulating various aspects of the immune system (Coico and Sunshine, 2009; Tizard, 2009). Two types of cytokines that can be released during an immune response are pro-inflammatory cytokines, which promote inflammation, and anti-inflammatory cytokines, which reduce inflammation (Coico and Sunshine, 2009).

Pro-inflammatory cytokines are cytokines that stimulate an immune response when a host is immunologically challenged (Coico and Sunshine, 2009). Examples of pro-inflammatory cytokines that may be increasingly expressed following exposure to pathogens are IL-6 and TNF-α (Coico and Sunshine, 2009). Primarily phagocytes, which include innate immune cells such as macrophages and neutrophils, produce pro-inflammatory cytokines (Coico and Sunshine, 2009). The main role of pro-inflammatory cytokines is to modulate an inflammatory response (Coico and Sunshine, 2009). For example, TNF-α is known for attracting neutrophils to sites of tissue damage, activating macrophages, stimulating macrophage phagocytosis, and killing tumor cells whereas IL-6 is known for activating T-cells and stimulating Ig production in B cells (Coico and Sunshine, 2009; Tizard, 2009). However, increased levels of pro-inflammatory cytokines can lead to tissue damage and impaired growth because nutrients are being directed towards the immune system instead of growth (Grijo et al., 2010; Li et al., 2005). Therefore, in attempt to avoid the overproduction of pro-inflammatory cytokines, the body also produces anti-

inflammatory cytokines. An example of an anti-inflammatory cytokine is IL-10. This anti-inflammatory cytokine is produced by macrophages and Type 2 helper T cells (Coico and Sunshine, 2009). Anti-inflammatory cytokines are known for inhibiting macrophage production, inhibiting Type 1 helper T cells, and balancing the amount of pro- and anti-inflammatory mediators (Coico and Sunshine, 2009; Li et al., 2005). Therefore, the balance of pro-inflammatory and anti-inflammatory cytokine production in the intestine as it relates to weaning and PWD is an important issue to hog production.

2.1.2 Immunoglobulin A. Another key factor in immune responses is immunoglobulins or antibodies. In pigs, there are five classes of immunoglobulins (IgA, IgD, IgG, IgM, IgE) (Tizard, 2009). Since IgA has been found to be important in mediating immunological lines of defenses within the mucosal immune system, the discussion will be focused on IgA (Ushida et al., 2008). IgA is an antibody that is produced by plasma B cells in the intestinal tract and they line the mucosa, which prevents the attachment of infectious pathogens to the enteric epithelium (Chau et al., 2009; Tizard, 2009; Ushida et al., 2008). A study conducted by Inoue and Nakano (1984), found that IgA levels in the intestines of piglets was the lowest at 3 weeks of age, which suggests this age is the critical time when the piglets need to be protected against pathogenic enteric diseases. It was also reported that three weeks post-weaning, luminal IgA production was still extremely low, which suggests that at around 50 days of age, piglet immune systems are still not yet fully developed and properly functioning (Ushida et al., 2008).

2.2 Active and Passive Immunity

Active immunity occurs when an infection or an administered antigen, as done in vaccination, triggers an immune response (Tizard, 2009). In contrast, passive immunity occurs

when specific antibodies from an individual who has been previously immunologically challenged, either by immunization or natural infection, are transferred to a non-immunized individual (Coico and Sunshine, 2009). Passive immunity can be achieved from the actual administration of antibodies to an individual, but more commonly, passive immunity is acquired immediately after birth when newborns consume their mothers' colostrum and milk (Coico and Sunshine, 2009; Rooke and Bland, 2002).

During the first few weeks of life, the adaptive immune system of mammals is not fully developed (Rooke and Bland, 2002); therefore, animals rely on passive immunity (transfer of the immunoglobulins IgG, IgM, and IgA) acquired from their mothers' colostrum and milk until their adaptive immune systems develop (Rooke and Bland, 2002). IgA present in the colostrum and milk of sows provides protection of the mucosal surfaces in young piglets until the piglets reach 3-4 weeks of age when they are able to start producing enough IgA to protect themselves against infection or disease (Chau et al., 2009; Inoue and Nakano, 1984). Unlike humans, most livestock species cannot transfer immunoglobulins across their placentas because of the epitheliochorial nature of the placenta, which makes livestock even more reliant on passive immunization via the colostrum and milk (Rooke and Bland, 2002).

2.3 The Effect of Early Weaning on Intestinal Health

The practice of the commercial swine industry is to wean piglets at approximately 21 days of age to produce more piglets per sow each year (Guthrie et al., 1978); however, piglets weaned at this age have weak and immature immune systems. In addition to immature immune systems, during the time of weaning, they are also moved to a new environment, without the sow, where they must adapt to living and eating on their own (Lyutskanov et al., 2011; Vondruskova et al., 2010). These changes cause stress, which exuberates the weakening of their

immune systems and makes them more vulnerable to disease (Lyutskanov et al., 2011; Vondruskova et al., 2010). One disease that piglets are prone to developing, particularly when they are weaned before four weeks of age, is PWD. Symptoms associated with PWD include watery diarrhea, weight loss, dehydration, and in severe cases, death (Bruins et al., 2011; Ding et al., 2006).

Stress, morphological and functional changes in the small intestine, a weak immune system, and environmental and diet changes can all contribute to the development of PWD (Kyriakis et al., 1999; Vondruskova et al. 2010). When intestinal microflora become imbalanced, pathogens such as Enterotoxigenic *Escherichia coli* strains and rotaviruses start multiplying, which cause the diarrhea to occur (Kyriakis et al., 1999). Several other pathogens that may cause diarrhea include *Lawsonia* spp., *Clostridium* spp., *Brachyspira* ssp., *Campylobacter* ssp., *Salmonella* ssp., coronaviruses, and transmissive gastroenteritis viruses (Jacobson et al., 2003; Vondruskova et al., 2010); however, in the swine industry, the most common cause of post-weaning diarrhea is Enterotoxigenic *Escherichia coli* (Bruins et al., 2011; Krause et al., 2010). Typically, the colonization of the gut with pathogenic microorganisms occurs at the time of weaning when the piglets are experiencing many physical and environmental changes (Vondruskova et al. 2010). Moreover, Ushida et al. (2008) reported that enteric pathogens readily colonized the intestines post-weaning when IgA concentrations in the intestines were low and that this decrease allowed for increased prevalence of diarrhea causing enteropathogens to adhere to the mucosal lining of the piglets' intestines.

2.4 Preventing Post-Weaning Diarrhea Using Antibiotics

Since weaned piglets are experiencing many environmental and physical changes, keeping them healthy during the time of weaning is crucial. In an attempt to keep these animals

healthy and prevent PWD, piglet diets are being supplemented with sub-therapeutic levels of antibiotics (Ding et al., 2006). Sub-therapeutic levels of antibiotics have often been administered in animal feed to enhance the overall performance of the animal, increase feed efficiencies, promote growth, and prevent diseases (Gustafson, 1991; Langlois et al., 1978). However, sub-therapeutic use of antibiotics is thought by many to be a contributing factor in the arising issue of antibiotic resistance.

Bacteria have the potential to become antibiotic resistant through mutations or by conjugation (Langlois et al., 1978; Rambhia et al., 2009). In a study conducted by Mathew et al. (1998), the researchers investigated antibiotic resistance on commercial swine farms by examining the presence of *Escherichia coli* resistant bacteria. They focused on five antibiotics that are commonly administered on swine farms to control bacterial infections, promote increased growth rates, and increase feed efficiencies (Mathew, 1998). These antibiotics included: Apramycin, Carbadox, Gentamicin, Neomycin, and Tetracycline (Mathew, 1998). From their study, they concluded that antibiotic resistance is a widespread issue on commercial swine farms and that antibiotic resistance increases with increased usage (Mathew, 1998). This data coincides with a study conducted by Langlois et al. (1978) where they also observed antibiotic resistance after the use of tetracycline, penicillin, sulfamethizole, streptomycin, neomycin, kanamycin, and ampicillin.

When bacteria become resistant, diseases and illnesses are challenging to treat with the regular prescribed antibiotics (Vondruskova et al., 2010); therefore, alternatives to antibiotic growth promoters need to be evaluated. Several possible alternatives to using antibiotics include: probiotics, prebiotics, synbiotics, organic acids, zinc oxide, and plant extracts (Vondruskova et al., 2010).

2.5 Probiotics

Probiotics are live microorganisms that can provide health benefits to the host (Reid and Friendship, 2002). Several of these benefits include the following: regulation of intestinal microbial homeostasis, enzymatic activity that induces absorption and nutrition, stabilization of the gastrointestinal barrier function, stimulation of gastrointestinal immunity, prevention of pathogens colonizing the mucosa, and increased natural resistance to infectious enteric diseases (Gaggia et al., 2010; Marinho et al., 2007). Probiotics do not produce drug residues or resistances like those seen with the overuse of antibiotics (Ross et al., 2010). The main issue with beneficial microorganisms is they must be continually fed for the host to continue to benefit from them because within a few days or weeks after the treatment has been stopped, the number of probiotic bacteria begin to decline (Kleta et al., 2006).

Several examples of probiotic genera include the following: *Lactobacillus, Enterococcus, Saccharomyces, and Bifidobacteria* (Reid and Friendship, 2002). *Lactobacillus*, *Entercoccus*, and *Bifidobacterium* are all bacteria while *Saccharomyces* are yeasts. These bacteria and yeasts are typically administered in their viable forms and are given orally or mixed in the feed (Kleta et al., 2006).

Studies have shown that probiotics when supplemented in swine diets have positive effects on overall performance and health (Kyriakis et al., 1999; Ross et al., 2010; Taras et al., 2007; Veizaj-Delia et al., 2010). For example, several studies revealed when piglets are fed probiotics, they have better growth performances, feed efficiencies, and average daily gains (Kyriakis et al., 1999; Ross et al., 2010; Taras et al., 2007; Veizaj-Delia et al., 2010). Taras et al. (2007) reported that weanling piglets given probiotics increased their average daily gain by 11% and improved their feed efficiencies by 8%. When piglets are fed probiotics post-weaning,

increased counts of beneficial bacteria such as lactobacilli have been observed in the intestines, which leads to the potential suppression-reduction of pathogenic bacteria (Mallo et al., 2010). This information coincides with other studies, which have shown reduced incidences of diarrhea in piglets fed probiotics (Kyriakis et al., 1999; Taras et al., 2007). Research also suggests that when animals are fed probiotics, beneficial bacteria are capable of colonizing the intestines and preventing pathogenic microorganisms from adhering to the intestinal walls by forming a protective membrane (Yu et al., 2008).

Other studies have evaluated the effects that probiotics have on the swine immune system. In a study conducted by Daudelin et al. (2011), the researchers found that probiotics have an effect on cytokine expression when piglets are challenged with enterotoxigenic *Escherichia coli*. When challenged with enterotoxigenic *Escherichia coli*, the researchers observed piglets consuming probiotics have increased expression levels of pro-inflammatory cytokines; however, these results do not coincide with other studies where researchers have found probiotics decrease the expression of pro-inflammatory cytokines (Daudelin et al., 2011; Zanello et al., 2011). Therefore, the effect of probiotics on pro-inflammatory cytokines levels needs to be further investigated, which is one reason why this study investigated pro-inflammatory cytokines levels in the intestinal segments. Aside from cytokine expression levels, researchers have also observed improvements in mucosal immunity (Yoshida et al., 2009). In a study conducted by Yoshida et al. (2009), increased IgA levels were observed in the intestines of piglets fed probiotics.

When sows are fed probiotics during gestation and lactation, beneficial effects are also observed regarding her performance and the performance of her offspring. Studies reveal sows fed probiotics during gestation and lactation consume more feed and lose less body weight while

nursing their piglets (Alexopoulos et al., 2004; Bohmer et al., 2006). These sows also have increased litter sizes and produce heavier piglets at birth and weaning (Bohmer et al., 2006). Even piglet mortality is reduced when sows' diets are supplemented with probiotics (Alexopoulos et al., 2004; Bohmer et al., 2006). Bohmer et al. (2006) report piglet mortality decreased by 2% when sows were fed probiotics. Alexopoulus et al. (2004) also observed decreased incidences of diarrhea in piglets whose dams received probiotics. According to these studies, piglet performance is positively influenced when sows are supplemented probiotics in their diets during gestation and lactation (Alexopoulos et al., 2004; Bohmer et al., 2006). However, no studies were found regarding piglet IgA and cytokine production when the sows were fed the probiotic, YC.

2.5.1 Yeast culture. Yeast culture (YC) is one example of a probiotic. YC is a dried product that contains *Saccharomyces* and metabolites of fermentation (Shen et al., 2009). Yeast fermentation produces enzymes, saccharides, and vitamins that have the ability to benefit the health of swine along with their growth and metabolism (Shen et al., 2009). Shen et al. (2009) revealed supplementation of piglet diets with YC has beneficial effects on growth performance (Shen et al., 2009). In sows, diets supplemented with YC produced piglets that were heavier piglets at weaning (Kim et al. 2008). Kim et al. (2008) suggested that the improvements observed in the piglets may be due to improvements in the nutrient digestibility, milk production, and milk quality of the sows, but it needed to be further investigated Piglets that are directly fed YC have increased average daily gains and better mucosal immunity (Bontempo et al., 2006). Studies show YC not only increases the production of IgA by stimulating gut-associated-lymphoid tissue, but piglets supplemented with YC also have higher proliferating cell counts,

more mucosal macrophages, and increased levels of pro-inflammatory cytokines, which are beneficial to the piglet's health (Botempo et al., 2006; Shen et al., 2009).

2.6 Prebiotics

Prebiotics are nondigestible food ingredients that are fermented by the intestinal microbiota and are known to stimulate the growth and activity of beneficial bacteria (Gibson and Roberfroid, 1995). Even though the effects of prebiotics are indirect, they still increase populations of beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* (Heinrichs et al., 2009; Wang, 2009). These bacteria stimulate the immune system, inhibit the growth of pathogens, and reduce the incidence of diarrhea and constipation (Wang, 2009). The most studied prebiotics are nondigestible oligosaccharides such as inulin, fructooligosaccharides, galacto-oligosaccharides, and glucooligosaccharides (Wang, 2009). These prebiotics can be synthesized from various plant sources and from breast milk (Modesto et al., 2009; Wang, 2009).

Studies have shown that supplementation of prebiotics in piglet diets prevent chronic inflammatory intestinal issues and reduce incidences of intestinal disorders (Pouillart et al., 2010). Prebiotics fed to swine have increased the expression levels of the anti-inflammatory cytokine, IL-10, in the colonic tissues along with restoring immune homeostasis that is associated with the overproduction of pro-inflammatory cytokines (Pouillart et al., 2010). These feed additives have also been shown to heighten the innate immune system and stimulate IgA production in the intestines, which promotes good intestinal health and aids in preventing diseases (Henderson, 2012; Pouillart et al., 2010).

2.6.1 Oats. Oats are a source of a prebiotic and are a type of whole grain cereal that is known for containing large amounts of fermentable carbohydrates (Connolly et al., 2010). In relation to oats and their effects on swine, results from studies have varied. According to a study

conducted by Rivera et al. (1978), weanling piglets whose diets were supplemented with 10% oats were 5-11% heavier twenty-eight days post-weaning compared to piglets fed a standard control diet. Rivera et al. (1978) also concluded that dietary treatment of oats did not have a positive or negative effect on the incidence and severity of diarrhea. However, Lindemann et al. (1983) reported swine diets supplemented with 0%, 20%, 40%, or 60% oats resulted in decreased average daily weight gains with increased supplementation. Lindemann et al. (1983) attributed the decreased average daily gains to the possible decreased palatability of the oat diets or the change in the amount of lysine since soybean meal levels were decreased with increased oat supplementation. These researchers also found that swine supplemented with 40% or 60% oats required more feed per gain when compared to animals supplemented with 20% or less of oats in their diets (Lindemann et al., 1983). This data coincided with the study conducted by Rivera et al. (1978) where they also found that swine supplemented with 30% oats had higher feed per gain ratios, which further suggests increased levels negatively impacts swine performance.

Another study showed sows that consumed diets supplemented with approximately 90% oats during gestation consumed more feed during lactation, had decreased body weights, and less backfat thickness than sows fed other gestation diets (Matte et al., 1994). In the same study, first parity sows that were supplemented with oats during gestation produced more live piglets at birth than the other sows, but their overall piglet litter weights were lower (Matte et al., 1994). These litters of piglets also had slower average litter growth rates, which suggest that oat supplementation to sow gestation diets may negatively impact their piglets (Matte et al., 1994).

Overall, most studies conducted regarding supplementation of oats in growing piglet diets have focused primarily on weight gain and feed to gain ratios (Lindemann et al. 1983; Rivera et al. 1978). These data suggest that for growing swine to benefit from oats, their diets should not

contain more than 30% oats since increased supplementation of oats negatively impacted animal performance (Lindemann et al. 1983; Rivera et al. 1978).

In regards to the immune system, oat extracts contain anti-inflammatory properties (Sur et al. 2008). Guo et al. (2007) revealed polyphenols such as Avenanthramides from oats decrease pro-inflammatory cytokine expression. This data coincides with another study where Sur et al. (2008) revealed TNF-α production was inhibited when cells were treated with avenanthramides from oats. However, no studies were found regarding sows being supplemented with oats and its effect on the intestinal immunity and cytokine production of their offspring.

2.7 Synbiotics

Synbiotics is the term used to describe the consumption of probiotics with prebiotics (Vondruskova et al., 2010). Studies suggest synbiotics may work synergistically and provide increased health benefits compared to when prebiotics and probiotics are consumed individually (Vondruskova et al., 2010). Diets supplemented with synbiotics have been shown to increase the persistence of probiotic bacteria in the intestinal tract and increase the number of beneficial bacteria such as *Bifidobacteria* (Rastall and Maitin, 2002; Shim et al., 2005). These attributes are thought to positively impact the health status of the animal by preventing the colonization of pathogenic bacteria in the intestines, which leads to improved gut health and increased growth performance of the animals (Shim et al., 2005). Nevertheless, research regarding the efficacy of synbiotics is limited. Therefore, more studies need to be conducted regarding the immune health benefits associated with administering synbiotics in animal diets.

2.8 Impact of Mother's Diet on the Health of Her Offspring

A mother's diet has the potential to positively or negatively affect the overall health of her offspring. In swine, nutrients consumed during gestation and lactation play an important role in the viability of their offspring at birth (Alexopoulos et al., 2004). Variances in the performance of offspring have also been seen when sows are fed different diets. In a study conducted by Ariza-Nieto et al. (2011), piglets had increased average daily gains when sows were fed oregano essential oils; however, the diet had no effect on immunoglobulin concentrations, T lymphocytes, and natural killer cell activity (Ariza-Nieto et al., 2011). Other studies have revealed that when sow diets are supplemented with probiotics during gestation and lactation, their offspring have increased body weights and reduced incidences of diarrhea whereas sows that have consumed oats produce piglets that weigh less at birth and have slower growth rates (Alexopoulos et al., 2004; Bohmer et al., 2006; Matte et al., 1994). In humans, studies have also shown that mothers who consume probiotics are capable of transferring the beneficial bacteria to the intestinal tract of their infants both during and after pregnancy (Sanz, 2011). However, no studies have been conducted regarding the effect of feeding YC+O in sow diets on the mucosal immunity of their piglets.

The diet of the mother can also affect the quality and quantity of milk production. Several studies suggest a sow that consumed diets supplemented with probiotics may not only produce more milk, but also milk that is of better quality, which positively influences the performance of their offspring (Alexopoulos et al., 2004; Kim et al., 2008). According to Alexopoulos et al. (2004), sows supplemented with probiotics produced milk with more fat and protein. This attribute suggests that milk from sows fed probiotics may be of higher nutritional value to the piglets (Alexopoulos et al., 2004). Breast milk from humans who have consumed

probiotics contains fewer pro-inflammatory cytokines and their children have less gastrointestinal issues, which show another beneficial role of a mother's diet (Sanz, 2011).

Since a mother's diet can play an important role in the performance of her offspring, her diet should be formulated to positively influence the health of her offspring. Moreover, like most mammals, when piglets are born their immune systems are naïve, which makes them susceptible to diseases (Rooke and Bland, 2002). Due to the piglets being unable to initially produce effective levels of antibodies, they can acquire passive immunity through their dams' colostrum (Rooke and Bland, 2002). A sow's colostrum is a major source of IgA, IgG, and IgM and studies have shown these immunoglobulins protect the piglet during the first few weeks of life until they start producing their own antibodies (Chau et al., 2009; Franz and Corthier, 1980; Rooke and Bland, 2002; Ushida et al., 2008). Therefore, a study was designed that investigated the effect of dietary supplementation on the intestinal immunity of piglets. Specifically, this study focuses on the use of probiotics, prebiotic sources, and synbiotics as potential alternatives to using antibiotics in the swine industry during the time of weaning. YC, O, and a combination of YC and O fed to sows during gestation and lactation were evaluated to determine whether they positively affect the intestinal health of their offspring. The hypothesis was that YC and O would function to modulate intestinal immunity. The objective of this study was to investigate the expression levels of the mucosal antibody, IgA, the pro-inflammatory cytokines, IL-6 and TNF-α, and the anti- inflammatory cytokine, IL-10, in the intestines of piglets whose dams were fed probiotics, prebiotic sources, or synbiotics in their diets during gestation and lactation.

CHAPTER 3

Materials and Methods

3.1 Animals and Feed

The sows used in this study were housed in individual gestation crates at the Swine Research Unit at North Carolina Agricultural & Technical State University. For the study, 25 sows were randomly divided and placed into one of two trials. For trial #1, 13 sows (Yorkshire or Yorkshire crosses) with an average age of 17 months and an average parity of 3.3 were used for the study. During their gestation, these sows received the standard gestational diet approved by the North Carolina Agricultural & Technical State University's Swine Research Unit. Upon farrowing, the sows, in trial #1, were fed one of the following experimental rations throughout the lactation period: 1) control diet without antibiotic growth promoters (CON), 2.) control diet + YC (CON+YC), 3.) control diet + 15% oat (CON+O), and 4.) control diet + YC+ 15% oat (CON+YC+O).

In trial #2, 12 sows (Yorkshire, Yorkshire crosses, Landrace, Landrace crosses, or Berkshire) with an average age of 19 months and an average parity of 3.25 were used in the study. During gestation, these sows received a standard gestational diet approved by North Carolina Agricultural & Technical State University's Swine Research Unit until the last 30 days of gestation. During the last 30 days of gestation, the sows were given one of the following experimental gestational rations: 1) control diet without antibiotic growth promoters (CON), 2.) control diet + YC (CON+YC), 3.) control diet + 15% oat (CON+O), and 4.) control diet + YC+ 15% oat (CON+YC+O). Once the sows farrowed, they were fed the same lactation rations as described in trial #1. See Appendix B for all diet formulations. A detailed list of sow ages, parity, and breeds can be found in Appendix C.

3.2 Weaning

Piglets (n=72) were weaned at an average age of 21 days in trial #1 and 22 days in trial #2. Once weaned, the piglets were randomly assigned to pens based on the dietary treatment their dams received. All piglets whose dams received the same dietary treatment were assigned to the same pens. After weaning, all piglets, regardless of the dietary treatment of their dams, received a standard grower diet (See Appendix B). Nine piglets per dietary treatment group were used, for a total of 36 piglets per trial. All experiments were approved by the Institutional Animal Care and Use Committee of North Carolina Agricultural & Technical State University.

3.3 Milk Collection

On the day of farrowing, milk was collected from each sow in sterile 50 ml conical tubes and kept on ice until brought back to the laboratory. The milk samples were aliquoted into 1.5 ml microcentrifuge tubes and stored at -80ºC to preserve the samples until they were analyzed for IgA levels.

3.4 Necropsy

Necropsy of piglets was performed on the day of weaning (day 0) and days 7, 14, 21, and 28 post-weaning. On day 0, one piglet per treatment group was sacrificed while two piglets per treatment group were sacrificed on all other days during the study. Prior to sacrificing the piglets, an enclosed box was filled with CO_2 for 5 minutes. The piglets were then placed into the enclosed box containing CO_2 for approximately 5 minutes or until the piglet ceased respiration. When removed from the chamber, the piglets were exsanguinated to ensure death. The intestines were extracted from the body cavity by cutting the intestines at the pylorus and at the large intestinal and rectal junction. Once the intestines were removed, the small and large intestines

were separated. The small intestines were placed into sterile nalgene containers and put on ice until transported to the laboratory.

3.5 Intestinal Lavage

In the laboratory, lavage was completed on the intestines. Lavage fluid was made by diluting Halt Protease Inhibitor cocktail (100X) and 5M EDTA (100X) from Thermo Scientific (Rockford, Il, USA) in sterile 1X PBS. This dilution resulted in a final concentration of 1X Halt Protease Inhibitor cocktail and EDTA. Lavage was completed on the duodenum, jejunum, and ileum. These segments were measured and cut accordingly with clean surgical scissors. The duodenal segment was extracted by measuring 6 inches from the pylorus and then cutting the intestines. Next, the ileal segment was extracted by measuring 6 inches above the small and large intestinal junction and then cutting the intestines. Lastly, the jejunal segment was cut by measuring 3 feet above where the ileal segment had been removed (See figure in Appendix D for a visual and better understanding of the procedure).

The lumen of each segment of the intestine was then washed with 5 ml of lavage fluid using a sterile syringe. An alligator clamp was used to clamp one end of the intestine while filling it with the 5 ml of lavage fluid. Once filled with lavage fluid, the intestinal segment was gently massaged to remove cells and debris from the sides of the intestinal wall. After washing, the lavage fluid was drained into individual 50 ml conical tubes. The tubes were then centrifuged at 2500 rpm for 10 minutes at 4º C to remove the debris. Lavage fluid was aliquoted into 1.5 ml microcentrifuge tubes and stored at -80ºC.

3.6 ELISA

Porcine IgA ELISAs from Bethyl Laboratories (Montgomery, Tx, USA) were completed on the milk and intestinal lavage samples according to the manufacturer's instructions. The

plates were coated with antibody, incubated, washed, and then blocked for non-specific binding sites. Next, the plates were washed and the samples were added. After incubation, the plates were washed again, the HRP detection antibody was added, and allowed to incubate before washing the plate. Finally, TMB substrate was added and allowed to incubate before adding the stop solution. The plates were read using a VersaMax microplate reader. See Appendix D for the IgA ELISA protocol.

Porcine TNF-α, IL-6, and IL-10 ELISAs from R& D Systems (Minneapolis, Mn, USA) were completed on the intestinal lavage fluid, per the manufacturer's instructions. The plates were coated with antibody, incubated, washed, and then blocked for non-specific binding sites. Next, the plates were washed and the samples were added. After incubation, the plates were washed again, the detection antibody was added and allowed to incubate. The plate was then washed and diluted Streptavidin-HRP was added. The diluted Streptavidin-HRP was allowed to incubate and then the plate was washed. Lastly, TMB substrate was added and allowed to incubate before adding the stop solution. The plates were read using a VersaMax microplate reader. See Appendix D for the TNF-α, IL-6, and IL-10 ELISA protocols.

3.7 Statistical Analysis

Statistical analysis was completed on the IgA levels in the milk using GraphPad Prism. In GraphPad Prism, a one-way ANOVA with a Bonferroni post-test was completed on the samples.

CHAPTER 4

Results and Discussion

4.1 IgA Levels in the Milk of Sows on the Day of Farrowing

IgA levels in the milk were measured within a day after birth (See Figure 1 in Appendix A). In this study, IgA levels were expected to be higher in sows fed a probiotic, prebiotic source, and synbiotic diet when compared to the sows receiving the standard or control diet because prebiotics and probiotics have been shown to increase IgA levels (Henderson et al. 2012; Pouillart et al., 2010; Yoshida et al. 2009). However, no significant differences were observed between the dietary treatment groups regarding IgA levels present in the milk on the day of farrowing. This data suggests that feeding a CON+YC, CON+O, or CON+YC+O diet to gestational sows 30 days prior to farrowing may not affect IgA production in the milk on the day they give birth.

4.2 IgA Levels in the Duodenum, Jejunum, and Ileum

In trial #1, sows were only fed supplemented diets during lactation. IgA levels appeared to be the greatest at day 0 where n=1 as compared to all other days examined (See Figure 2 in Appendix A). In addition, on day 0, IgA levels appeared to be the highest in the jejunum of piglets whose dams were fed either a CON or a CON+O diet and in the ileum of piglets that were fed a CON+YC and a CON+YC+O diet. No other differences were observed in IgA levels within the intestine on days 7, 14, and 21. However, by day 28, a 2-fold increase was observed in IgA levels of the ileum of the piglets whose dams were fed a CON diet. This data suggests that YC and O supplementation during lactation may not contribute to IgA production.

In trial #2, IgA levels on day 0 appeared to be higher in the jejunum of the piglets whose dams were fed the CON+YC and the CON+YC+O diets when comparing them to all the other

segments (See Figure 3 in Appendix A). Although IgA was present in all intestinal segments, no differences were observed on days 7, 14, 21, and 28 post-weaning, which coincide with the absence of significant differences in IgA levels present in the milk. Interestingly, like trial #1, IgA levels appeared to be the lowest in the duodenum for all dietary treatment groups.

4.3 TNF-α Levels in the Duodenum, Jejunum, and Ileum

Analysis of lavage samples collected from trial #1 reveal that although TNF-α was detected in several of the intestinal segments, no trends or differences were observed (See Figure 4 in Appendix A). In contrast, the day 0 TNF-α levels within the lavage samples collected from the jejunum of the piglet whose dam was fed a CON+YC diet during gestation and lactation appeared to be dramatically higher (See Figure 5 in Appendix A). TNF-α production on all other days was low and no differences were observed. Therefore, the dietary supplements may not contribute to TNF-α production in offspring, but the possible effect of YC on TNF-α levels should be further investigated.

4.4 IL-6 Levels in the Duodenum, Jejunum, and Ileum

On the day of weaning, differences were observed in IL-6 levels in the jejunum of animals weaned from sows that were fed the CON+YC diet during lactation compared to several of the other intestinal segments (See Figure 6 in Appendix A). Similarly, in trial #2, IL-6 was consistently detected in piglets whose dams were fed CON+O and CON+YC+O diets (See Figure 7 in Appendix A). On day 0, IL-6 levels in the CON+YC+O piglet appeared to have dramatically higher IL-6 levels when compared to all other segments, but since only one piglet was sacrificed, the significance could not be determined. Furthermore, although IL-6 might have been detected in several intestinal segments, the detected levels did not appear to be dramatically higher when comparing them to the other intestinal segments for that particular day.

4.5 IL-10 Levels in the Duodenum, Jejunum, and Ileum

In trial #1, IL-10 was only detected in the intestines on day 7 and day 14 post-weaning (See Figure 8 in Appendix A). On day 7, IL-10 was present in the duodenum of piglets whose dams were fed the following diets: CON+O, CON+YC, and CON+YC+O, while on day 14, IL-10 was present in the duodenum of the piglets whose dams were fed CON+O. The detected levels did not appear to be dramatically higher when compared to the other dietary treatments. In trial #2, IL-10 was not detected in any of the intestinal segments. Together these data suggest that the piglets in this study may not have been experiencing an overwhelming inflammatory response.

4.6 Discussion

To date, not many studies have been conducted in regards to IgA and cytokine production in piglets whose dams received a probiotic, prebiotic, and synbiotic diet. More specifically, no studies were found where researchers had reviewed IgA and cytokine production in piglets after the sows were fed YC, O, or YC+O diets. Most studies that have been conducted where the sows were fed probiotic, prebiotic, and synbiotic diets focused primarily on piglet weight gains, feed efficiencies, litter sizes, and the incidences of diarrhea (Kim et al., 2008; Matte et al., 1994; Rivera et al., 1978; Shen et al., 2009). The studies where improved intestinal immunity was observed, the piglets had been directly fed the probiotic or prebiotic diet (Bontempo et al., 2006; Henderson et al., 2012; Pouillart et al., 2010). Therefore, the purpose of this study was to determine whether feeding a probiotic, prebiotic source, or synbiotic diet to sows during lactation or during both gestation and lactation would affect the intestinal health of their piglets post-weaning. It was hypothesized that feeding a probiotic, prebiotic source, or synbiotic diet to gestational and lactational sows would enhance the intestinal health of their offspring. However,

while the data is suggestive that dietary treatment of the sows may not play a role in improving the intestinal health of their piglets post-weaning, we are unable to make conclusive statements.

4.6.1 IgA. When the piglets were first born, IgA levels were measured in the milk. Since piglets depend on the passive transfer of antibodies during the first 24 hours after birth to protect them until they start producing their own immunoglobulins, IgA was measured to determine if YC, O, and YC+O fed to gestational sows would increase milk IgA levels (Rooke and Bland 2002). Based on the literature, IgA levels were expected to be higher in sows fed a probiotic, prebiotic source, and synbiotic diet when compared to the sows receiving a standard or control diet (Henderson et al. 2012; Pouillart et al., 2010; Yoshida et al. 2009). However, no significant differences in IgA levels on the day of farrowing were observed between the dietary treatments. This data suggest YC, O, and YC+O may not enhance IgA production.

Ideally, milk should have been collected throughout the lactation period. Even though it was attempted, milk collection was unsuccessful. During the first few hours after birth, milk can easily be collected, which is due to milk ejections occurring every 10-20 minutes (Klopfenstein et al., 2006). However, after the first few hours, milk ejections occur in cycles and the piglets must massage the udder to elicit the neuroendocrine milk ejection reflex (Klopfenstein et al., 2006). The neuroendocrine milk ejection reflex then stimulates the pituitary to release oxytocin, which causes the let down of milk (Klopfenstein et al., 2006). Therefore, after the first few hours, it is more difficult to collect milk unless oxytocin is exogenously administered to the sows (Klopfenstein et al., 2006). In this study, oxytocin was not exogenously administered to the sows, which resulted in the inability to successfully collect milk after the day of farrowing.

Following weaning, IgA levels were also measured in the small intestines of the piglets. IgA is an important mucosal antibody and plays an important role in preventing pathogens from

adhering to the mucosal surfaces (Chau et al., 2009; Ushida et al., 2008). In this study, IgA levels appeared to be the lowest in the duodenum regardless of the dietary treatment. This observation coincides with a study where IgA levels were also the lowest in the duodenum when compared to the jejunum and ileum (Inoue and Nakano, 1984).

When reviewing the different dietary treatment groups, it was expected that IgA production in the intestinal tract would be higher in the probiotic, prebiotic, and synbiotic dietary treatment groups since studies reveal that probiotics and prebiotics increase IgA production levels (Henderson et al. 2012; Pouillart et al., 2010; Yoshida et al. 2009). Although several differences in IgA levels were observed on day 0, no other differences were possibly observed except for day 28 in trial #1. On day 28, the CON piglets, whose dams received their assigned diet during lactation only, had a 2-fold increase in IgA in their ileums compared to the other segments. This data suggests that YC and O fed to sows may only affect IgA production in piglets early after weaning. This observation coincides with a study conducted by Duncker et al. (2006) who reported that the probiotic strain, *Escherichia coli* Nissle 1917, did not affect IgA production in the intestines of young adult piglets. In contrast, other studies have found that IgA production increases after administering probiotics and prebiotics to piglets (Henderson et al., 2012; Yoshida et al., 2009).

4.6.2 Pro-inflammatory cytokines. Cytokines are also an important part of an immune response. Studies have shown that pro-inflammatory cytokine levels increase following pathogenic exposure (Opapeju et al., 2010; Zanello et al., 2011); however, probiotics and prebiotics have been shown to reduce and prevent chronic inflammatory intestinal issues (Pouillart et al., 2010; Zanello et al., 2011). Therefore, we wanted to determine if piglets whose dams were fed probiotics, prebiotic sources, and synbiotics would have decreased levels of pro-

inflammatory cytokines as compared to piglets whose dams were fed a control diet. In this study, no differences were observed except for possibly on day 0. On day 0, the piglets whose dam received the CON+YC diet during lactation appeared to have slightly higher levels of IL-6 when compared to several of the intestinal segments. Therefore, in this study, IL-6 levels may have played a beneficial role in the health of these piglets and not posed a health risk. Typically, the pro-inflammatory cytokine, IL-6, is associated with acute inflammation, which produces beneficial responses following pathogenic exposure (Gabay, 2006). Harmful effects are usually not observed unless inflammation and cytokine production persists, which will result in tissue damage and detrimental effects on animal health (Gabay, 2006).

Another observation was the differences on day 0 in IL-6 levels in the ileum of piglets whose dams received the CON+YC+O and the TNF-α levels in the jejunum of piglets whose dams received the CON+YC diet during gestation and lactation when compared to other dietary treatments. The observed differences, suggest that YC and YC+O may possibly promote intestinal inflammation in piglets, which coincides with a study conducted by Shen et al. (2009) where they also observed increases in pro-inflammatory cytokines following supplementation of YC in swine diets. Therefore, the probiotic and prebiotic source fed in this study may not be the most suitable choice for reducing inflammatory responses since pro-inflammatory cytokines can be detrimental to the animal's well being and cause consumed nutrients to be directed away from growth and towards the immune system if they are overproduced (Li et al., 2005). However, studies found animals that were infected with pathogenic bacteria had IL-6 expression levels around 4 ng/ml and TNF-α expression levels around 2 ng/ml during the peak of infection (Jeong et al., 2010; Sofi et al., 2007). In this current study, pro-inflammatory cytokine production was typically lower, 0.50 ng/ml or less, than the previous mentioned expression levels with the

exception of day 0. On day 0, IL-6 production appeared to be slightly higher in the jejunum of the piglet whose dam received the CON+YC during lactation only and the ileum of the piglet whose dam received the CON+YC+O during gestation and lactation. However, since pro-inflammatory cytokine levels did not appear to be dramatically increased post-weaning, it is speculated that the piglets were not experiencing an overwhelming inflammatory response.

Differences in inflammatory cytokines may not have been observed due to the lack of pathogenic exposure. If the piglets in this study were exposed to only low levels of pathogens, the pathogenic exposure may not have been enough to stimulate an immune response. In a study conducted by Li et al. (2005), they fed piglets β-glucan extracted from *Saccharomyces cerevisiae*. β-glucan is known for initiating immune responses during infection; however, in their study, an immune response was not initiated (Li et al., 2005). They concluded β-glucan did not modulate an immune response in treated animals due to the lack of pathogenic exposure (Li et al., 2005). Therefore, if the piglets in this study were not exposed to infectious pathogens then an immune response would not have been initiated, which would explain the absent to low levels of pro-inflammatory and anti-inflammatory cytokines in the intestinal segments.

4.6.3 Anti-inflammatory cytokines. In trial #1, IL-10 was only detected on day 7 and day 14; however, observed differences were not present as IL-10 levels were either absent or extremely low. This data suggests that YC and O may not affect anti-inflammatory cytokine levels. The low to absent levels of IL-10 also coincide with the study conducted by Shen et al. (2009) where they observed increased levels of pro-inflammatory cytokines following YC supplementation, but no differences in IL-10 production. Another possibility is that the pro-inflammatory cytokine levels may have been increased, but were not being overproduced. This possibility may also explain the low to absent levels of anti-inflammatory cytokines since anti-

inflammatory cytokines are responsible for balancing pro- and anti-inflammatory mediators to prevent the overproduction of pro-inflammatory cytokines (Li et al., 2005).

4.6.4 Incidence of diarrhea. In a companion study performed by a fellow graduate student in the lab, post-weaning diarrheal scores were recorded. This data shows that on the day of weaning, the piglets appeared to have normal feces; however, all piglets regardless of the dietary treatment of their dams had diarrhea on all other days post-weaning. This data coincides with Ushida et al. (2008) where PWD was typically observed following weaning when intestinal IgA production was low. It also coincides with the data collected within this study, which shows that IgA levels may have been elevated in several intestinal segments on the day of weaning, but were relatively low on all other days post-weaning. Bacterial counts from the piglets' feces are also being evaluated to determine whether pathogenic bacteria counts were different between the dietary treatment groups.

4.6.5 Future directions. Throughout both trials, we note that the sows' diets may have played the largest role in the intestinal health of their piglets at the time of weaning; however, statistical analysis of the data could not be performed due to the lack of animals sacrificed per treatment group on day 0. Therefore, it is recommended that more piglets per treatment group are sacrificed per day. In this study, statistical analysis also could not be completed on days 7, 14, 21, and 28 post-weaning because only 2 piglets were sacrificed per treatment group. Therefore, this study should be repeated using more piglets per dietary treatment to allow for statistical evaluation of the data, and more focus should be on time points closer to weaning since sows' diets appeared to play the largest role at the time of weaning.

Several issues impacted the number of piglets that were sacrificed per treatment group. The original proposed study was to feed the sows their experimental diets during both gestation

and lactation. However, in trial #1, the gestation ration was unavailable at the time it was needed. This resulted in the sows only consuming the experimental diet during lactation. Since the sows, in trial #2, were fed both gestation and lactation diets, the data collected from trial #1 could not be compiled with the data from trial #2. Therefore, for each trial, the data only represents one piglet per dietary treatment group on day 0 and two piglets per treatment group on day 7, 14, 21, and 28 post-weaning. If the sows in trial #1 and trial #2 would have both consumed the experimental rations during gestation and lactation, the data would have been representative of two piglets per treatment group on day 0 and four piglets per treatment group on day 7, 14, 21, and 28 post-weaning. Statistical analysis would have then been able to have been completed on all days except for the day of weaning.

The sows also played a role in the decision to only sacrifice one piglet on day 0 and two piglets on day 7, 14, 21, and 28 post-weaning. During the study, two sows unexpectedly died before the piglets were weaned and two sows quit lactating during the lactation period. Therefore, unfortunately these piglets could not be used in the study due to the inability to cross foster them to sows that were on the same dietary treatment. Ultimately, this resulted in a lack of piglets for certain dietary treatment groups.

Another issue that confounds the data is the weaning age of the piglets. In both trials, the age of the piglets at weaning varied. Even though the average weaning age was 21 days in trial #1 and 22 days in trial #2, some piglets were weaned at 27 days of age while others were weaned at 17 days of age. The age of weaning will also play a major role in the development of the immune system and their ability to fight infection. Previous studies have shown that piglets rely on passive immunity from the sow until they start producing their own antibodies and that most animals do not start producing their own immunoglobulins until they are at least 3 weeks of age

(Chau et al., 2009; Inoue and Nakano, 1984). Therefore, passive immunity along with age may explain why IgA levels appeared typically higher on the day of weaning and then appeared to be lower by day 7 post-weaning. At the time of weaning, the piglets would have still been consuming the sows' milk, which is a source of IgA whereas by day 7 the IgA received from the sow would have been depleted and they would have been solely relying on the production of their own antibodies, which is typically low depending on the exact age of the piglet (Chau et al., 2009; Inoue and Nakano, 1984). The age differences may also explain the variability in the data. For example, it was not uncommon for one animal in a specific dietary treatment to have detectable levels of a certain cytokine, while another animal in the same treatment group did not.

To gain a better understanding of the effect a probiotic, prebiotic source, and synbiotic diet fed to gestational and lactational sows has on the mucosal immunity of their piglets, it is recommended, that in future studies, the piglets be weaned in groups with similar ages. Reducing the variations in age will allow for a better understanding of the effect the sows' diets have on their piglets' immune systems. To reduce the age differences, synchronizing the sows to where they will be bred at the same time may be one potential option. It would also be beneficial to collect milk at the end of the lactation period to see if the diet plays a role in IgA secretion in the milk and whether it influences IgA levels in the intestines of the piglets immediately following weaning. Therefore, to successfully collect milk, the use of oxytocin should be considered.

These issues need to be addressed in future studies. In addition, pathogenically challenging piglets to see if any differences are present within the dietary treatment groups may be another option. As previously mentioned, if an immune response is not initiated then differences between the dietary treatments may not be observed especially in regards to cytokine

production. It is also suggested that the sows used are similar in age, parity, and breed. Research reveals that when the same diets are given to primiparous and multiparous sows, the diet may have a different effect on the performance of their offspring (Kim et al. 2008; Matte et al. 1994). The breed also has the potential to greatly affect the overall performance of the piglets (Tang et al., 2008).

CHAPTER 5

Conclusion

PWD has a major economic impact on the swine industry (Opapeju et al., 2010). It often causes large economic losses due to the morbidity and mortality associated with the disease (Krause et al., 2010; Opapeju et al., 2010). Piglets that acquire PWD have decreased growth rates and in severe cases, death may occur (Zanello et al., 2011). In 2007, it was estimated that the swine industry lost approximately 90 million dollars due to piglets dying from PWD caused by enterotoxigenic *Escherichia coli* (Zhang, 2007). If death does not occur, economic losses can still be present because the decreased growth rates and stunted growth associated with PWD often leads to longer time required for pigs to reach slaughter weight (Lyutskanov, 2011). To reduce economic losses associated with PWD, the current practice of the industry is to supplement piglet diets with sub-therapeutic levels of antibiotics in attempt to prevent the onset of post-weaning diarrhea (Krause et al., 2010; Opapeju et al., 2010). However, due to the increased bacterial resistances to sub-therapeutic antibiotics, alternatives to using antibiotics are being evaluated. Although this study finds that feeding a YC, O, and YC+O diet to gestational and lactational sows may not alter the intestinal immunity of their piglets, it is recommended that further investigation is done before concluding that the diets do not affect the immunity of the piglets and are not a good potential alternative to using antibiotics.

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Appendix A

Figures

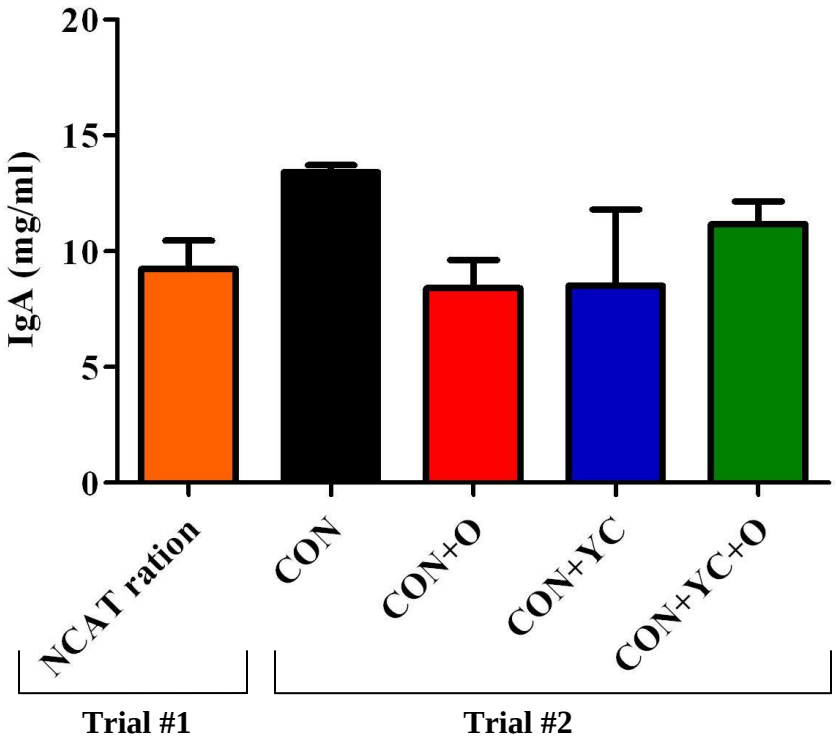


Figure 1. IgA levels in milk on day of farrowing. Each column represents n=3 except for the NCAT ration column which is n=11. No significant differences were observed.

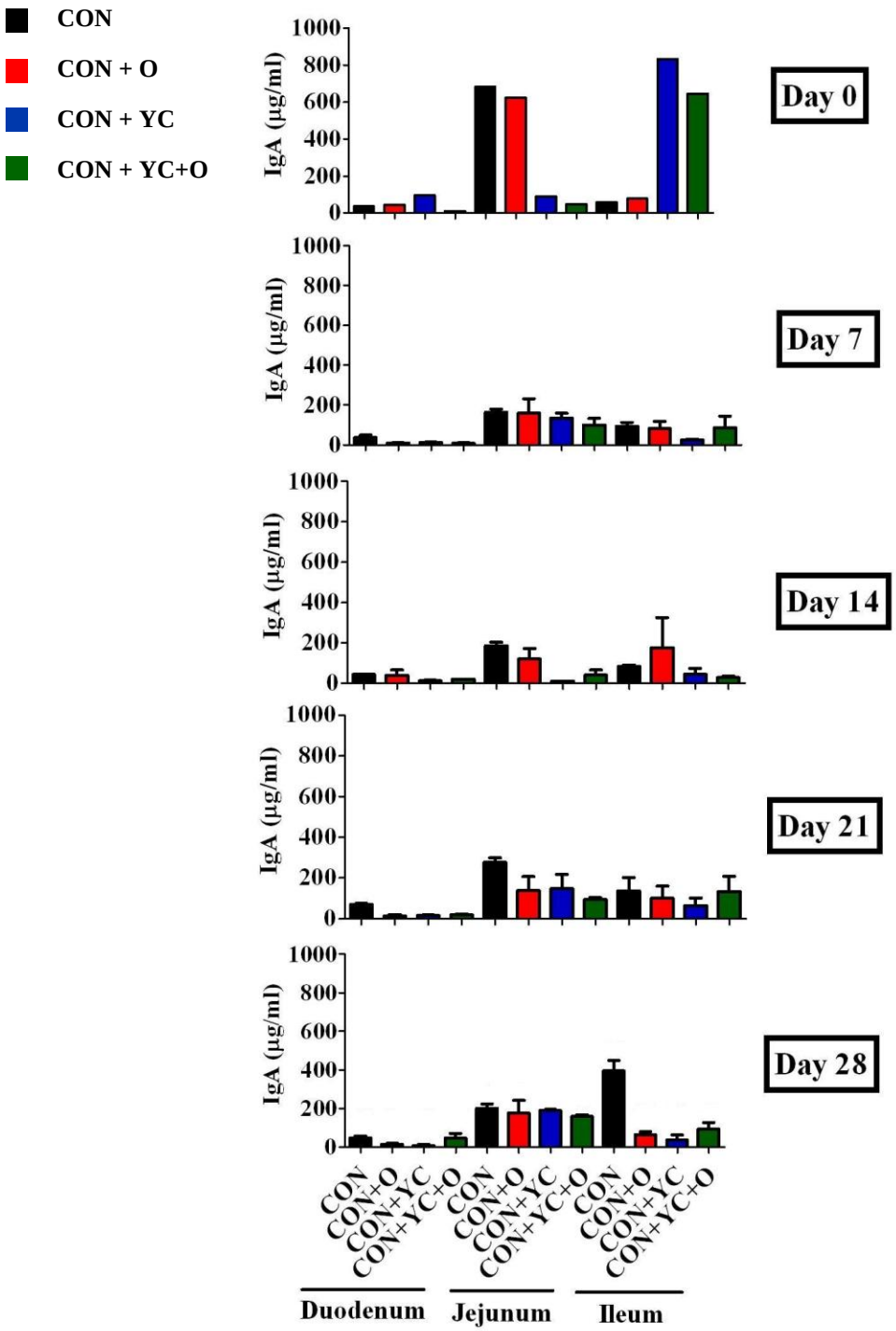


Figure 2. Trial #1: IgA levels in intestinal lavage. On day 0, each column represents n=1 and on day 7, 14, 21, and 28, each column represent n=2. Data are shown as Mean±SD.

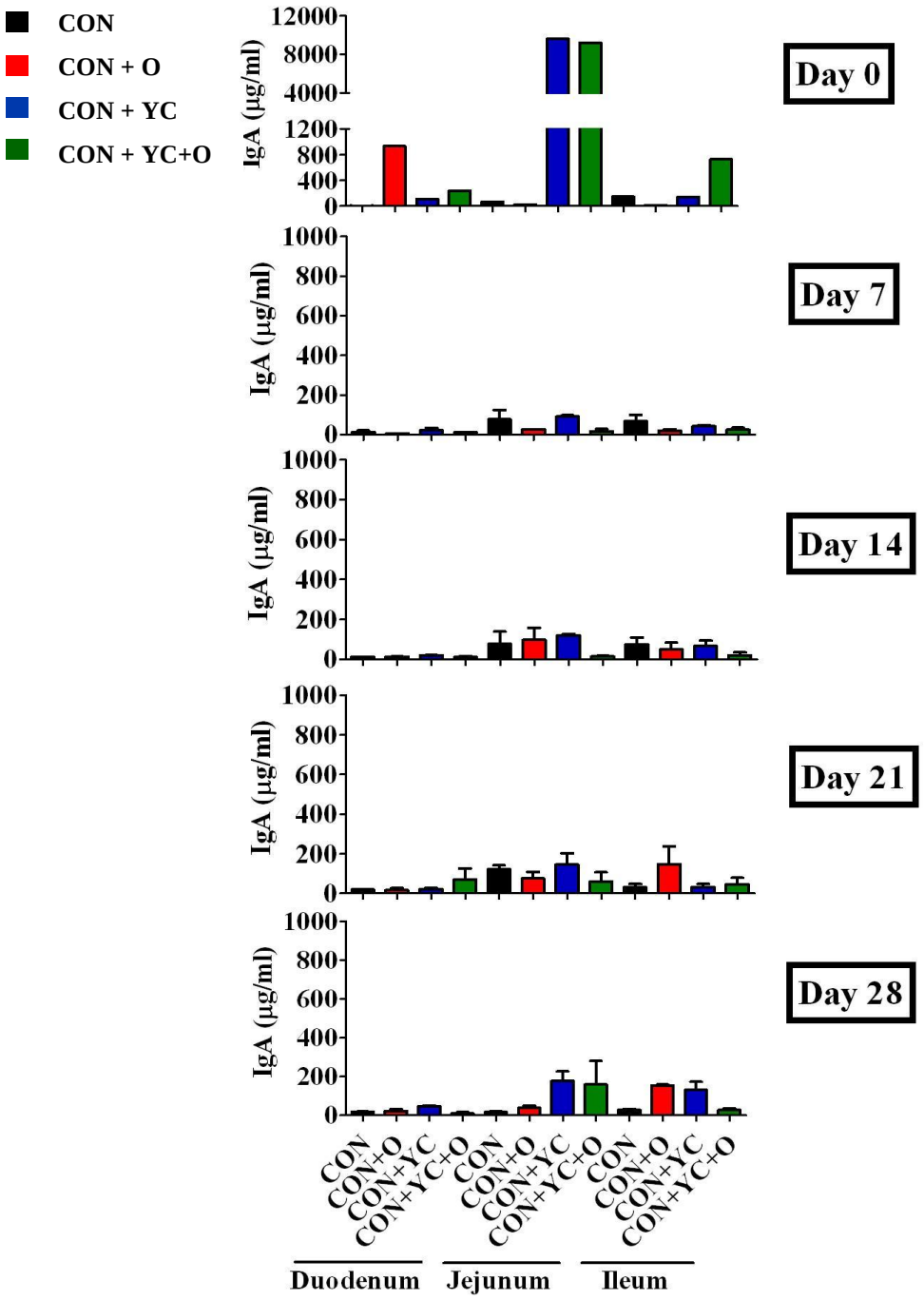


Figure 3. Trial #2: IgA levels in intestinal lavage. On day 0, each column represents n=1 and on days 7, 14, 21, and 28, each column represent n=2. Data are shown as Mean±SD.

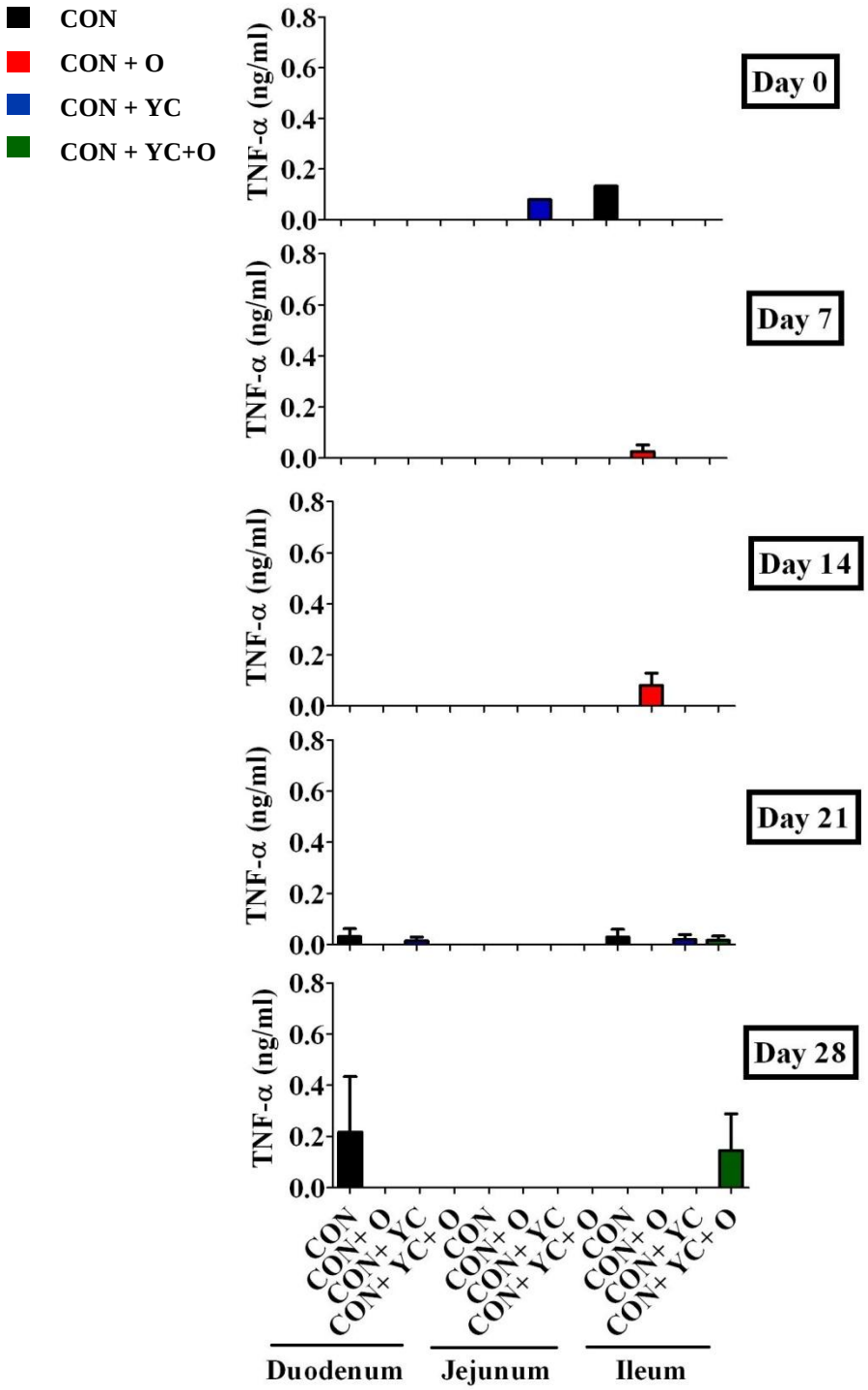


Figure 4. Trial #1: TNF-α levels in intestinal lavage. On day 0, each column represents n=1 and on day 7, 14, 21, and 28, each column represent n=2. Data are shown as Mean±SD.

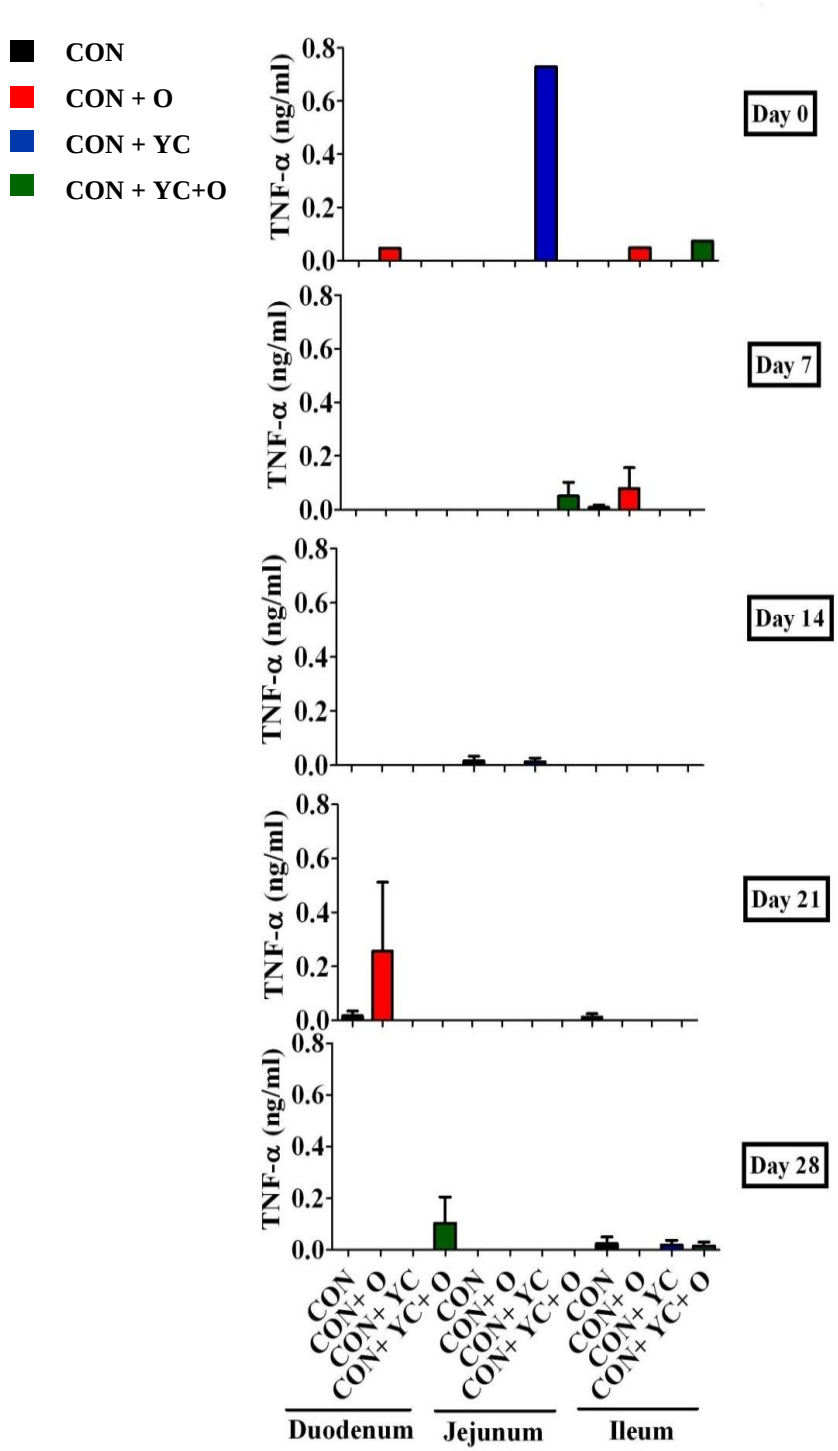


Figure 5. Trial #2: TNF-α levels in intestinal lavage. On day 0, each column represents n=1 and on day 7, 14, 21, and 28, each column represent n=2. Data are shown as Mean±SD.

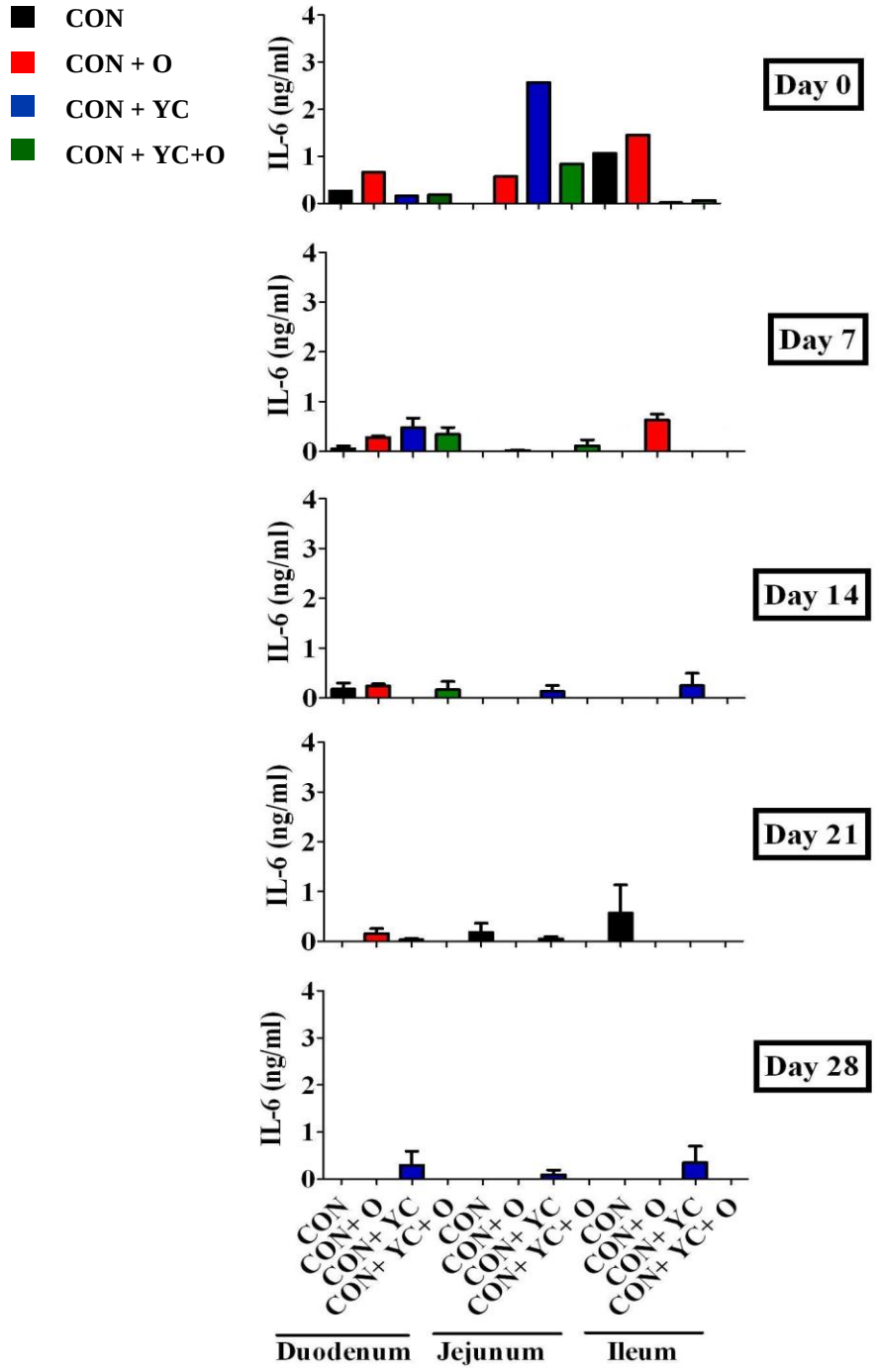


Figure 6. Trial #1: IL-6 levels in intestinal lavage. On day 0, each column represents n=1 and on day 7, 14, 21, and 28, each column represent n=2. Data are shown as Mean±SD.

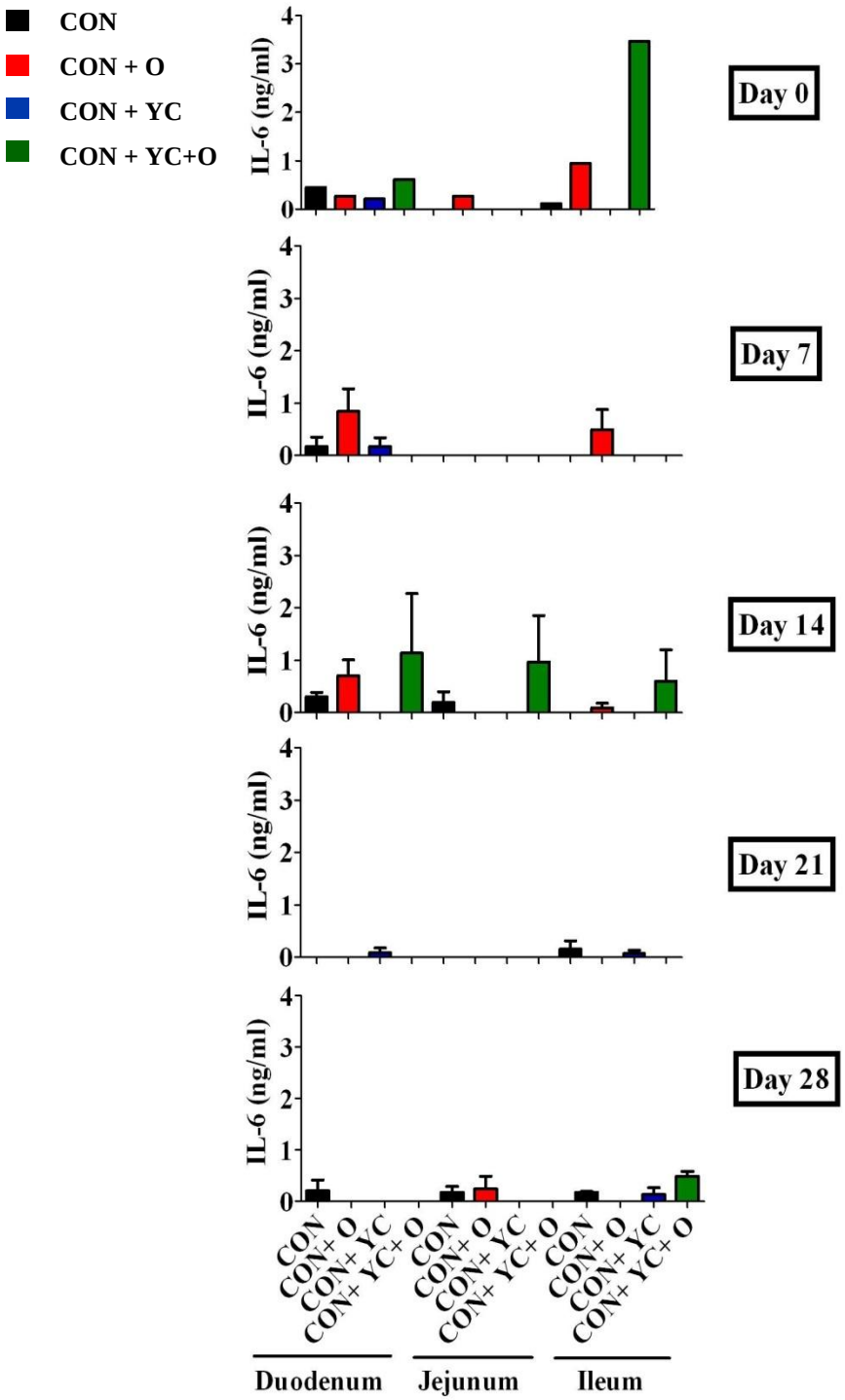


Figure 7. Trial #2: IL-6 levels in intestinal lavage. On day 0, each column represents n=1 and on day 7, 14, 21, and 28, each column represent n=2. Data are shown as Mean±SD.

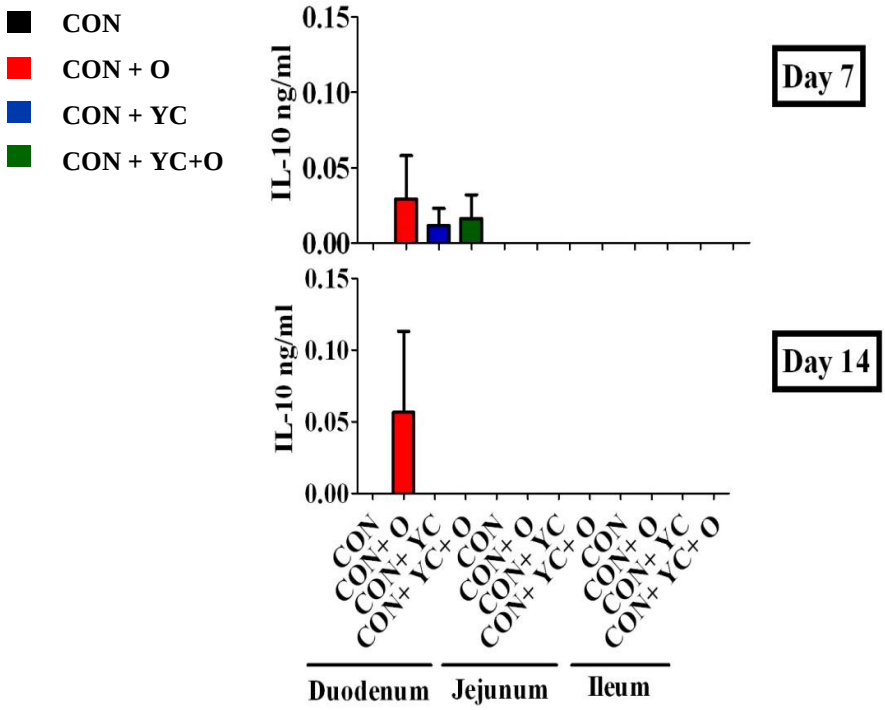


Figure 8. Trial #1: IL-10 levels in intestinal lavage. Each column represents n=2. Data are shown as Mean±SD.

Appendix B

Sow and Piglet Diet Formulations

Table 1

CON Diet Ration

Feed Ingredients	**Gestation Diet (% of diet)**	**Lactation Diet (% of diet)**
11102 NCDA Corn (Rolled)	80.30	n/a
Soybean Meal	13.80	n/a
Soybean Meal 48%	n/a	17.60
Corn NCDA	n/a	73.00
Corn (1/8) Micro-Flush	1.00	1.00
Limstone Fine	1.11	1.10
MON-CAL 21% P	2.05	2.40
Salt	0.50	0.50
Swine TM PX (KSU)	0.15	n/a
Swine Sow-Pig VIT	0.04	n/a
Threonine	n/a	0.01
BIOLYS 50%	n/a	0.25
Swine TM Prmx (KSU)	n/a	0.15
Swine VTM Prmx	n/a	0.04
Poultry Fat	1.00	4.00

Table 2

CON+YC Diet Ration

Feed Ingredients	**Gestation Diet (% of diet)**	**Lactation Diet (% of diet)**
11102 NCDA Corn (Rolled)	79.90	n/a
Soybean Meal	13.80	n/a
Soybean Meal 48%	n/a	17.50
Corn NCDA	n/a	72.60
Corn (1/8) Micro-Flush	1.00	1.00
Limstone Fine	1.10	1.07
MON-CAL 21% P	2.04	2.37
Salt	0.50	0.50
Swine TM PX (KSU)	0.15	n/a
Swine Sow-Pig VIT	0.40	n/a
Diamond V Mill Yeast Culture	0.50	0.50
Threonine	n/a	0.009
BIOLYS 50%	n/a	0.25
Swine TM Prmx (KSU)	n/a	0.15
Swine VTM Prmx	n/a	9.70
Poultry Fat	1.00	4.00

Table 3

CON+O Diet Ration

Feed Ingredients	**Gestation Diet (% of diet)**	**Lactation Diet (% of diet)**
11102 NCDA Corn (Rolled)	65.30	n/a
Soybean Meal	13.80	n/a
Soybean Meal 48%	n/a	10.85
Ground Whole Oat 15%	15.00	15.00
Corn NCDA	n/a	64.80
Corn (1/8) Micro-Flush	1.00	1.00
Limestone Fine	1.10	1.07
MON-CAL 21% P	2.05	2.38
Salt	0.50	0.50
Swine TM PX (KSU)	0.15	n/a
Swine Sow-Pig VIT	0.037	n/a
Threonine	n/a	0.009
BIOLYS 50%	n/a	0.25
Swine TM Prmx (KSU)	n/a	0.15
Swine VTM Prmx	n/a	0.04
Poultry Fat	1.00	4.00

Table 4

CON+YC+O Diet Ration

Feed Ingredients	**Gestation Diet (% of diet)**	**Lactation Diet (% of diet)**
11102 NCDA Corn (Rolled)	65.00	n/a
Soybean Meal	13.80	10.80
Ground Whole Oat 15%	15.00	15.00
Corn NCDA	n/a	64.50
Corn (1/8) Micro-Flush	1.00	1.00
Limestone Fine	1.10	1.10
MON-CAL 21% P	2.03	2.37
Salt	0.50	0.50
Swine TM PX (KSU)	0.15	n/a
Swine Sow-Pig VIT	0.04	n/a
Diamond V Mills Yeast Culture	0.50	0.50
Threonine	n/a	0.009
BIOLYS 50%	n/a	0.25
Swine TM Prmx (KSU)	n/a	0.15
Swine VTM Prmx	n/a	0.40
Poultry Fat	1.00	4.00

Table 5

NCAT Ration

Feed Ingredients	Concentration of Diet
Crude Protein	15.00% minimum
Lysine	0.65% minimum
Crude Fat	4.25 % minimum
Crude Fiber	6.00% minimum
Calcium	0.93-1.42%
Phosphorus	0.85% minimum
Salt	0.15%-0.65%
Selenium	0.30 ppm minimum
Zinc	165 ppm minimum

Table 6

Piglet Grower Ration

Feed Ingredients	**Concentration of Diet**
Crude Protein	18.00% minimum
Lysine	1.10% minimum
Crude Fat	5.00 % minimum
Crude Fiber	4.00% minimum
Calcium	0.61-1.11%
Phosphorus	0.55% minimum
Salt	0.15%-0.65%
Selenium	0.30 ppm minimum
Zinc	150 ppm minimum

Appendix C

Sow and Sire Information

Table 7

Trial #1: Sow and Sire Information

Sow #	**Breed**	**Sow Age (months)**	**Litters (#)**	**Sire breed**
125	Yorkshire	21	5	Duroc
115	Yorkshire	19	4	Yorkshire
503	Landrace/Duroc	26	6	Berkshire
532	Yorkshire/Duroc	9	1	Duroc
504	Yorkshire	11	2	Yorkshire
502	Yorkshire	14	2	Yorkshire
530	Yorkshire	16	3	Yorkshire
143	Yorkshire	22	3	Yorkshire
121	Yorkshire	24	4	Duroc
142	Yorkshire	9	1	Yorkshire
80	Yorkshire	25	5	Yorkshire
506	Yorkshire/Hampshire	9	1	Yorkshire
110	Yorkshire	22	6	Yorkshire

Table 8

Trial #2: Sow and Sire Information

Sow #	**Breed**	**Sow Age (months)**	**Litters (#)**	**Sire breed**
541	Landrace/Hampshire	26	3	Duroc
79	Yorkshire	18	2	Berkshire
76	Yorkshire/Landrace	29	5	Yorkshire
147	Yorkshire	16	3	Duroc
138	Yorkshire	21	4	Yorkshire
527	Landrace	27	4	Yorkshire
512	Landrace/Hampshire	32	5	Yorkshire
1022	Berkshire	19	4	Yorkshire
596	Yorkshire	14	4	Yorkshire
529	Landrace	12	3	Berkshire
130	Yorkshire	9	1	Yorkshire
529	Yorkshire	7	1	Yorkshire

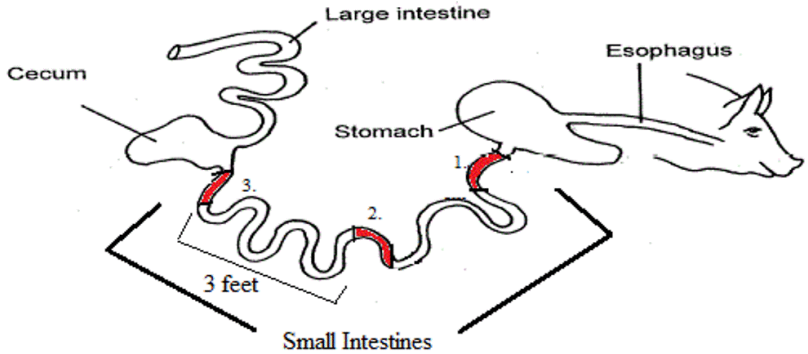
Appendix D

Protocols

Extraction of the Intestines

1. Extract the intestines from the body cavity by cutting the intestines where the stomach and small intestines connect and where the large intestines and rectum connect. Prior to cutting the small intestine where it attaches to the stomach, tie off the intestine with a small piece of colored sewing thread. Once the intestines are removed, separate the small and large intestine by cutting the intestinal tract above the cecum. When the small intestine is separated from the large intestine, tie off the end of the small intestine with a different color of sewing thread. By using different colored thread, it will allow one to know which end of the small intestine is the duodenum and which end is the ileum when the lavage is completed.

2. Cut the intestinal tract into the following 6 inch segments as shown in the diagram below:



Adapted version of the figure located at: http://www.thepigsite.com/articles/2749/digestive-system-of-the-pig-anatomy-and-function

a. Segment #1: Duodenum

1. Measure 6 inches from where the small intestine is connected to the

stomach and then cut the intestines.

b. Segment #2: Jejunum

    1. Measure 3 feet above where the segment was taken for the ileum and then cut the intestines. Next, measure 6 inches and cut the intestines again.

c. Segment #3: Ileum

    1. Measure 6 inches above where the small and large intestine connect and then cut the intestines.

Lavage of the Intestinal Segments

1. Clamp one end of the intestinal segment with an alligator clamp.
2. Using a syringe, fill the intestinal segment with 5 ml of ice cold lavage fluid.
3. While holding the open end of the intestinal segment, massage the intestine with one finger to remove cells and debris from the side of the intestinal lumen.
4. Drain the lavage fluid into a labeled 50 ml conical tube.
5. Centrifuge the lavage fluid at 2500 rpm for 10 minutes.
6. Pipette out 1 ml of lavage fluid and put it in a microcentrifuge tubes (at least 3-4 aliquots). Regardless of dietary treatment and intestinal segment, do not pool samples.
7. Store microcentrifuge tubes at -80ºC until needed.

Porcine IL-6 ELISA Protocol

A. Materials:

- Capture Antibody
- Detection Antibody
- Standard
- Streptavidin-HRP
- 96 well High-binding plate

B. Solutions:

- PBS
- Wash Buffer: PBS+ 0.05% Tween 20
- Reagent Diluent: 1% BSA in PBS
- TMB Substrate Solution
- Stop Solution: 2N H_2SO_4

C. Procedure:

1. Capture Antibody:
    a. Dilute capture antibody in PBS
    b. Coat 96 well plate with 50 µl of diluted capture antibody per well
    c. Incubate overnight at room temperature
2. Wash 3 times with PBS+ 0.05% Tween
3. Block plate with 150 µl per well of reagent diluent (1% BSA in PBS) and incubate at room temperature for 1 hour
4. Wash 3 times with PBS+ 0.05% Tween

5. Standards and Samples:
    a. Standards: Label 8 tubes
        i. Tube #1: 17 µl of standard+ 483 µl of reagent diluent
        ii. Tube #2: 250 µl part i + 250 µl of reagent diluent
        iii. Tube #3: 250 µl part ii + 250 µl of reagent diluent
        iv. Tube #4: 250 µl part iii + 250 µl of reagent diluent
        v. Tube #5: 250 µl part iv + 250 µl of reagent diluent
        vi. Tube #6: 250 µl part v + 250 µl of reagent diluent
        vii. Tube #7: 250 µl part vi+ 250 µl of reagent diluent
        viii. Tube #8: 250 µl of reagent diluents
    b. Samples
        i. Samples were left undiluted
    c. Add 50 µl of standard or sample per well
    d. Incubate at room temperature for 2 hours
6. Wash 3 times with PBS+0.05% Tween
7. Detection Antibody:
    a. Dilute detection antibody in reagent diluent (1% BSA in PBS)
    b. Add 50 µl per well
    c. Incubate at room temperature for 2 hours
8. Wash 3 times with PBS+0.05% Tween
9. Diluted Streptavidin-HRP (1:200):
    a. Add 50 µl per well
    b. Incubate at room temperature for 20 minutes

10. Wash 3 times with PBS+0.05% Tween

11. TMB Substrate:

    a. Add 50 µl of TMB per well

    b. Incubate at room temperature in the dark for 20 minutes

12. Stop solution:

    a. Add 25 µl of stop solution per well

13. Record optical density at 450 nm using a microplate reader

Porcine IL-10 ELISA Protocol

A. Materials:

- Capture Antibody
- Detection Antibody
- Standard
- Streptavidin-HRP
- 96 well High binding plate

B. Solutions:

- PBS
- Wash Buffer: PBS+ 0.05% Tween 20
- Reagent Diluent: 1% BSA in PBS
- TMB Substrate Solution
- Stop Solution: 2N H_2SO_4
- Normal Goat Serum

C. Procedure:

1. Procedure Capture Antibody:
    a. Dilute capture antibody in PBS
    b. Coat 96 well plate with 50 µl of diluted capture antibody per well
    c. Incubate overnight at room temperature
2. Wash 3 times with PBS+ 0.05% Tween
3. Block plate with 150 µl per well of reagent diluent (1% BSA in PBS) and incubate at room temperature for 1 hour

4. Wash 3 times with PBS+ 0.05% Tween
5. Standards and Samples:
    a. Standards: Label 8 tubes
        i. Tube #1: 25 µl of standard+ 475 µl of reagent diluent
        ii. Tube #2: 250 µl part i + 250 µl of reagent diluent
        iii. Tube #3: 250 µl part ii + 250 µl of reagent diluent
        iv. Tube #4: 250 µl part iii+ 250 µl of reagent diluent
        v. Tube #5: 250 µl part iv + 250 µl of reagent diluent
        vi. Tube #6: 250 µl part v + 250 µl of reagent diluent
        vii. Tube #7: 250 µl part vi+ 250 µl of reagent diluent
        viii. Tube #8: 250 µl of reagent diluents
    b. Samples
        i. Samples were left undiluted
    c. Add 50 µl of standard or sample per well
    d. Incubate at room temperature for 2 hours
6. Wash 3 times with PBS+0.05% Tween
7. Detection Antibody:
    a. Dilute detection antibody in reagent diluent (1% BSA in PBS) containing normal goat serum
    b. Add 50 µl per well
    c. Incubate at room temperature for 2 hours
8. Wash 3 times with PBS+0.05% Tween
9. Diluted Streptavidin-HRP (1:200):

a. Add 50 µl per well

b. Incubate at room temperature for 20 minutes

10. Wash 3 times with PBS+0.05% Tween

11. TMB Substrate:

a. Add 50 µl of TMB per well

b. Incubate at room temperature in the dark for 20 minutes

12. Stop solution:

a. Add 25 µl of stop solution per well

13. Record optical density at 450 nm using a microplate reader

Porcine TNF-α Protocol

A. Materials:

- Capture Antibody
- Detection Antibody
- Standard
- Streptavidin-HRP
- 96 well High binding plate

B. Solutions:

- PBS
- Wash Buffer: PBS+ 0.05% Tween 20
- Block Buffer: 1% BSA in PBS
- Reagent Diluent: PBS+ 0.1% BSA+0.05% Tween 20
- TMB Substrate Solution
- Stop Solution: 2N H_2SO_4

C. Procedure

1. Capture Antibody:
    a. Dilute capture antibody in PBS
    b. Coat 96 well plate with 50 µl of diluted capture antibody per well
    c. Incubate overnight at room temperature
2. Wash 3 times with PBS+ 0.05% Tween
3. Block plate with 150 µl per well of reagent diluent (1% BSA in PBS) and incubate at room temperature for 1 hour

4. Wash 3 times with PBS+ 0.05% Tween
5. Standards and Samples:
    a. Standards: Label 8 tubes
        i. Tube #1: 4.5 µl of standard+ 1495.5 µl of reagent diluent
        ii. Tube #2: 250 µl part i + 250 µl of reagent diluent
        iii. Tube #3: 250 µl part ii + 250 µl of reagent diluent
        iv. Tube #4: 250 µl part iii + 250 µl of reagent diluent
        v. Tube #5: 250 µl part vi + 250 µl of reagent diluent
        vi. Tube #6: 250 µl part v+ 250 µl of reagent diluent
        vii. Tube #7: 250 µl part vi + 250 µl of reagent diluent
        viii. Tube #8: 250 µl of reagent diluent
    b. Samples
        i. Samples were left undiluted
    c. Add 50 µl of standard or sample per well
    d. Incubate at room temperature for 2 hours
6. Wash 3 times with PBS+0.05% Tween
7. Detection Antibody:
    a. Dilute detection antibody in reagent diluent (1% BSA in PBS)
    b. Add 50 µl per well
    c. Incubate at room temperature for 2 hours
8. Wash 3 times with PBS+0.05% Tween
9. Diluted Streptavidin-HRP (1:200):
    a. Add 50 µl per well

b. Incubate at room temperature for 20 minutes

10. Wash 3 times with PBS+0.05% Tween

11. TMB Substrate:

a. Add 50 µl of TMB per well

b. Incubate at room temperature in the dark for 20 minutes

12. Stop solution:

a. Add 25 µl of stop solution per well

13. Record optical density at 450 nm using a microplate reader

IgA ELISA Protocol

A. Materials:

- Affinity Purified Antibody
- Standard
- HRP Detection Antibody
- 96 well High binding plate

B. Solutions:

- Coating Buffer : 0.05 M Carbonate-Bicarbonate
- Wash Buffer: PBS+ 0.05% Tween 20
- Blocking Buffer: Tris-Buffered Saline (TBS)+ 1% BSA
- Reagent Diluent: Tris-Buffered Saline (TBS)+ 1% BSA+ 0.05% Tween 20
- TMB Substrate Solution
- Stop Solution: 0.18M H_2SO_4

C. Procedure:

1. Coating Plate with Antibody:
    a. Dilute 0.5 µl of affinity purified antibody to 50 µl of coating buffer per well
    b. Add 50 µl of diluted antibody to each well
    c. Incubate at room temperature for 1 hour
2. Wash 5 times with PBS+0.05% Tween
3. Block plate:
    a. Add 100 µl of blocking buffer per well
    b. Incubate at room temperature for 30 minutes
4. Wash 5 times with PBS+0.05% Tween

5. Standards and Samples:
    a. Standards: Label 8 tubes
        i. Tube #1: 2.5 µl of pig reference serum+ 1625 µl of reagent diluent
        ii. Tube #2: 250 µl part i + 250 µl of reagent diluent
        iii. Tube #3: 250 µl part ii + 250 µl of reagent diluent
        iv. Tube #4: 250 µl part iii + 250 µl of reagent diluent
        v. Tube #5: 250 µl part iv + 250 µl of reagent diluent
        vi. Tube #6: 250 µl part v + 250 µl of reagent diluent
        vii. Tube #7: 250 µl part vi + 250 µl of reagent diluent
        viii. Tube #8: 250 µl of reagent diluents
    b. Samples
        i. Dilute samples:
            1. Milk
                a. Dilute 1:80,000
            2. Lavage Fluid was diluted accordingly to individual samples:
                a. 1:100
                b. 1:1000
                c. 1:2000
                d. 1:4000
    c. Add 50 µl of standard or sample per well
    d. Incubate for 1 hour at room temperature
6. Wash 5 times with PBS+0.05% Tween
7. HRP Detection Antibody:

   a. Dilute HRP detection antibody in reagent diluents
      i. Dilution: 1:40,000
   b. Add 50 µl of diluted HRP detection antibody per well
   c. Incubate at room temperature for 1 hour
8. Wash 5 times with PBS+0.05% Tween
9. TMB Substrate:
   a. Add 50 µl of TMB substrate per well
   b. Incubate at room temperature in the dark for approximately 15 minutes
10. Stop Solution:
   a. Add 50 µl of stop solution per well
11. Record optical density at 450 nm using a microplate reader