IMPACT OF EARLY NUTRITION ON THE DEVELOPMENT OF LUNG IMMUNITY IN THE PIGLET

BY

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THESIS

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Abstract

This thesis covers a variety of topics and analyses related to early nutrition on the impact it has on the development of lung immunity in the piglet. In the first section, an introduction is given. In short, neonates are susceptible to infection early in life, especially respiratory infections (Murphy *et al.* 2008). Respiratory infections are a major cause of morbidity and mortality in infants and children world-wide. The immune system exists to protect the host against infection and to help the neonate respond appropriately to critical transition periods of life, namely birth and weaning where the infant is exposed to a variety of new environmental and food antigens. Deficiencies of the immune system, both innate and adaptive immunity contribute to impaired host defense (Marodi and Notarangelo 2007) which can lead to increased susceptibility to infection. Exposure to dietary antigens influences the rate of maturation of the immune system (Kelly and Coutts 2000) and can even help provide a protective effect against infection. Breast milk is the optimal form of nutrition and is thought to help the immune system develop by providing signals to the immune system (Kelly and Coutts 2000), contributing bioactive components and stimulating the release of cytokines in peripheral blood mononuclear cells (PBMC) (Bessler *et al.* 1996) thus decreasing the risk of pneumonia (Chantry *et al.* 2006), upper respiratory, lower respiratory and gastrointestinal infections (Duijts *et al.* 2010). Despite the benefits of breast milk, only about 13% of infants are exclusively breastfed by 6 months of age (CDC 2010), therefore, increased understanding of lung immune characteristics and how they differ between breastfed and formula infants is necessitated.

The next section of the thesis looks at developmental differences in lung, mediastinal lymph nodes, and thoracic lymph nodes in breastfed compared to formula-fed piglets. In this study, colostrum-fed newborn piglets were either fed medicated sow milk replacer formula beginning at 48 hours of life (n=11) or remained with the sow (n=12) throughout the duration of the study. On d7 and d21 postpartum, approximately half of the piglets in each group were euthanized and blood and tissue samples were collected. Immune cells in the lungs, MSLN and TLN were analyzed through a variety of techniques. T lymphocyte subpopulations were identified using flow cytometry, cytokine mRNA expression was evaluated via RT-PCR, and total IgG, IgM, and IgA concentrations in serum were analyzed using enzyme linked immunoabsorbant assay (ELISA). Both dietary (SR vs. FF) and developmental effects on immunological development were observed. Through flow cytometry, it was found that NK cells were affected by diet in TLN, but not in PBMC or MSLN. However, an effect of day (e.g. development) was seen in PBMC NK cells. CD4+CD8+ T cell ratios were not different between FF and SR piglets in PBMC; however, diet affected MSLN at d21 and TLN at d7. Expression of CD4+CD8+ double positive T cells in PBMC were affected by day, while diet effects were seen in TLN on d7 and MSLN on d21. mRNA expression was investigated in whole tissue samples from the lung, TLN, and MSLN. Diet also affected the mRNA expression of IL-1β and TNF-α in TLN, dectin, IFN-α, and TGF-β2, in MSLN and IFN-β in lung tissue in which FF animals had higher mRNA expression than the SR counterpart. In addition, the expression of TLN IL-12 and dectin and MSLN IFN-α decreased over time while lung IL-6, TGF-β1, INF-α, and TNF-α increased over time. Turning to systemic immunity, serum IgG concentrations were lower in the SR piglets

than FF piglets ($p<0.05$), and IgG levels in d7 animals were higher than at d14 and d21 ($p<0.05$). Serum IgM concentrations were not significantly different in SR piglets compared to FF piglets nor did the concentrations exhibit developmental changes. Serum IgA levels were lower in the SR piglets when compared to the FF piglets ($p<0.05$), and IgA levels in d7 animals were higher than on d14 and d21 ($P<0.05$). The findings of this study have established a set of baseline measurements that establish the developmental changes in immune cells populations and cytokine expression in bronchial associated lymph tissues. Furthermore, these data demonstrated that differences exist between SR and FF piglets and provide a framework for future respiratory challenge studies to continue to pinpoint diet/immunological factors that increase the neonate's ability to resist respiratory infections and recover more quickly from pathogenic invasion.

This developmental study also established a foundation of normative changes over time for future studies to probe effectiveness of various formula components on mucosal lung immune development. The next section of the thesis discusses one component, β-glucan, and the effect it has on mucosal lung immune development. In this study, piglets (n=5-6/group) were fed formula containing 0 (control), 5 (WGP5), 50 (WGP50), or 250 (WGP250) mg/L formula. Half of the piglets in each treatment were vaccinated (FV) by i.m. injection against influenza (Fluzone™, Sanofi Pasteur, Swiftwater, PA) on d7 and received a booster on d14. Piglets were euthanized on d7 and d21. Weight gain and formula intake were unaffected by diet or vaccination. Fluzone-specific serum IgG concentrations was measured by ELISA. FV piglets had higher ($p<0.0001$) fluzone-specific IgG titer at d14 and 21 than non-V piglets independent of diet. Vaccination response were unaffected by oral WGP supplementation. TNF-α,

dectin, IL-1α, -2, -4, and -12 mRNA expression in lung were unaffected by age or dietary WGP. Lung TGFβ-1 mRNA expression was greater ($p<0.05$) at d21 than d7, and lung TGFβ-2 mRNA was lower ($p<0.01$) in all WGP diets compared to control. TNF-α, dectin, TGFβ-1, IL-2, -4, -6, or -12 mRNA in mediastinal lymph nodes (MSLN) were unaffected by age or dietary WGP. In MSLN, TGFβ-2 mRNA expression increased from d7 to d21 ($p<0.05$). TNF-α, TGFβ-1, TGFβ-2, IL-4, -6, or -12 mRNA in thorasic lymph nodes (TLN) were unaffected by age or dietary WGP. Dectin mRNA expression in TLN was lower at d21 compared to d7 ($p<0.05$). T-cell phenotypes were examined in MSLN and TLN by flow cytometry. In MSLN and TLN, CD4+ T-cells decreased, while CD8+ T-cells increased between d7 and d21 piglets ($p<0.001$), but these developmental patterns were unaffected by dietary WGP. Total serum IgG, IgM and IgA concentrations were also analyzed via ELISA. Total serum IgG, IgM and IgA were unaffected by WGP but followed typical developmental patterns. Thus, with the exception of reducing TGFβ-2 mRNA in lung, dietary WGP did not affect cytokine expression, T-cell phenotypes or vaccination response in piglets. The thesis comes to a close with a discussion of overall conclusions and future directions for this work.

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List of Abbreviations

BALT	Bronchus-associated lymphoid tissue
βG	β-Glucan(s)
BRM	Biological Response Modifiers
BW	Body weight
CR3	Complement Receptor 3
d	Day
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DP	Double positive
FDA	Food and Drug Administration
FF	Formula fed
FV	Fluzone vaccinated
GIT	Gastrointestinal tract
GALT	Gut-associated lymphoid tissue
h	Hour
HA	Hemagglutinin
HMOs	Human Milk Oligosaccharides
I.M.	Intramuscular
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
LRP	Lipoprotein receptor-related protein
LPS	Lipopolysaccharide
Mø	Macrophage

M	Membranous
mAbs	Monoclonal antibodies
MALT	Mucosal-associated lymphoid tissue
MSLN	Mediastinal lymph nodes
N	Neuraminidase
NALT	Nasopharynx-associated lymphoid tissues
NF-kB	Nuclear factor-kappa B
NK	Natural killer
NOAEL	No observed adverse effects
PAMPS	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
RIN	RNA integrity number
RNA	Ribonucleic acid
PP	Peyer's Patches
PPRS	Porcine reproductive and respiratory virus
PRRS	Pattern-recognition receptors
RT	Reverse transcription
SD	Standard deviation
sIgA	Secretory Immunoglobulin A
SIV	Swine influenza virus
SR	Sow-reared
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-alpha
TLN	Thorasic lymph nodes
TLR	Toll-like receptors
WGP	Wellmune WGP
V	Vaccinated

Chapter 1

Literature Review

Neonatal Immune Development

The immune system exists to protect the host against pathogenic organisms. This defense is particularly critical during periods of transition when neonates are subjected to major stressful events such as birth and weaning. Neonates are susceptible to infections early in life, especially respiratory infections. Deficiencies of both innate and adaptive immunity contribute to impaired host defense (Marodi and Notarangelo 2007). Highly complex pathways of recognition, response, elimination and memory have evolved in order to help protect the host especially at these vulnerable times in life. In addition to protecting the infant from infection, the immune system acts to ensure tolerance to 'self', to food, and to other environmental components. Development and maintenance of tolerance begins at an early age, sometimes even prenatally (Calder *et al.* 2006). A breakdown of tolerance can lead to inflammatory diseases

Neonates are born with an impaired production of T_H1-T-cell associated cytokines due to a maternal T_H2 bias during pregnancy, which is necessary to prevent the mother from generating an immune response against the developing fetus. This T_H2 bias creates a T_H2 dominance in the fetus. However, this bias against T_H1 cytokines leaves the newborn susceptible to microbial infection and, if the neonate is not able to effectively down-regulate the pre-existing T_H2 dominance and overcome low levels of allergen-specific IgE antibodies, an allergic phenotype may develop (Calder *et al.* 2006). Classic

T_H1 cytokines are interleukin (IL)-2, IL-12, and IFN-γ whereas the classic $T_H2$cytokines are IL-4 and IL-10.

Exposure to bacteria during delivery and on the mother's skin, as well as the introduction to formula and eventually solid foods, are key events in stimulating the maturation of the infant's gastrointestinal and immune systems. Early nutrition exposes the infant to novel food antigens and may be the source to which the immune system must become tolerant. These factors influence the development of the intestinal microbiota, which, in turn, affects antigen exposure, immune maturation, and immune responses (Calder *et al.* 2006).

Neonatal Piglet Immune System

The piglet is a good model to study neonatal immune development for several reasons. Unlike some other mammals, the pig can be reared independently of their mothers and can survive on bovine milk-based sow-milk replacer formula. They are sufficiently large in body size at birth, which allows for extensive surgical manipulation and long-term dietary treatment protocols (Calder *et al.* 2006). Piglets have similar respiratory and digestive system physiology and development as human infants. Furthermore, due to the piglet's rapid growth rate compared to the infant, the effects of experimental dietary treatments can be observed more rapidly.

Piglets are susceptible to similar diseases and pathogens as humans. For example, clinical signs of influenza in pigs are comparable to those in humans (Haesebrouck and Pensaert 1986). Pigs are a particularly good model because they are susceptible to human and avian influenza viruses (Ito *et al.* 1998).

Piglets secrete defense proteins, peptides, cytokines, and chemokines that are homologous to those of other mammals (Butler *et al.* 2009). The lymphoid cell populations, cells that mediate the production of immunity, of the pig are shown to be consistent to other vertebrates, especially humans (Boeker *et al.* 1999), with the exception of expressing greater proportions of CD4+/CD8+ double positive (DP) lymphocytes, comprising between 8 to 64% of the circulating pool of small resting T-lymphocytes (Pescovitz *et al.* 1985; Pescovitz *et al.* 1994; Zuckermann and Husmann 1996; Zuckermann and Husmann 1996) compared to <3% (Zuckermann 1999) in the human counterpart, in peripheral blood and secondary lymphoid organs.

One primary difference between the piglet and the human infant involves the transfer of passive immunity. The pig is born with virtually no serum immunoglobulins (Ig) due to the lack of placenta transfer (Gaskins and Kelley 1995) and, therefore, are dependent upon ingestion of colostrum and milk to obtain Ig (Bourne 1976). In contrast, human infants receive passive immunity both *in utero* via placental transfer of Ig and postnatally via consumption of colostrum and milk postpartum (Bourne 1976; Sangild 2006).

Maternal immunity, including specific systemic humoral immunity, involving mostly IgG, is transferred from the maternal circulation into colostrum and is typically absorbed within the neonatal intestine by macromolecular absorption. In addition, secretory IgA (sIgA), produced by the mammary gland, provides local immune protection within the piglet intestine. These antibodies are produced by the sow in response to intestinal and respiratory antigens and are passed on to the piglet to help protect the piglet against local pathogens (Salmon *et al.* 2009). Immunoglobulins play a

key role in host defense by forming IgA and antigen complexes in the lumen of the intestine. The content of IgA antibodies from milk gradually declines throughout lactation, allowing bacterial colonization to occur (Salmon *et al.* 2009). In addition to IgG, transfer of cytokines from colostrum to the bloodstream of the piglet peaks at two days after birth, which coincides with gut permeability; typically, gut closure occurs 24-36 h after birth (Nguyen *et al.* 2007)**.**

Lung Mucosal Immunology

The first point of contact for inhaled substances such as environmental pollutants, cigarette smoke, airborne allergens and microorganisms are the epithelial lining of the upper airways and lungs (Diamond *et al.* 2000). The lungs and upper airways are mucosal surfaces that have dual function; they protect the organism from invasion of foreign antigens and bacteria, while allowing for the exchange of materials with the environment. Due to the high exposure of the lungs and upper airways to airborne pathogens, they are common sites for infection. Mucosal defense mechanisms are, therefore, critical for preventing colonization of the respiratory tract by pathogens and penetration of antigens through the epithelial barrier.

The mucosal surfaces provide both nonimmune and immune mucosal defense. Tight junctions connect the epithelial cell layer covering most of the mucosal surfaces creating a physical barrier for pathogens and antigens. A mucus layer forms that retards the movement of microorganisms and allows for the sequestering of pathogens and antigens. If bacteria stick to the mucus layer before they can reach the target cell

receptor, they are swept away from the organism by other nonspecific defense mechanisms such as coughing, sneezing and mucociliary clearance.

The goal of the mucosal innate immune system is to discriminate between pathogen-associated and 'harmless' antigens, expressing active responses against pathogens and tolerance to harmless antigens (Bailey *et al.* 2005). Components of the innate immune system include phagocytes, such as neutrophils and macrophages (mø), natural killer (NK) cells, basophils, mast cells and eosinophils (Bals and Hiemstra 2004). The mucosal immune system is organized such that lymphoid elements of different mucosal tissues are collectively known as mucosal-associated lymphoid tissue (MALT), which then is separated into several components. These components included gut-associated lymphoid tissue (GALT), the bronchus-associated lymphoid tissue (BALT), the nasopharynx-associated lymphoid tissues (NALT), the mammary glands, and the genitourinary lymphoid tissue (Mestecky and McGhee 1987). BALT is made up of peribronchial, mediastinal, and cervical lymph nodes, mucosal epithelia, and lymphoid follicles sites, where foreign antigens are encountered and selectively taken up for initiation of immune responses (Vancikova 2002).

In recent years, it has become clear that airway epithelial cells not only provide a passive barrier function, but also actively contribute to the innate immune system (Diamond *et al.* 2000; Holgate *et al.* 2000). The airway epithelium senses bacterial exposure and responds by increasing its defenses. This response consists of an increase in the release of antimicrobial peptides into the lumen of the airways, and the release of chemokines and cytokines into the submucosa that initiate an inflammatory reaction. This inflammatory reaction includes the recruitment of phagocytes that serve to remove

microorganisms, and dendritic cells and lymphocytes that may aid in mounting an adaptive immune response (Bals and Hiemstra 2004).

There are several proposed mechanisms by which pathogens are recognized by the airway epithelium that lead to a protective response of the innate immune system. Cells of the innate immune system use pattern recognition molecules to bind to conserved molecular patterns that are present on microorganisms. Toll-like receptors (TLR) are one such family of pattern recognition receptors. The TLRs help shape the adaptive immune response by directing the way that DC 'educate' T-cells. Activation of TLRs also regulates the expression of a variety of genes, including cytokines and chemokines. Airway epithelial cells secrete molecules that are involved in inflammatory and immune processes (Diamond *et al.* 2000; Holgate *et al.* 2000). These include cytokines, chemokines, leukotrienes, calprotectin, β-defensins and other factors (Bals and Hiemstra 2004). By secreting these molecules, the airway epithelium is capable to chemoattract and activate cells of the innate and adaptive immune system, to immobilize and kill microorganisms, to induce wound healing and angiogenesis in response to injury and to orchestrate the initiation of and adaptive immune response (Bals and Hiemstra 2004).

Porcine Response to Vaccination

While the immune system of the piglet is anatomically developed at birth, it is functionally immature and several weeks are necessary for full functional maturation for many components of the immune system (Gaskins 1998). It is important that active immunity develops rapidly and appropriately in the piglet as immune protection acquired from colostrum does not confer protection against antigens to which the sow had not been

exposed. The rate of maturation of the immune system is influenced by exposure to the intestinal microflora and to dietary antigens (Kelly and Coutts 2000). The components of the immune system are present before birth as the piglet has the ability to synthesize IgG in response to an antigen challenge *in utero* and spontaneously IgG-secreting thymic B-cells are detectable in 67-day-old pig fetuses (Gaskins 1998; Rooke and Bland 2002). The age at which the suckling piglet accumulates appreciable concentrations of IgG by *de novo* synthesis appears to be at ~7 days of age in both naturally-suckled (Rooke *et al.* 2003) and artificially-reared piglets (Drew and Owen 1988).

Vaccines can play an integral role in protecting the young pig against various diseases. It requires 10 to 21 days after vaccination for the pig to mount a protective immune response. The exact length of time depends on the pig's age, the vaccine administered, and whether the pig had been vaccinated with the same vaccination before (Haesebrouck *et al.* 2004). Vaccination against swine influenza virus can greatly reduce or prevent virus replication after challenge and the resulting disease (Van Reeth *et al.* 2002). Reeth and colleagues (2002) showed that piglets vaccinated against swine influenza virus (SIV) and then challenged had lower virus titers than the non-vaccinated pigs, reduced production of cytokines in the lungs upon challenge, and had clear reductions in disease severity.

Influenza Vaccination

Influenza is primarily a respiratory tract infection involving the inflammation of the respiratory tract, which can progress to life-threatening pneumonia, hypercytokinemia, edema, acute lung injury, respiratory failure and death. Influenza is

contagious and occurs worldwide. About 20% of children and 5% of adults develop influenza A or B every year (Nicholson *et al.* 2003), which kills about 36,000 Americans every year (Fiore *et al.* 2008). Young children are more likely to catch influenza than older children or adults (Nicholson *et al.* 2003). Symptoms of influenza include croup, bronchiolitis, asthma, bronchitis, and otitis media.

There are three types of influenza viruses: A, B and C. Human influenza A and B viruses cause seasonal epidemics of disease while influenza type C infections cause a mild respiratory illness and are not generally associated with seasonal epidemics [CDC http://www.cdc.gov/flu/about/viruses/types.htm accessed Feb. 2, 2011]. Influenza type A viruses can be divided into two subtypes based on two proteins on the surface of the virus: the hemagglutinin (HA) and the neuraminidase (N). There are two different subtypes of influenza A recognized in people at this time. They are influenza A (H1N1) and influenza A (H3N2) viruses.

Vaccination remains the most effective first line of defense for people of all age groups against seasonal influenza (Wong *et al.* 2010). The risk of infection and associated morbidity and mortality is significantly reduced by vaccination against the pathogen *Haemophilus influenza* (*H. influenza*) (Fiore 2010). The benefits of annual immunization via intramuscular injection of high-risk populations with injectable trivalent inactivated influenza vaccines have been well established over the years.

Fluzone® vaccines (Sanofi Pasteur, Swiftwater, PA) are given for active immunization against influenza disease caused by influenza viruses: influenza A (H1N1), influenza A (H3N2), and type B (Fiore 2010). Fluzone® is an inactivated vaccine, also known as the flu shot, which is given by injection into the muscle. Influenza vaccines

have been known to produce a protective effect, which is largely dependent on the vaccine's ability to stimulate circulating antibody to the HA (Hobson *et al.* 1972). Vaccination against *H. influenza* is currently recommended for all children 6 months and older. In addition, a recent randomized double blind placebo controlled study conducted in 1375 healthy U.S. infants demonstrated that Fluzone® administered to young infants beginning at 6 to 12 weeks of age was safe and immunogenic (Englund *et al.* 2010). Another option for vaccination delivery is through a nasal spray FluMist® (MedImmune Vaccines, Inc. Gaithersburg, MD). The FluMist® offers an effective line of defense against seasonal influenza without having needle shots. In contrast to the injectable vaccine, FluMist® is a live trivalent vaccine which has been shown to induce protective immunity in the respiratory tract (McCarthy and Kockler 2004).

Nutritional Regulation of Immune Function

Evidence suggests that the composition of the diet influences the functioning of our immune system (Volman *et al.* 2008). Innate immune defenses are critical, especially immediately following birth and during the weaning period when the neonate is subject to a vast array of potentially pathogenic microorganisms that were not encountered in utero or during the first few months of life, respectively. The intestine is the largest immune organ in the body and, therefore, is a location that is exposed to vast quantities of dietary and microbial antigens (Kelly and Coutts 2000). The development of the normal immune function of the intestine is, therefore, vital for survival and is dependent on appropriate dietary and pathogenic antigen exposure among other things (Kelly and Coutts 2000).

It is generally accepted that the generation of appropriate immune responses and the development of immune regulatory networks in the neonate are dependent on the development of a normal intestinal flora as well as exposure to dietary antigen (Brandtzaeg 1996). Furthermore, immunological response following antigen exposure is determined by a number of factors including genetic background, nature, timing, and dose of the administered antigen (Strobel and Mowat 1998). As previously mentioned, the innate immune system plays an important role in the elimination of infectious agents while minimizing the damage they cause and, at the same time, they play an equally important role in processing harmless antigens. Failure to regulate the processing of harmless antigens correctly can lead to disease such as food-related allergy, autoimmunity, and inflammatory bowel disorders (Kelly and Coutts 2000). Due to the impact that antigens have on development of the immune system, it is critical that neonates are exposed to appropriate antigens through nutrition suited for immune development.

It is generally accepted that infants who are breast-fed have fewer infections compared with those who are formula fed. There is consistent evidence, in both developed and developing countries, that breast-feeding provides a protective effect in the first 4-6 months of life (Golding *et al.* 1997). In addition to supplying nutrition, maternal colostrum and milk also protect the neonate against gastrointestinal and respiratory diseases (Kelly and Coutts 2000) and have been shown to promote the maturation of the developing intestinal epithelium (Burrin *et al.* 1992; Wang and Xu 1996).

There is also a considerable body of literature describing immunosuppressive effects of breast-feeding and maternal antibodies on the development of active immune responses (Rennels 1996; Hodgins *et al.* 1999). During the early stages of life, maternal milk provides signals to the immune system that help develop appropriate response to antigens (Kelly and Coutts 2000). Breast milk also contains several bioactive components including human milk oligosaccharides (HMO), cytokines such as interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-18, IFN-γ, TNF-α, and TGF-β, as well as, immune cells. Breast-fed infants also have increased NK cells counts and higher antibody titres than formula-fed infants (Grimble and Westwood 2001). The majority of lymphocytes in milk are activated T-cells that express the surface antigen CD45RO+ and are often associated with immunological memory (Goldman and Goldblum 1997). There is evidence to suggest that the lymphocytes from milk can attach and traverse the neonatal intestine, and can remain locally with the intestine or migrate to enter circulation (Goldman and Goldblum 1997; Xanthou 1997).

Bioactive Components in Human Milk

Human colostrum has been shown to stimulate the release of cytokines from peripheral blood mononuclear cells (PBMC), thus altering the cytokine background against which immunological events are instigated (Bessler *et al.* 1996). The CDC report card suggests that 75% of infants in the U.S. are breastfed at birth but within the first 6 months it drops to 43% and only 33% of infants in the U.S. are exclusively breastfed for the first 3 months of life (CDC 2010). Therefore, it is important to supplement formula in such a way that neonates can experience similar immunological advantages to those

that are breast-fed. Several bioactive components are being investigated to more closely mimic human milk.

Lactoferrin: Lactoferrin (Lf), an iron-binding glycoprotein functions to transport iron in milk and other secretions (Gonzalez-Chavez *et al.* 2009). It is found in most biological fluids such as saliva, tears, bile, nasal and bronchial secretions, urine, and most highly in milk and colostrum (7 g/L) (Gonzalez-Chavez *et al.* 2009) and is considered an innate defense protein. It is synthesized by epithelial cells and granuloctyes (Kruzel *et al.* 2007) and is released by mucosal epithelia and neutrophils during inflammation (Puddu *et al.* 2009).

Lactoferrin is involved in several physiological functions, including immune response, antioxidant and anti-inflammatory properties, and protection against microbial infection (Gonzalez-Chavez *et al.* 2009). It also aids in controlling the initial reactions to infections, trauma, and injury (Kruzel *et al.* 2007). It is recognized by low-density lipoprotein receptor-related protein-1 and -2 (LRP1 and LRP2) receptors that have been identified on macrophages, platelets, and intestinal cells (Kruzel *et al.* 2007).

Lactoferrin acts as an antimicrobial agent by limiting the proliferation and adhesion of microbes and/or by phagocytosis (Puddu *et al.* 2009) due to the interaction of Lf with anionic molecules on some bacterial, viral, fungal, and parasite surfaces (Gonzalez-Chavez *et al.* 2009). These properties are mainly related to its ability to sequester iron in biological fluids, to destabilize the membranes of microorganisms (Puddu *et al.* 2009), or to bind exogenous pro-inflammatory bacterial components, such as lipopolysaccharides and their receptors (Legrand and Mazurier 2010). Lactoferrin seems to exert its antibacterial properties by direct interaction with microbes or by

competition with bacteria for iron which then inhibits the growth of the bacteria (Jenssen and Hancock 2009; Lonnerdal 2009). Lactoferrin also exerts anti-viral activity against several viruses that infect the gastrointestinal tract including rotaviruses, enteroviruses, and adenoviruses (Oguchi *et al.* 1995; Firth *et al.* 2005). Lactoferrin has also been shown to stimulate mucosal cell proliferation and differentiation in Caco-2 cells (Oguchi *et al.* 1995) which may enhance nutrient uptake.

Nucleotides: Nucleotides are low molecular weight intracellular compounds that consist of a nitrogenous base (purine and pyrimideine bases), a five-carbon sugar, and one to three phosphate groups, and are considered the foundation of RNA and DNA. Nucleotides are sources of cellular energy, signal transduction, physiologic mediators, coenzyme factors, and regulation of enzyme activity. They are naturally found in all foods of animal and vegetable origin as free nucleotides and nucleic acids (Gil 2002) and are also commonly found in human breast milk and cow's milk. Bovine milk has significantly lower nucleotide content than human milk resulting in infant formula with significantly lower nucleotide content than human milk. Since the goal is to mimic human milk as closely as possible, nucleotides have been added to infant formulas. Although 13 nucleotides have been described in human milk, only five of them have been added to formula (Yu 2002).

Proteases and nucleases degrade dietary nucleoproteins and nucleic acids into nucleotides. Intestinal alkaline phosphatases and nucleotidases cleave the phosphate groups from nucleotides to from nucleosides which are absorbed in the small gut. The absorbed nucleosides are mainly degraded to uric acid and allantoin, but some are reconverted to nucleotides (Yu 2002). Nucleotides also can be synthesized *de novo* but

this process is metabolically costly. Supplementation of formula with nucleotides may prevent the neonate from resorting to the *de novo* synthesis of nucleotides therefore optimizing physiological function.

Dietary nucleotides have been reported to be beneficial for infants since they positively influence lipid metabolism, immunity, and tissue growth, development and repair (Gil 2002). Dietary nucleotides have also been shown to play a significant role in the immunological development of neonates. Research indicates that supplementation of infant formula with nucleotides leads to improved growth and reduced susceptibility to infection (Grimble and Westwood 2001). In animal research, nucelotides have been shown to increase immune responses and beneficially affect gastrointestinal development.

In particular, it has been demonstrated, in a rat model, that nucleosides contribute to enhanced growth and maturation of the gut by increasing villus height and enzyme activities (Iwasa *et al.* 2000). Another study showed that mice that were fed a diet supplemented with 0.5% nucleotide mixture were less susceptible to mortality, had increased cell proliferation, greater production of Il-2 and IFN-γ when exposed to *Cryptosporidium parvum* given orally when compared to the counterpart non-nucleotide supplemented mice (Adjei *et al.* 1999). In another study, weanling mice fed chow plus water supplemented with 0.035% nucleotides exhibited increased NK cell activity and lower mø activation compared to the mice fed chow plus nonsupplemented water (Carver *et al.* 1990). Other animal studies have shown that mucosal DNA and protein synthesis, disaccharidase activity, villus cell number and height (Uauy *et al.* 1990) and small

intestinal wall thickness and weight are increased (Carver 1994) with nucleotide supplementation.

Human milk oligosaccharides (HMO): These complex carbohydrates are unique because of their structural diversity and high concentrations in human milk. Human milk contains 7-12 g HMO/L, making them the third largest component of human milk (Newburg 1997). The oligosaccharide content in the milk of most domestic mammals is 10- to 100-times lower than that found in human milk (Boehm and Stahl 2003).

HMOs are synthesized in the mammary gland starting with lactose at the reducing end and are comprised of D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and sialic acid (Bode 2009). The type of oligosaccharides produced in the mammary gland is believed to be influenced by maternal genetics, including the mother's Lewis blood group (Macfarlane *et al.* 2008). There are thought to be 12 core structures made up of glucose, galactose, and N-acetylglucosamine (Newburg *et al.* 2005), with over 200 structures characterized by mass spectroscopy (Ninonuevo *et al.* 2006). HMOs differ not only in structure but also in size, charge, sequence, and abundance (Ninonuevo *et al.* 2006). Human milk contains mainly neutral compared to the acidic HMO (Boehm *et al.* 2005). Neutral HMOs contain galactose, N-acetylglucosamine, fucose, and lactose core or anionic, whereas acidic HMOs contain the same oligosaccharide composition with N-acetylneuraminic acid (Ninonuevo *et al.* 2006).

Oligosaccharides in human milk are not digestible by small intestinal enzymes (German *et al.* 2008). The nutritional function that is most associated with HMOs is to serve as a prebiotic or indigestible carbohydrate that is fermented by the gut microbiota (German *et al.* 2008). Microbial degradation and fermentation of HMOs takes place in

the colon, providing the resident bacterial flora with the required carbon and energy for growth (Crociani *et al.* 1994). Evidence suggests that HMOs also act as receptor analogs to inhibit the adhesion of pathogens on the epithelial surface and interact directly with immune cells (Boehm and Stahl 2007), and protect the breastfed infant against infections and diarrhea (Newburg *et al.* 2005). HMOs have been demonstrated to selectively nourish the growth of highly specific strains of bifidobacteria, thus establishing the means to guide the development of the unique gut microbiota in infants fed breast milk (Ward *et al.* 2006). The presence of bifidobacteria in the infant gastrointestinal tract (GIT) has been associated with a number of important health-promoting effects, including reduced incidence of diarrheal illnesses, improved lactose digestion, and enhanced immunomodulatory functions (Leahy *et al.* 2005).

(1,3/1,6)-β-D-Glucan

Although β-Glucans are not normally found in human milk, they are thought to potentially influence neonatal immune development. The focus of my research was to determine the bioactivity of (1,3/1,6)-β-D-Glucan added to formula fed to piglets.

β-glucans are polysaccharides that occur as a principal component of cellular walls. Yeast, fungi, seaweeds, mushrooms, and some cereals such as oats and barley contain large amounts of β-glucans. β-glucans have been shown to lead to a variety of biological responses, including activation of neutrophils (Zhang and Petty 1994), macrophages (Adachi *et al.* 1994; Lebron *et al.* 2003), complement (Saito *et al.* 1992) and possibly eosinophils (Mahauthaman *et al.* 1988).

β-glucans stimulate the immune system, modulate humoral and cellular immunity, and, therefore, help the host to fight infections. β-(1,3)-glucans belong to the group of "Biological Response Modifiers" (BRM), meaning that they do not have direct cytotoxic activities, but are able to boost the natural defense mechanisms of the host (Zekovic *et al.* 2005; Descroix *et al.* 2006). The biological effects of β-glucans are measured through activation of natural killer (NK) cells, T-cells, and nuclear factor-kappaB (NF-kB), phagocytic activity, stimulation of secretion of cytokines, and production of reactive oxygen species (Descroix *et al.* 2006).

β-glucans are also antigenic substances that contain pathogen-associated molecular patterns (PAMPS), which are identified by pattern-recognition receptors (PRR) present on immune cells. These PRRs include at least four receptors that recognize β-glucans and allow the innate immune system to mount an immune response against it, including dectin-1, complement receptor 3 (CR3; CD11b/CD18), lactosylceramide, and scavenger receptors (Descroix *et al.* 2006). Several studies support the CR3 receptor as the primary receptor mediating the physiological response to yeast (1,3/1,6)- β-D-glucan (Baran *et al.* 2007; Li *et al.* 2007). Factors such as solubility, degree of branching, and primary structure impact the biological activity of β-glucans. β-glucans with a degree of branching between 0.2 and 0.33, a molecular weight between 100 and 200 kDa, and a triple-helix structure are most biologically effective (Zekovic *et al.* 2005).

Further, Beier and Gebert have shown that the uptake of particulate antigenic matter, including microorganisms and vaccine-bearing microspheres, by the intestinal mucosa is achieved by membranous (M) cells in the domes of Peyer's patches (PP) (Beier and Gebert 1998). Following uptake, the particles continue through the dome

epithelium into the subepithelial lymphoid tissue where an immune response is often initiated (Beier and Gebert 1998). Using Baker's yeast (*Saccharomyces cerevisiae*) as a tracer, Beier and Gebert investigated the kinetics of particle uptake in the PP of pigs (Beier and Gebert 1998). Transcytosis of yeast particles by M cells occurred within 1 h. Without significant phagocytosis by intraepithelial macrophages, the particles migrated across the basal lamina within 2.5-4 h where they were quickly phagocytosed and transported out of the PP domes (Beier and Gebert 1998).

In our study, Wellmune WGP® (Biothera, Eagan, MN) was extracted from cell walls of purified *Saccharromyces cerevisiae* using a proprietary technology, which produced a whole glucan particle in which the outer surface of mannoprotein and inner cellular contents were removed to expose the β 1,3/1,6 glucan. WGP is a linear glucose molecule with a beta 1,3/1,6 branch point extended by a long β 1,3 oligosaccharide **[**http://immunehealthbasics.com/HowItWorks.cfm**,** accessed Feb 2, 2011]. WGP yeast particles are approximately 2-5 mm in size, which is ideal for uptake into M-cells and PP. When WGP is ingested, it is transported to immune organs where it is taken up by PP. Macrophages then digest the WGP into smaller fragments that are released over a few days **[**http://immunehealthbasics.com/HowItWorks.cfm**,** accessed Feb 2, 2011]. Yeast β-glucan binds to the CR3 receptor (Thornton *et al.* 1996) and activates or enhances neutrophil anti-microbial activity, including migration of immune cells to the site of a foreign challenge and increased phagocytosis and oxidative burst. Several animal studies have shown beneficial effects of β-glucan. In one such study, mice were given a subcutaneous injection of yeast β-glucan (PGG-glucan) and whole glucan particle (WGP) β-glucan (Kournikakis *et al.* 2003). β-glucan increased survival rate, diminished

bacterial load in the lungs, and increased the proportion of bacteria-free animals after infection with anthrax in mice (Kournikakis *et al.* 2003). In another study, bacterial counts in blood of *Staphylococcus. aureus* (*S. aureus*)-challenged rats treated intramuscularly with PGG-glucan were lower than in control rats, and also the number of monocytes and neutrophils were increased (Liang *et al.* 1998). Additionally, Rice and colleagues (2005) found an increased survival in mice challenged with *S. aureus* or *Candida albicans* after oral administration of glucan phosphate (GluP). Furthermore, Kournikakis et al. (2003) showed that orally administrated WGP β-glucan increased survival in mice challenged with the anthrax bacteria.

In addition, yeast β-glucans have been shown to improve the humoral immunity of pigs and modulate cellular immunity of weanling pigs. Li and coworkers (2005) supplemented pigs diets with 50 mg/kg β-glucan. Pigs were injected with ovalbumin on day 14 and lymphocytes were isolated on day 28 and challenged with lipopolysaccharide (LPS) *ex vivo* to assess the impact of β-glucan on cellular immunity of pigs (Li *et al.* 2005). They found that β-glucan mitigated the elevation of pro-inflammatory cytokines, IL-6, and tumor necrosis factor-alpha (TNF-α), and enhanced the increase of an anti-inflammatory cytokine (IL-10) after LPS challenge (Li *et al.* 2005).

In another study, dietary yeast β-glucans exerted anti-viral effects against SIV in piglets that were fed 50 mg/kg β-glucan daily (Jung *et al.* 2004). On day 3, piglets were inoculated intra-nasally with SIV. Piglets that received the oral β-glucan had significantly lower microscopic lung lesion scores and significantly less nucleic acid in the lungs than those that did not receive the supplemented feed. Pre-administered β-glucan also reduced the pulmonary lesion score and viral replication scores in SIV-

infected piglets (Jung *et al.* 2004). Yeast β-glucans also had an anti-viral effect against porcine reproductive and respiratory virus (PPRS) in weanling pigs (Xiao *et al.* 2004). In both studies, yeast β-glucan administration was associated with an up-regulation of the T_H1 cytokine IFN-gamma. Indeed, a hallmark of WGP therapy is a switch from a T_H2 to a T_H1 response.

β-D-Glucan has had mixed effects on lung function and airway responsiveness. In one study, *S. cerevisiae* β-glucan fed orally (50 mg/pig) reduced the pulmonary lesion score and viral replication rate on pneumonia induced by swine SIV in piglets (Jung *et al.* 2004). In another study, pigs were fed diets with 0.025 or 0.5% β-glucan. Dietary β -glucan increased average daily food intake and average daily growth compared to control animals; however, pigs fed β-glucan had decreased plasma haptoglobin but more pigs fed 0.025% β -glucan died by d12 after *Streptococcus suis* challenge (Dritz *et al.* 1995).

Chapter 2

Research Objective, Specific Aims, and Hypothesis

Respiratory infections are a major cause of morbidity and mortality in infants and children world-wide. Nutrition during infancy impacts the infant's immunological development and ability to ward off respiratory infections and respond appropriately to antigens. Epidemiological evidence and clinical studies support the concept that 'breast is best', due in part to the effects of maternal milk on the immunological development of the neonate. Maternal milk provides signals to the immune system that support development of an appropriate antigen response (Kelly and Coutts 2000), as well as contributing several bioactive components including HMO, cytokines, and immune cells. Human colostrum has been shown to stimulate the release of cytokines from PBMC, thus altering the cytokine background against which immunological events are instigated (Bessler *et al.* 1996). Breastfed infants also have been acknowledged to have increased natural killer cells counts, higher antibody titers (Grimble and Westwood 2001), and activated T-cells that are often associated with immunological memory (Goldman and Goldblum 1997).

Despite the clear immunological advantages of breast feeding infants and the implications associated with those advantages, not every infant is breastfed, thus, establishing a need to identify not only how immune development differs between breastfed and formula-fed neonates, but also which bioactive components trigger these immunological benefits.

The ***overall goal of this thesis research*** was to investigate how early nutrition influences immune development. The overall hypothesis guiding this research was that

immune development differs between breast- and formula-fed neonates and that the addition of the BMR β-glucan to formula would mediate immune development of formula-fed piglets.

Specific Aim 1 investigated the development of immune cells in the lungs, mediastinal (MSLN) and thoracic lymph nodes (TLN), serum Ig profiles, and tissue cytokine mRNA expression in sow-reared (SR) pigs compared to those that were formula-fed (FF). The ***working hypothesis*** of this specific aim was that SR piglets would exhibit an earlier and more robust immunological development than the FF counterpart, which would be manifest through greater percentages of double positive T cell populations, greater percentages of NK cells, higher expression of T_H1 related cytokines, and greater concentrations of total serum IgG, IgA and IgM in SR piglets compared to FF piglets. It is also hypothesized that developmental changes will be observed; double positive T cells will likely increase over time, while CD4+ T cells will likely decrease over time, CD8+ T cells will likely increase over time, NK cells will likely increase over time. It is also hypothesized that T_H1-associated cytokines will likely increase over time, while T_H2-assoicated cytokines will decrease over time. It is also likely that total serum IgG, IgM and IgA concentrations will decrease from initial d7 concentrations and then at some point begin to increase towards d7 concentrations. Chapter 3 compared serum IgG, IgA and IgM concentrations, T-cell subpopulations and NK cells, and cytokine gene expression in lung, MSLN and TLN tissue.

Specific Aim 2 investigated the effect of yeast β-glucan supplemented to infant formula on the development of mucosal immunity in the lung as well as the immune response to immunization with an influenza vaccine. The ***working hypothesis*** of this aim

was that the addition of yeast β-glucan would enhance the immune response by enabling a switch from a T_H2 to a T_H1 response and increase cytokine secretion. Chapter 4 compared T-cell subpopulations in TLN and MSLN, gene expression in lung, TLN and MSLN tissues, total and Fluzone-specific IgG concentrations in serum, lung histomorphology and T-cell immunohistochemistry, and ex vivo proliferation of T-cells isolated from the TLN of 7-and 21-day-old piglets fed formula or formula supplements with WGP β-glucan.

Chapter 3

Impact of Diet on Development of Lung Immunity in the Neonatal Piglet

Abstract

Lung immune development is critically important in order to protect neonates from foreign pathogens that could lead to respiratory infection. We hypothesized the development of immune cells in the lungs of sow-reared piglets differs from that of formula-fed piglets. In this study, colostrum-fed newborn piglets were either fed medicated sow milk replacer formula beginning at 48 hours of life (n=11) or remained with the sow (n=12) throughout the duration of the study. On d7 and d21 postpartum, approximately half of the piglets in each group were euthanized and blood and tissue samples were collected. Immune cells in the lungs, MSLN and TLN were analyzed through a variety of techniques. T lymphocyte subpopulations were identified using flow cytometry, cytokine mRNA expression was evaluated via RT-PCR, and total IgG, IgM, and IgA concentrations in serum were analyzed using enzyme linked immunoabsorbant assay (ELISA). Both dietary (SR vs. FF) and developmental effects on immunological development were observed. Through flow cytometry, it was found that NK cells were affected by diet in TLN, but not in PBMC or MSLN. However, an effect of day (e.g. development) was seen in PBMC NK cells. CD4+CD8+ T cell ratios were not different between FF and SR piglets in PBMC; however, diet affected MSLN at d21 and TLN at d7. Expression of CD4+CD8+ double positive T cells in PBMC were affected by day, while diet effects were seen in TLN on d7 and MSLN on d21. mRNA expression was investigated in whole tissue samples from the lung, TLN, and MSLN. Diet also affected

the mRNA expression of IL-1β and TNF-α in TLN, dectin, IFN-α, and TGF-β2, in MSLN and IFN-β in lung tissue in which FF animals had higher mRNA expression than the SR counterpart. In addition, the expression of TLN IL-12 and dectin and MSLN IFN-α decreased over time while lung IL-6, TGF-β1, INF-α, and TNF-α increased over time. Turning to systemic immunity, serum IgG concentrations were lower in the SR piglets than FF piglets ($p<0.05$), and IgG levels in d7 animals were higher than at d14 and d21 ($p<0.05$). Serum IgM concentrations were not significantly different in SR piglets compared to FF piglets nor did the concentrations exhibit developmental changes. Serum IgA levels were lower in the SR piglets when compared to the FF piglets ($p<0.05$), and IgA levels in d7 animals were higher than on d14 and d21 ($P<0.05$). The findings of this study have established a set of baseline measurements that establish the developmental changes in immune cells populations and cytokine expression in bronchial associated lymph tissues. Furthermore, these data demonstrated that differences exist between SR and FF piglets and provide a framework for future respiratory challenge studies to continue to pinpoint diet/immunological factors that increase the neonate's ability to resist respiratory infections and recover more quickly from pathogenic invasion.

Introduction

Neonates are susceptible to infection early in life, especially respiratory infections (Murphy *et al.* 2008). In fact, respiratory infections are one of the leading causes of morbidity in children (Duijts *et al.* 2010). Breastfeeding has been shown to provide a protective effect against respiratory infections compared to formula feeding in infants (Chantry *et al.* 2006; Duijts *et al.* 2010). Breast milk plays several roles in regulating the immune system. It provides signals to the immune system to help initial immune development (Kelly and Coutts 2000), contributes bioactive components such as cytokines and human milk oligosaccharides, and stimulates the release of cytokines in PBMC(Bessler *et al.* 1996). Infants that are breastfed have also been shown to have increased natural killer cell counts (Grimble and Westwood 2001), higher antibody titers (Grimble and Westwood 2001), greater T helper cell phenotype development (Murphy *et al.* 2008), increased vaccination response (Dorea 2009) and lower morbidity and mortality rates (Kelly and Coutts 2000).

The piglet is an excellent model in which to study development of the BALT, because they have similar immune and respiratory system physiology and development when compared to human infants. In addition, piglets are susceptible to similar diseases and pathogens as humans (Haesebrouck and Pensaert 1986; Ito *et al.* 1998). Lastly, the pig can be easily reared independently of their mothers and can survive on bovine milk-based sow-milk replacer formula, which provides an exceptional opportunity to screen potential additives to human infant formula.

Piglets secrete defense proteins, peptides, cytokines and chemokines that are homologous to those of other mammals (Butler *et al.* 2009). The lymphoid cell

populations in the pig are shown to be consistent to other vertebrates, especially humans (Boeker *et al.* 1999). An exception is the fact that pigs express greater proportions of CD4+CD8+ double positive (DP) lymphocytes. In pigs, this population comprises between 8 to 64% of the circulating pool of small resting T-lymphocytes (Pescovitz *et al.* 1985; Pescovitz *et al.* 1994; Zuckermann and Husmann 1996; Zuckermann and Husmann 1996) compared to <3% in the human counterpart (Zuckermann 1999).

In one analysis it was shown that infants who were fully breastfed for 4 to <6 months were at greater risk for pneumonia than those who were fully breastfed for ≥ 6 months (Chantry *et al.* 2006). Another study showed that infants who were breastfed exclusively until 4 months of age and partially thereafter had lower risks of infections in the upper respiratory, lower respiratory and gastrointestinal tracts in infancy (Duijts *et al.* 2010). Approximately 80% of infants in the U.S. receive infant formula at some time in their first year of life. Optimally, bovine milk-based formulas should be formulated to mimic, as closely as possible, the biological actions of breast milk on immune system development. This is particularly important in achieving optimal development of mucosal immunity. However, a detailed analysis of the development of the lungs and BALT of breast-fed and formula-fed infants is lacking. The purpose of this study was to use the neonatal piglet model to gain a better understanding of the development of immune cellularity and cytokine expression in the lung and investigate the impact of neonatal nutrition by comparing SR and FF piglets. This model then can be used to probe the effectiveness of various formula components on mucosal lung immune development. In addition, peripheral blood cells and serum immunoglobulins were included as markers of systemic immune development.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Dietary Treatment and Animal Protocol

Piglets or sows were obtained from the Imported Swine Research Lab (ISRL) on the University of Illinois campus. The formula fed (FF) piglets (n=11) were obtained at 48 h postpartum to allow for consumption of colostrum. They were transferred to ERML animal care facility. Piglets were individually housed in environmentally controlled rooms (25°C) in cages, that maintain six piglets separated by Plexiglas partitions. Radiant heaters were attached to the tops of the cages to maintain an ambient temperature of 30°C. A commercially-available medicated sow milk-replacer formula (Advance Baby Pig Liquiwean, Milk Specialties Global Animal Nutrition, Carpentersville, IL) was provided as 14 equal feedings at a total volume of 360 ml/kg BW/day. Sow-reared (SR) piglets (n=12) were vaginally-delivered and remained with their birth mother in an environmentally-controlled room at ERML. Piglets were allowed to nurse *ad libitum* and were not given creep feed. However, by d21 piglets could be observed consuming small amounts of feed from the sow's bin. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

Sample Collection

On d7 (FF, n=5, SR, n=5) or d21 (FF, n=6, SR, n=7) postpartum, piglets were sedated with intramuscular injection of Telazol (3.5 mg/kg BW each tiletamine HCl and zolazepam HCl, Pfizer Animal Health, Fort Dodge, IA). After sedation, blood was collected by cardiac puncture into non-coated vacutainer tubes (BD Biosciences, Franklin Lakes, NJ) for serum isolation. Piglets were then euthanized by an intravenous injection of sodium pentobarbital (72 mg/kg BW Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). After death, a thoracotomy was performed and mediastinal lymph nodes (MSLN) and thoracic lymph nodes (TLN) were quickly excised. A portion of the lung, MSLN and TLN were snap frozen in liquid nitrogen. The remaining MSLN and TLN were collected for isolation of mononuclear cells.

Isolation and Phenotypic Identification of Mononuclear Cells from Tissues

Mononuclear cells from TLN and MSLN were obtained by cutting tissues into pieces and dissociating using a GentleMACS Dissociator (Miltenyi Biotec, Auburn, CA). Cells then were sequentially passed through 100 µm and 40 µm cell strainers (BD Biosciences, Bedford, MA) to form single cell suspensions. Cells were counted using a Countess automated cell counter (Invitrogen, Carlsbad, CA). The number of viable cells was assessed by trypan blue (Invitrogen Gibco) exclusion. The phenotype of T lymphocyte subpopulations from MSLN and TLN were monitored by flow cytometry using a panel of fluorescently labeled mAbs. Lymphocytes were identified by anti-swine CD45 (Clone K252-1E4, AbD Serotec, Raleigh, NC). Anti-CD45 was conjugated to

Alexa 647 with a Zenon Mouse Antibody Labeling Kit (Invitrogen Molecular Probes, Eugene, OR). T lymphocytes were identified by mouse anti-pig CD3:biotin (Clone BB23-8E6, Southern Biotech, Birmingham, AL) that was visualized with streptavidin:PE-Cy7 (Southern Biotech). To further differentiate T cell populations, cells were stained with mouse anti-pig CD4:FITC (Clone 74-12-4, Southern Biotech) and mouse anti-pig CD8:PE (Clone 76-2-11, Southern Biotech). All staining procedures took place on ice and care was taken to prevent unnecessary exposure to light. Briefly, one million cells per well were blocked with a mixture of 5% mouse serum (Southern Biotech) and 200 ug/ml purified mouse IgG (Invitrogen) for 5min. CD3 was added to the wells, incubated for 20 min, centrifuged, and then fluid was then aspirated. Cells were then incubated for 20 min in a total volume of 40 µl (10 µl of each: CD45, Strep-PECy7, CD4 and CD8). Cells were washed twice with PBS/1% BSA/0.1% sodium azide, and then fixed with 2% paraformaldehyde. Staining was assessed using an LSRII flow cytometer (BD™, Biosciences, San Jose, CA). The relative number of T cell subpopulations was determined using FlowJo 7.0 software (FlowJo, Ashland, OR). CD3+ events were considered T cells. CD45+CD3+CD4+ events were considered T helper cells. CD8+CD3+CD8+ events were considered cytotoxic T cells. NK cells were identified at CD3-CD4-CD8+ events.

Cytokine Expression

Total RNA was isolated and purified from snap frozen TLN, MSLN and lung samples with the TRIzol reagent (Invitrogen) following the manufacturer's protocol. RNA was quantified by spectrophotometry using a Nanodrop 1000 (Thermo Scientific,

Rockford, IL) at 260 nm. RNA concentration was adjusted to 0.25 µg/ml using RNase free water (Invitrogen). RNA quality was analyzed by a bioanalyzer (2100 Bioanalyzer, Agilent Technologies, Inc., Santa Clara, CA). All samples had an RNA integrity number (RIN) greater than 6.0.

Reverse transcription (RT) was performed on 3 µg of total RNA in a reaction involving 100 mM deoxyribonucleotide triphosphate (dNTP), 10X RT Buffer, 10X RT Random Primers, MultiScribe Reverse Transciptase, RNAse inhibitor (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA) and Diethylpyrocarbonate-treated (DEPC) water (Invitrogen). The final RT product was quantified by spectrophotometry as above. The final RT product was adjusted to a concentration of 1:150 or 1:200 using RNase-free water. Quantitative real-time PCR was conducted using SYBR-Green (Roche Diagnostics GmbH, Mannheim, Germany) and fluorescence intensity was collected using the Taqman ABI 7900 machine (Applied Biosystems Inc., Foster City, CA). A total of 40 PCR cycles were run. The primers used are listed in **Table 3.1,** and final primer concentrations were 300nM. Beta-actin was used as an internal standard reporter gene. Results are expressed using the Relative Standard Curve Method. In short, serial dilutions from a stock of pooled porcine spleen cDNA (range: 1:5 to 1:15,625) were made and run on each plate. Each sample was run with primers to assess the target gene and beta-actin. Normalized values for each target were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold-difference was calculated for each measurement by dividing the normalized target values by the normalized calibrator sample (in this case the formula fed group average). All samples that were statistically compared to each other were run on the same plate.

Isolation of Mononuclear Cells from Peripheral Blood

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. Briefly 10 ml of heparinized blood was diluted in 25 ml of RPMI and layered over Ficoll-Paque PLUS lymphocyte separation medium (GE Healthcare, Uppsala, Sweden). The peripheral blood mononuclear cells (PBMC) were recovered after centrifugation (400 x g, 30 min) across the density gradient. Isolated PBMC were placed in complete culture medium (RPMI 1640 (Gibco Invitrogen, Grand Island, NY) containing 10% fetal calf serum (Gibco Invitrogen, Grand Island, NY), 2 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 50 µg/mL gentamycin).

Serum Immunoglobulin Levels

Total serum immunoglobulin levels were detected by ELISA using assays specific for porcine IgG, IgM and IgA Quantification Sets (Bethyl Laboratories, Montgomery, TX, USA). A 96-well, flat-bottomed ELISA plate (Nunc, Rochester, NY) was coated with 100 µl coating antibody (µg coating antibody as suggested by the manufacturer diluted in 0.05M carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. The antibody solution was poured off, and the plate was washed three times with PBS/0.05% Tween20. The plates were blocked with 300 µl of 3% BSA/PBS for 1 h at room temperature (RT). The plates were washed as before. Serum samples were serially diluted in 0.05% gelatin/PBS, added to the wells in duplicate (100 µl per well), and plates were incubated for 1 h at RT. Samples for standard curves were included with the quantification sets and were used as directed. Plates were washed as before, and 100 µl

HRP-conjugated detection antibody (concentration as recommended by the manufacturer) in 0.05% gelatin/PBS was added to each well. Plates were protected from light and incubated for 1 h at RT. Plates were washed four times with PBS-Tween 20. 100 µl TMB reagent solution (OptEIA, BD Biosciences, San Diego, CA) was added to each well and allowed to develop protected from light at RT for the time recommended by the manufacturer. The reaction was stopped with 100 µl 2N H_2SO_4 per well. The absorbance was then read at 450 nm with 570 nm correction using a microplate reader (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA). Total immunoglobulin values were determined based on a standard curve that was run on each plate.

Statistical Analysis

Analyses were performed using the PROC GLM (generalized linear model) procedure within SAS (Version 9.2, SAS Institute, Cary, NC). The initial model was day, diet and day*diet interaction. If the model was not significant, d7 and d21 data were combined. If diet was not significant, diet was excluded and SR and FF at d7 data were combined and SR and FF at d21 were combined. Statistical significance was defined as $p<0.05$, with trends reported as $p<0.10$. All data are expressed as mean ± SD.

Results

T Lymphocyte Populations

Flow cytometry was used to analyze T cell phenotype as well as NK cells in PBMC, MSLN and TLN. PBMC NK cell populations and CD4:CD8 ratios did not differ between SR and FF piglets, thus SR and FF samples were pooled at both d7 and d21 in order to make developmental comparisons. CD4+CD8+ double positive T cell data were not pooled because the model was significant. There were more CD4+CD8+ double positive T cells in the PBMC populations from d21 pigs than d7 pigs ($p<0.05$) (**Figure 3.1A**). NK cells were higher in the PBMC populations from d7 than those from d21 piglets ($p<0.05$) (**Figure 3.1B**). CD4:CD8 ratios did not differ between d7 and d21 piglets. Thus, diet did not significantly affect developmental profiles of T cell and NK cells in PBMC.

This was not true in the BALT. On d7, MSLN CD4:CD8 T cell ratios did not differ between SR and FF pigs. By d21, CD4:CD8 T cell ratios were higher ($p<0.0001$) in SR (7.9 ± 1.7%) than in FF piglets (4.2 ± 1.3%) at d21 ($p<0.0001$) (**Figure 3.2**). CD4+CD8- T cells were higher ($p<0.05$) at d7 than d21 and CD4+CD8- T cells were higher ($p<0.05$) in SR compared to FF piglets (**Figure 3.3A**). CD4-CD8+ T cells were higher ($p<0.01$) at d21 than d7 (**Figure 3.3B**). FF piglets had more double positive T cells compared to SR piglets ($p<0.01$) (**Figure 3.4**). NK cells were not significantly different between SR and FF piglets in MSLN at either age.

Diet effects were also observed in the TLN T cell populations. These effects, however, were observed earlier than those in the MSLN. Day 7 TLN CD4:CD8 T cell ratios were higher ($p<0.05$) in SR (10.5 ± 2.6%) than FF piglets (6.7 ± 1.9%) ($p<0.05$)

(**Figure 3.5A**). Additionally, d7 TLN double positive CD4+CD8+ T cells were higher ($p<0.05$) in SR (9.9 ± 0.02%) than in FF piglets (7.4 ± 0.01%) (**Figure 3.5B**). There were no differences between FF and SR groups for CD4:CD8 T cell ratio ($p=0.09$) or percentages of CD4+CD8+ T cells ($p=0.28$) in TLN at d21. Diet and developmental changes were observed in CD4-CD8+ T cells; CD8+ T cells were higher ($p<0.01$) at d21 than d7 and FF were greater ($p<0.01$) than SR (**Figure 3.6**). There were no differences in CD4+CD8- T cell populations. Diet also affected NK cells; NK cells were higher ($p<0.05$) in SR (7.4 ± 2.8%) than in FF piglets (3.4±3.1%) (**Figure 3.7**).

Cytokines mRNA Expression

The mRNA expression of various genes was investigated in whole tissue samples from the lung, TLN and MSLN in order to gain a better understanding of gene expression profiles in these tissues (see **Table 3.2** for a summary of the roles of these cytokines/receptor). No differences were observed between FF and SR piglets in mRNA expression of IL-6, TGF-β1, IFN-α, IFN-β or TGF-β2 in TLN. However, there were developmental differences seen in the expression of IL-12 (**Figure 3.8A**) and dectin (**Figure 3.8B**), as well as, diet effects for IL-1β (**Figure 3.9A**), and TNF-α (**Figure 3.9B**) expression. TLN IL-12 and dectin mRNA were higher ($p<0.05$) at d7 than at d21. TLN IL-1β and TNF-α were higher ($p<0.05$) in FF animals than in SR animals.

In MSLN, mRNA expression of IL-6, IL-12, TGF-β1, IL-1β, IFN-β, and TNF-α were not significantly different between SR and FF piglets. However, dectin mRNA expression was higher ($p<0.01$) in FF compared to SR piglets (**Figure 3.10A**). Dectin expression also tended ($p=0.051$) to be decreased from d7 to d21. A day*diet interaction

was also significant ($p<0.05$) in mRNA expression of dectin. TGF-β2 and IFN-α mRNA expression were higher ($p<0.05$) in FF compared to SR piglets (**Figures 3.10B and 3.11**).

In lung, mRNA expression of dectin, IL-12, TGF-β2, and IL-1β were not significantly different between SR and FF piglets. However, IL-6 (**Figure 3.12A**), TGF-β1 (**Figure 3.12B**), TNF-α (**Figure 3.13A**), and IFN-α (**Figure 3.13B**) mRNA expression were higher ($p<0.05$) at d21 than at d7. IFN-β mRNA expression was higher ($p<0.05$) in FF piglets compared to SR piglets (**Figure 3.14**). (See **Table 3.3** for a summary of cytokine/receptor results.)

Total Serum Immunoglobulin Levels

In order to determine if circulating immunoglobulins differed in SR and FF piglets, sera from both groups were analyzed for IgG, IgM, and IgA concentrations at d7, d14 and d21. The serum IgG concentrations were lower ($p<0.05$) in the SR piglets when compared to the FF piglets. IgG concentrations in d7 animals were higher ($p<0.05$) than in d14 and d21 animals (**Figure 3.15**). Serum IgG concentrations ranged from 16.0 ±7.9 mg/ml to 26.1 ± 12.4 mg/ml in d7, whereas d14 ranged from 12.6 ± 4.8 mg/ml to 15.3 ± 5.2 mg/ml. Day 21 total serum IgG ranged from 9.5 ± 4.3 mg/ml to 15.2 ± 10.2 mg/ml. Serum IgM levels were not significantly different in SR piglets compared to FF piglets. Day 7 total serum IgM ranged from 6.8 ± 3.0 mg/ml to 11.9 ± 4.4 mg/ml while day 14 was 10.3 ± 4.6 mg/ml to 13.0 ± 7.6 mg/ml, and d21 ranged from 7.6 ± 1.6 mg/ml, to 12.2 ± 3.7 mg/ml. The serum IgA concentrations were lower in the SR piglets when compared to the FF piglets ($p<0.05$), while IgA concentrations on d7 animals were higher than

those on d14 and d21 ($p<0.05$) (**Figure 3.16**). Serum IgA concentrations averaged 2.5 ± 1.2 mg/ml at d7, 0.5 ± 0.4 mg/ml at d14, and 0.2 ± 0.1 mg/ml at d21.

Discussion

The immune system is functionally immature at birth, the immediate postnatal period and during weaning are the developmental periods when the neonate is subjected to a vast array of potentially pathogenic microorganisms, especially respiratory infections, that were not encountered in utero or during the first few months of life, respectively. At these especially vulnerable times in the neonate's life, highly complex pathways of recognition, response, elimination and memory are important in order to help protect the host (Calder *et al.* 2006). Composition of the diet influences the development and competence of the immune system (Volman *et al.* 2008). In this study, we examined developmental differences in the BALT of SR and FF piglets.

Flow cytometry was used to assess immune development by characterizing the T cell subpopulations and NK cells present in PBMC, TLN and MSLN. Hawkes and colleagues compared PBMC from breast-fed and formula-fed human infants at 6 months of age. They found that the relative frequency of NK cells was greater in breast-fed than in formula-fed infants (9.7% vs. 7.1%; $p < 0.001$) (Hawkes *et al.* 1999). The population of NK cells within the PBMC in our study was not significantly different between SR was compared to FF, but were observed to decrease over time. In the same study, Hawkes and co-workers found that the CD4:CD8 ratio in breast-fed infants was lower than that in formula-fed infants (2.8 vs. 3.3; $p < 0.005$) (Hawkes *et al.* 1999). In contrast, the results of our study found that the CD4:CD8 ratio was not statistically different in SR compared to FF PBMC. However, the CD4:CD8 ratio was higher in SR piglets compared to FF piglets at d21 in MSLN and at d7 in TLN.

As expected (Zuckermann 1999), CD4+CD8+ double positive T lymphocytes increased from d7 to d21 in PBMC. While MSLN CD4+CD8+ double positive T lymphocytes were higher in FF piglets compared to SR piglets at d21, TLN CD4+CD8+ cells were higher in SR compared to FF piglets at d7. One possible explanation is that the TLN and MSLN drain different internal structures possibly resulting in varying levels of expression.

In both humans and swine, neonates are born with an impaired production of T_H1-cell associated cytokines due to a maternal T_H2 bias during pregnancy that protects the fetus from immune response or rejection (Calder *et al.* 2006). This T_H2 bias creates T_H2 dominance in the fetus. Because this comes at the expense of T_H1 cytokine production, it leaves the newborn susceptible to microbial infection. Additionally, if the neonate is not able to effectively down-regulate the pre-existing T_H2 dominance and overcome low levels of allergen-specific IgE antibodies, an allergic phenotype may develop (Calder *et al.* 2006).

In order to gain an overview of cytokine production in the lungs of SR versus FF piglets, mRNA expression was analyzed. This analysis included T_H1-associated cytokines (interleukin (IL)-12), pro-inflammatory cytokines (IL-6 and TNF-α), and T_H3 cytokines (TGF-β1, and TGF-β2). Expression of both dectin and IL-12 decreased over time in TLN. This may correspond with an initial aggressive attempt to establish T_H1 development, once a balance has been created between T_H1 and T_H2 expression the drive to establish this switch is decreased resulting in decreased expression of both dectin and IL-12. Expression of IL-6, TGF-β1, TNF-α, and IFN-α in lung all increased overtime as expected. These cytokines likely increased over time due to greater exposure of the lung

to various antigens. IL-1β, and TNF-α in TLN, dectin, TGF-β2, and IFN-α in MSLN, as well as IFN-β in lung all had greater expression in FF animals compared to SR animals. Perhaps this is due to stress associated with birth and subsequent separation from their mother a birth, or perhaps this pattern is due to an underlying inflammation since the FF piglets are less protected from environmental pathogens than the SR counterpart. These differences in expression could also be due to the differences in colostrum each piglet received.

In each tissue, we saw differing patterns of cytokine mRNA expression. These differences could be due to location of each tissue in the body and the proximity of each tissue to pathogens. Lung tissue would likely be the furthest target on the pathogen's route from the oral/naso-pharynx route into the body. Thus, lung tissue would be exposed to fewer foreign microorganisms leading it to mount a different type of immune response than the MSLN, which are closer to the point of airway branching and therefore in closer proximity to the foreign microorganisms route of entry. TLN are located superior to both the lung and MSLN and, therefore, encounter pathogens before the others in the course of ingestion/inhalation. TLN and MSLN also drain different internal structures and may therefore be exposed to different organisms causing dissimilar strengths of expression. Each lymph node excised in the TLN and MSLN groups differ in its location and therefore each may differ in level of expression thus causing noticeable variation around the mean.

To gain a perspective of systemic immune development of piglets in each of the dietary treatment groups, total serum IgG, IgM and IgA concentrations were measured. We found that the serum IgG concentrations were lower in the SR piglets when compared

to the FF piglets ($p<0.05$). Overall, IgG concentrations were higher on d7 than on d14 and d21 ($P<0.05$). Previous research suggested that concentrations of IgG in colostrum decrease 5-fold in the first 24 h of lactation and then IgG concentration in the sow's milk decreases 30-fold in the first week (Bourne 1973). The IgG concentrations in serum of young piglets followed this same pattern (Bourne 1973). It is likely that the sows that nursed the piglets that were eventually FF provided varying levels of IgG that subsequently created variation in immunoglobulin levels in the piglet serum.

Serum IgM concentrations were not significantly different in SR piglets compared to FF piglets. Bourne's research suggests that concentrations of IgM in colostrum decrease 2-fold in the first 24 h of lactation and then drop slightly through the course of the next 35 days (Bourne 1973). The IgM concentrations in the serum of young piglets decreased rapidly following the first 24 h of life to their lowest level at 8-14 d of age. IgA then began to increase (Bourne 1973).

Serum IgA concentrations were lower in the SR piglets when compared to the FF piglets ($p<0.05$). Overall, IgA serum concentrations were higher on d7 than on d14 and d21 ($P<0.05$). Bourne's research suggests that concentrations of IgA in colostrum decrease 3-fold in the first 24 h of lactation and then tend to fluctuate through the course of the next 35 days of age (Bourne 1973). The IgA concentrations in serum of young piglets varied in the first 24 h but then decreased rapidly after 24 h to their lowest values at 17-22 d of age (Bourne 1973). Curtis and Bourne concluded, based on half-life studies of naturally absorbed colostral immunoglobulins and ^{125}I-labelled immunoglobulins, that IgA production in the piglet did not contribute significantly to serum IgA concentrations in the first 7-12 d of life nor did IgG production during the first 2-3 weeks of life.

However, IgM produced in the first week of life contributed significantly to circulating IgM levels (Bourne and Curtis 1973). Ogawa and co-workers found that TGF-β1 and TGF-β2 in colostrum correlated with increased serum IgA concentrations in human infants during the first month of life (Ogawa *et al.* 2004). It was also found that high concentrations (10 ng/ml) of TGF-β1 suppressed immunoglobulin-secreting cell responses to LPS and rotavirus while low concentrations (0.1 ng/ml) promoted isotype switching to IgA antibody (Nguyen *et al.* 2007).

It has been generally recognized that infants who are breast-fed have fewer infections compared with those who are formula fed. There is consistent evidence, in both developed and developing countries, that breast-feeding provides a protective effect in the first 4-6 months of life (Golding *et al.* 1997). Maternal colostrum and milk, in addition to supplying the neonate with nutrition, also protect against gastrointestinal and respiratory diseases (Kelly and Coutts 2000) and have been shown to promote the maturation of the developing intestinal epithelium (Burrin *et al.* 1992; Wang and Xu 1996).

In conclusion, this research supports previous research that diet does, in fact, impact immune development. This research helps identify some of the lung-specific immunological differences associated with diet and development. Thus, this study has established a set of baseline measurements from which it will be possible to look at effects of pathogens or other insults to the system as well as dietary components that may bring the FF piglets bronchial-associated immune development more in line with that seen in the SR piglets. Through this study, we have established a foundation of normative changes over time for future studies to probe effectiveness of various formula

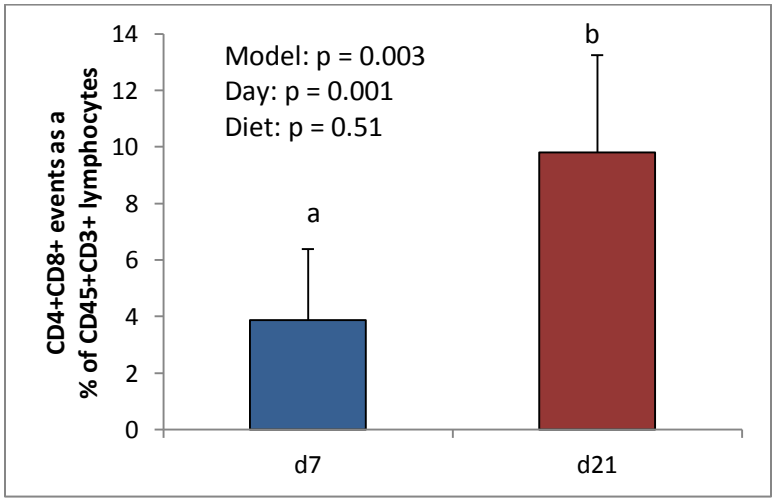
components on mucosal lung immune development and to identify potential effects of diet in a respiratory challenge model. These challenge experiments will determine if the difference in immune development seen in piglets fed sow-milk replacer or sow's milk itself affects the neonate's ability to resist respiratory infections or enables them to recover more quickly.

Table 3.1: Primers Used for Quantitative Real Time-PCR.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Gene Bank Accession ID
β-actin	CACGCCATCCTGCGTCTGGA	AGCACCGTGTTGGCGTAGAG	DQ845171.1
Dectin	CTCTCACAACCTCACCAGGAGAT	CAGTAATGGGTCGCCAATAAGG	FJ386384.1
IL-12	CGTGCCTCGGGCAATTATAA	CAGGTGAGGTCGCTAGTTTGG	NM_213993.1
IL-6	CTGGCAGAAAACAACCTGAACC	TGATTCTCATCAAGCAGGTCTCC	AB194100.1
IL-1β	AACGTGCAGTCTATGGAGT	GAACACCACTTCTCTCTTCA	NM_214055.1
IFN-α	TCTCATGCACCAGAGCCA	CCTGGACCACAGAAGGGA	NM_001166311.1
IFN-β	AGTGCATCCTCCAAATCGCT	GCTCATGGAAAGAGCTGTGGT	GQ415073.1
TGF-β1	CCTGCAAGACCATCGACATG	GCCGAAGCTTGGACAGAATC	AF461808.1
TGF-β2	TGTGTGCTGAGCGCTTTTCT	GAGCGTGCTGCAGGTAGACA	L08375.1
TNF-α	AACCTCAGATAAGCCCGTCG	ACCACCAGCTGGTTGTCTTT	EU682384.1

Figure 3.1: T cells and NK cells in PBMC. Samples were analyzed by flow cytometry, as described in Materials and Methods. SR and FF NK cells in PBMC were not significantly different; thus, both day 7 SR and FF samples were pooled and day 21 SR and FF samples were pooled for statistical analysis of NK cells. Data are presented as mean ± SD. Data with different letters are significantly different ($p<0.01$). A) **PBMC CD45+CD3+CD4+CD8+ T cells were significantly higher at d21 compared to d7**. B) **NK cells were significantly higher at d7 compared to d21 ($p<0.05$)**.

A



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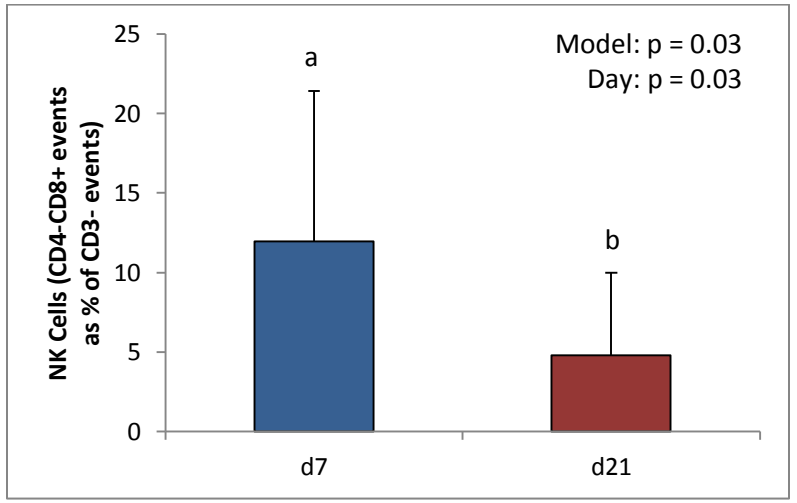


Figure 3.2: MSLN CD4:CD8 T cell ratios CD4+CD8+ cells are significantly different on d21 but not on d7. Piglets were euthanized on d7 or d21. MSLN were collected. Cells were isolated and stained with a panel of fluorescently labeled mAb including CD45, CD3, CD4, and CD8. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). **MSLN CD4:CD8 T cell ratios were significantly higher in SR compared to FF on d21 ($p=0.0256$)**. The overall model was significant and d7 was higher than d21 ($p<0.1$) and a day x diet interaction ($p<0.05$). Data are presented as CD4+events: CD8+ events ratio as a percentage of CD45+CD3+ lymphocytes.

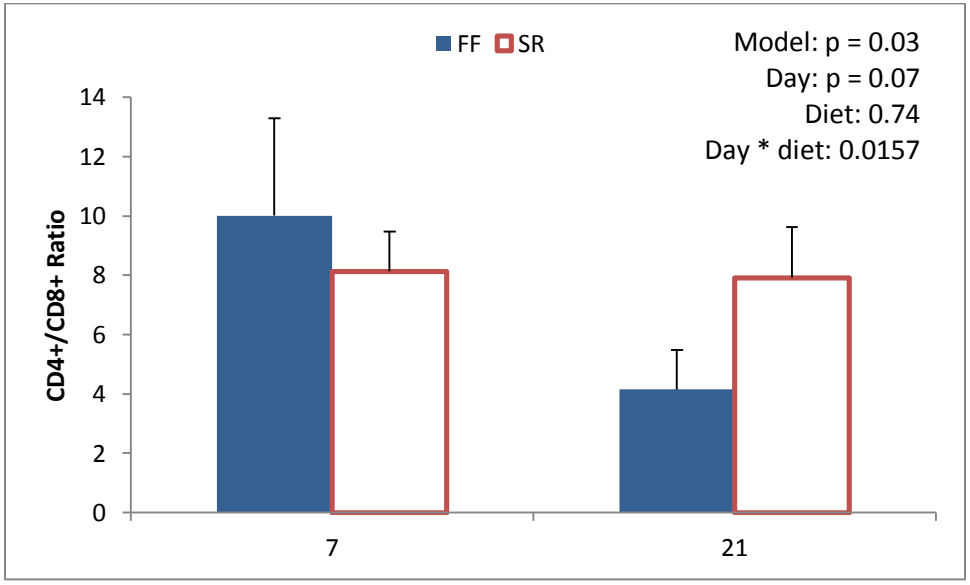
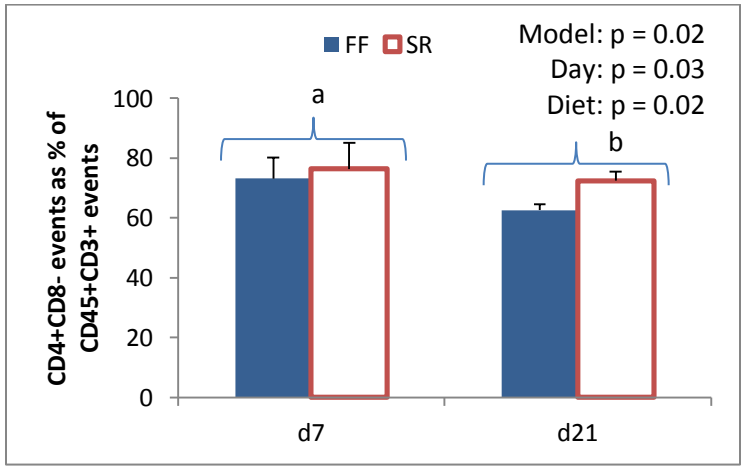


Figure 3.3: MSLN CD4+CD8- T cells are significantly higher at d7 than d21 and in SR compared to FF while CD4-CD8+ T cells are significantly higher at d21 than d7. Piglets were euthanized on d7 or d21. MSLN were collected. Cells were isolated and stained with a panel of fluorescently labeled mAb including CD45, CD3, CD4, and CD8. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). A) **MSLN CD4+CD8- T cells were higher on d7 than d21**. CD4+CD8- were also higher in SR compared to FF animals. B) **MSLN CD4-CD8+ T cells were higher on d21 than d7**.

A



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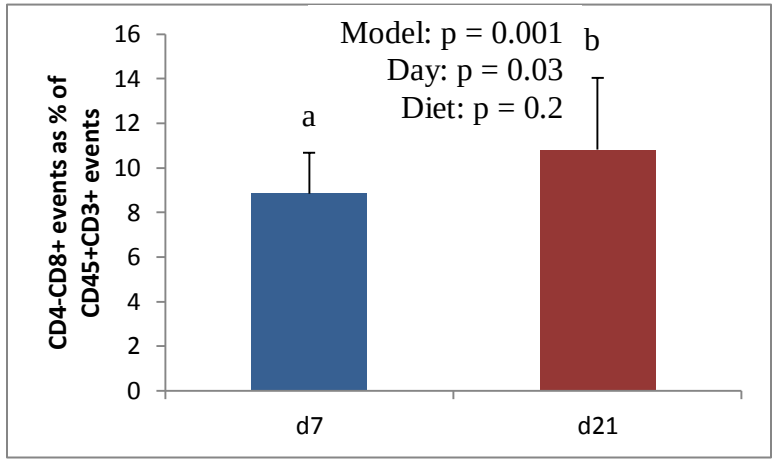


Figure 3.4: MSLN CD4+CD8+ cells are significantly higher in FF compared to SR. Piglets were euthanized on d7 or d21. MSLN were collected. Cells were isolated and stained with a panel of fluorescently labeled mAb including CD45, CD3, CD4, and CD8. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). **MSLN CD45+CD3+CD4+CD8+ T cells were significantly higher in FF piglets compared to SR ($p<0.01$).**

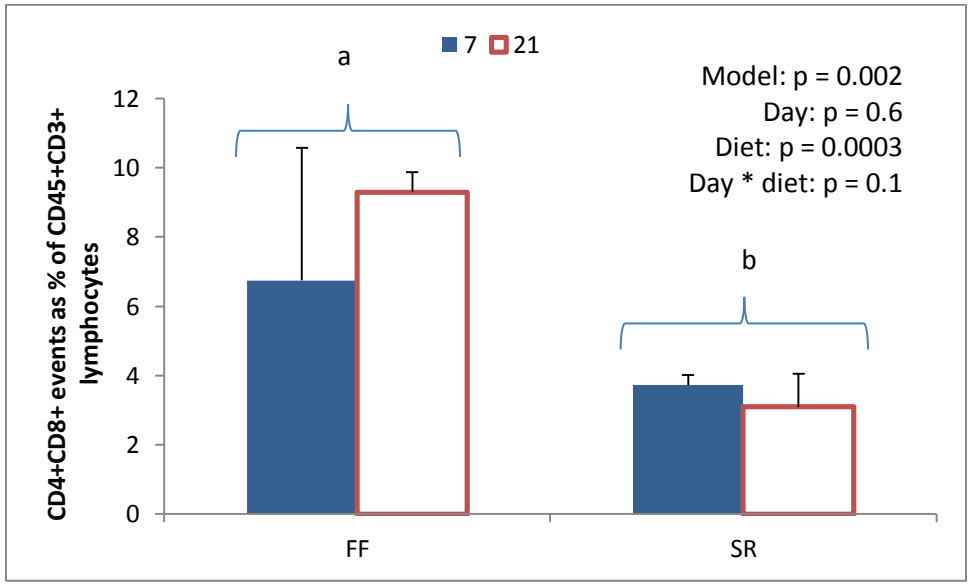
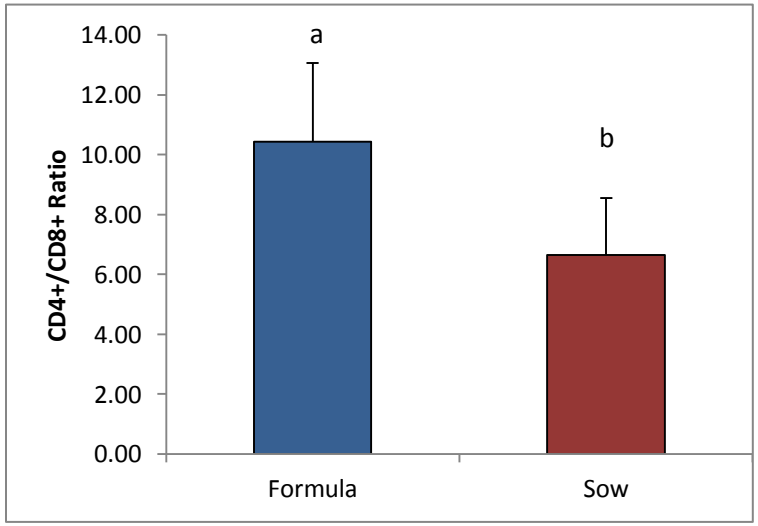


Figure 3.5: TLN CD4:CD8 T cell ratios and CD4+CD8+ cells are significantly different on d7 but not on d21. Piglets were euthanized on d7 or d21. TLN were collected. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). A) **TLN CD4:CD8 T cell ratios were significantly higher in FF than SR piglets at d7 (p=0.028).** Data are expressed as CD4+events: CD8+ events ratio as a percentage of CD45+CD3+ lymphocytes. B) **TLN CD4+CD8+ T cells were significantly higher in SR than FF piglets at d7 (p= 0.023)**.

A



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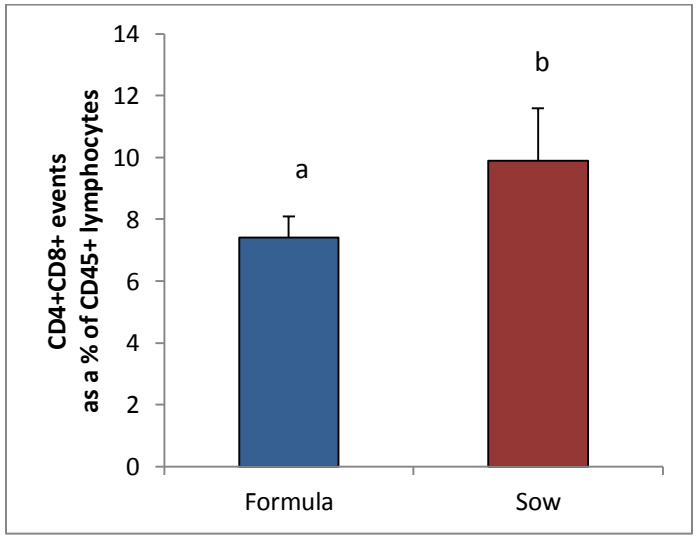


Figure 3.6: TLN CD4-CD8+ T cell populations were greater in FF compared to SR and were higher at d21 than d7. Piglets were euthanized on d7 or d21. TLN were collected. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). **TLN CD4-CD8+ T cell populations were significantly higher in FF than SR piglets and were higher at d21 than d7.**

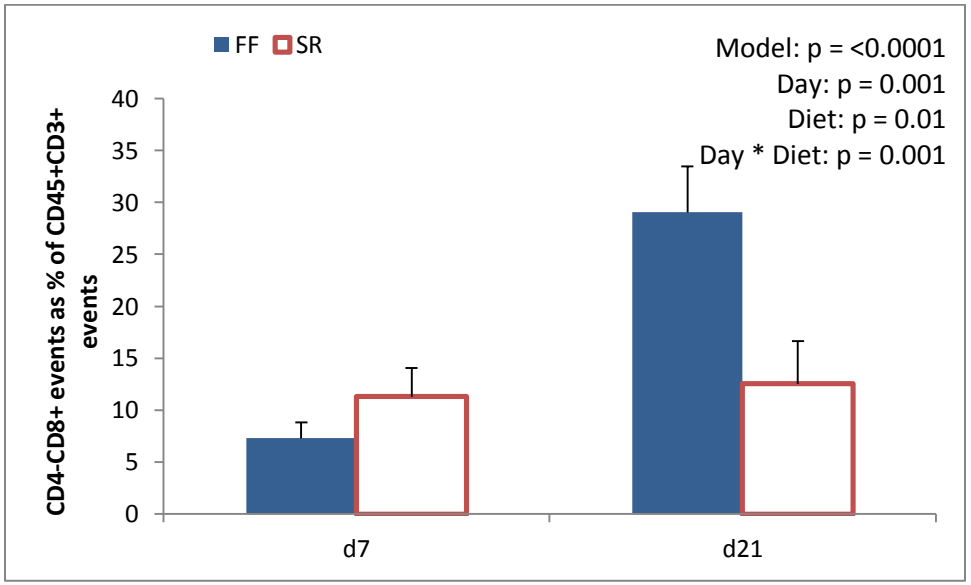


Figure 3.7: TLN NK cells are significantly higher in SR compared to FF piglets. Piglets were euthanized on d7 or d21. TLN were collected. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). NK cells are CD4-CD8+ events as a percentage of CD3- events.

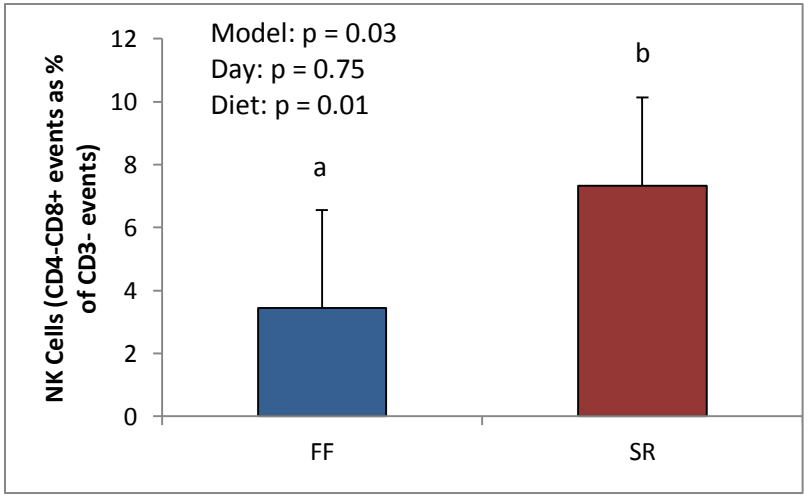


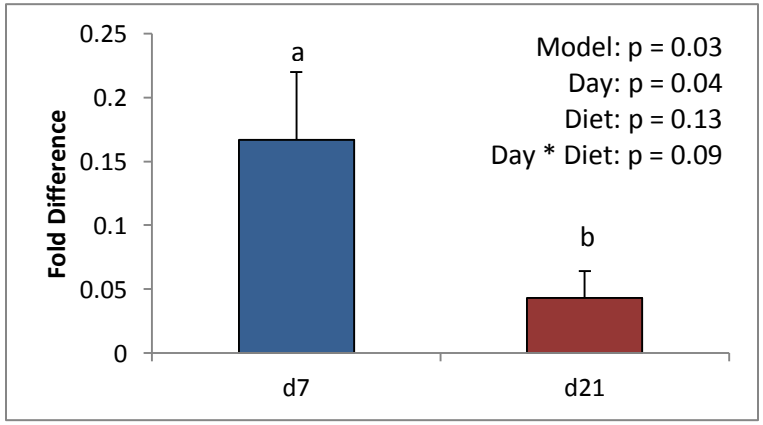
Table 3.2: Summary of the roles of cytokines/receptor.

Gene/Receptor	Major Roles
IL-12	**T_H1-cell development activation[1]**
Dectin	**Pattern recognition receptor, binds fungal species[2], role phagocytosis of apoptotic cells[2], mediates production of various cytokines and chemokines[2], stimulation of Dectin drives T_H1 development[2]**
IL-1β	**Phagocyte activation[1], increases endothelial adhesion molecules[1], T-cell and macrophage activation[1]**
TNF-α	**Promotes inflammation and endothelial activation[1], early inflammatory cytokine**
TGF-β1	**Inhibits cell growth and induces switch to IgA production[1], regulatory cytokine**
TGF-β2	**Promotes oral tolerance**
IL-6	**Lymphocyte growth and B-cell differentiation[1], early inflammatory cytokine**
IFN-α	**Antiviral, increased MHC class I expression[1]**
IFN-β	**Antiviral[1], increased MHC class I expression[1]**

[1](Murphy *et al.* 2008) [2](Willment and Brown 2010)

Figure 3.8: IL-12 and Dectin mRNA expression in TLN. Piglets were sow-reared or formula-fed and euthanized on d7 or d21. TLN were excised and snap frozen. TLN mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target gene were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for day 7 formula fed animals. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD of the fold difference relative to formula-fed animals. Data with different letters are significantly different ($p<0.05$). A) **IL-12 mRNA expression in TLN was greater on d7 than on d21.** B) **Dectin mRNA in TLN was higher on d7 than on d21**.

A



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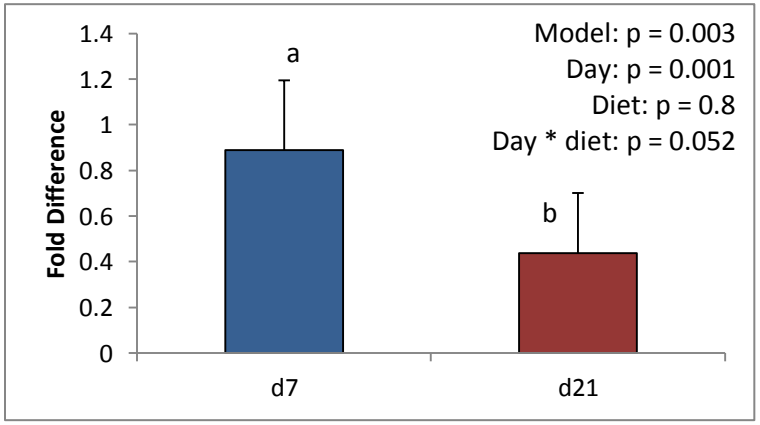


Figure 3.9: IL-1β and TNF-α mRNA expression in TLN. Piglets were sow-reared or formula-fed and euthanized on d7 or d21. TLN were excised and snap frozen. TLN mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target gene were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for day 7 formula fed animals. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD of the fold difference relative to formula-fed animals. Data with different letters are significantly different ($p<0.05$). A) **TLN IL-1β expression was higher in formula-fed animals than in sow-reared animals.** B) **TLN TNF-α expression was higher in formula-fed animals than in sow-reared animals.**

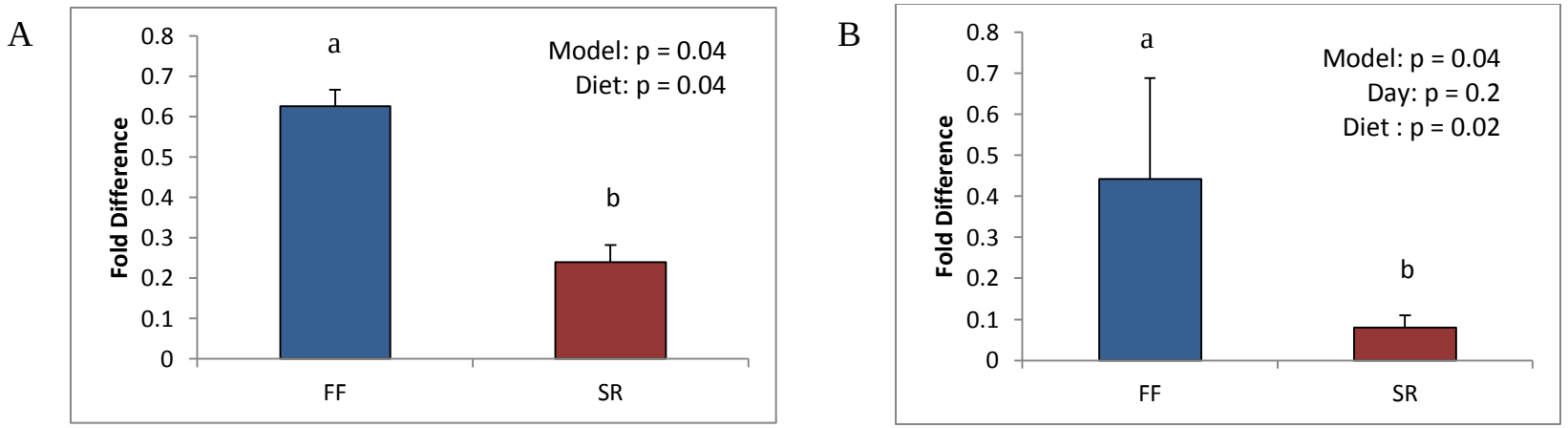


Figure 3.10: Dectin and TGFβ-2 mRNA expression in MSLN. Piglets were sow-reared or formula-fed and euthanized on d7 or d21. TLN were excised and snap frozen. MSLN mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target gene were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for day 7 formula fed animals. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD of the fold difference relative to formula-fed animals. Data with different letters are significantly different ($p<0.05$). A) **Dectin mRNA expression was higher in formula fed piglets compared to sow reared piglets and was trending towards a difference in day where d7 was higher than d21 (p=0.0505). Day*diet interaction was also significant.** B) **TGFβ-2 mRNA expression was significantly higher in formula fed piglets compared to sow reared piglets.**

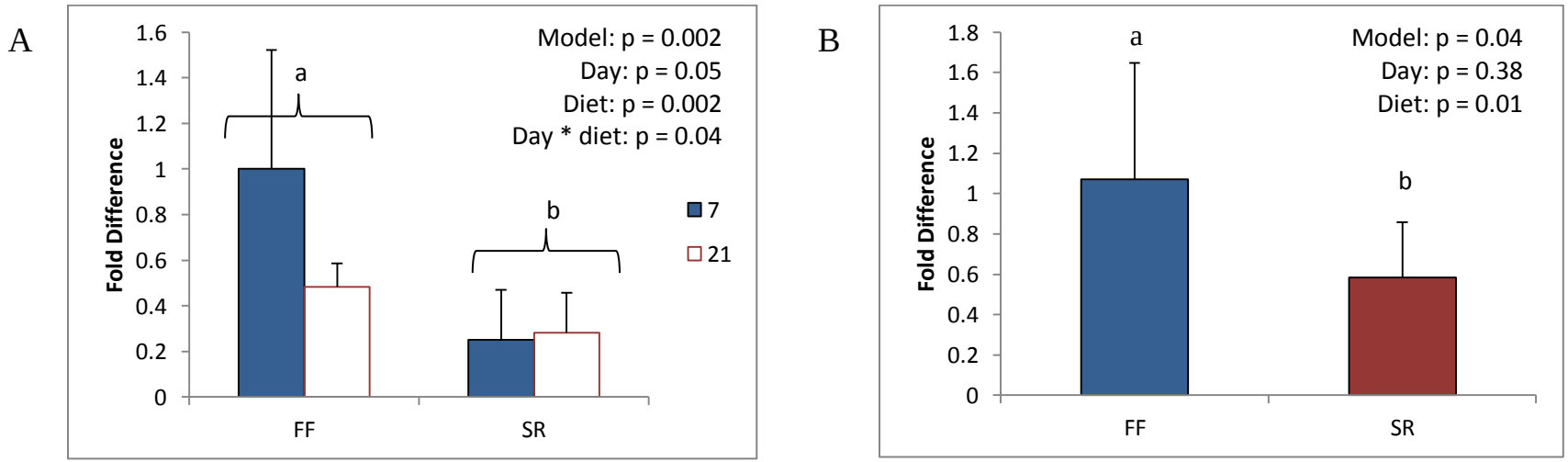


Figure 3.11: IFN-α mRNA expression in MSLN. Piglets were sow-reared or formula-fed and euthanized on d7 or d21. TLN were excised and snap frozen. MSLN mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target gene were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for day 7 formula fed animals. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD of the fold difference relative to formula-fed animals. Data with different letters are significantly different ($p<0.05$). **IFN-α mRNA expression was significantly higher in formula fed piglets compared to sow reared piglets.**

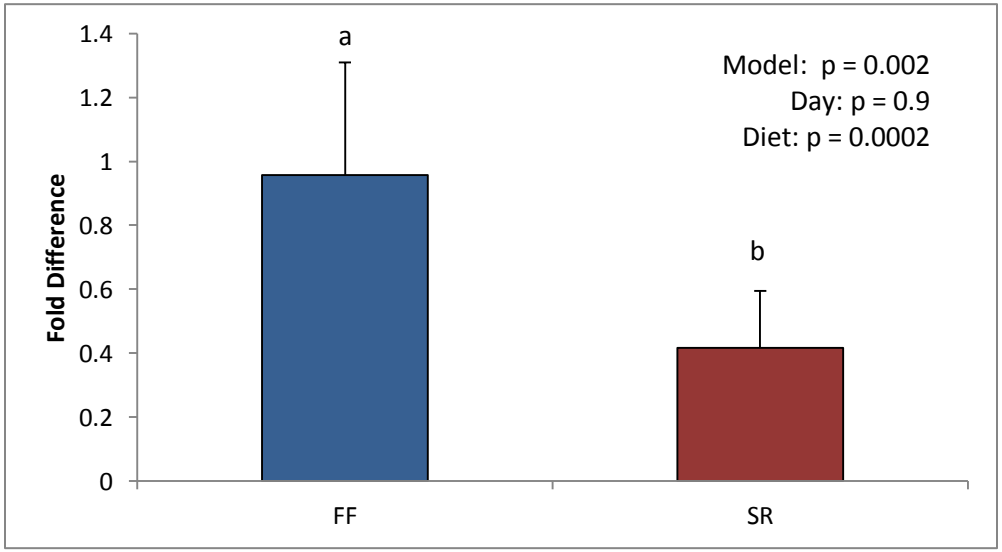
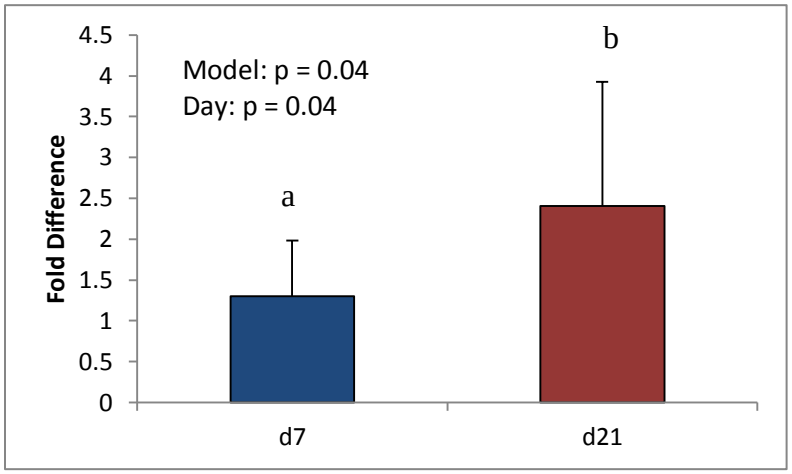


Figure 3.12: IL-6 and TGFβ-1 expression in Lung. Piglets were sow-reared or formula-fed and euthanized on d7 or d21. Lung were excised and snap frozen. Lung mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target gene were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for day 7 formula fed animals. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD of the fold difference relative to formula-fed animals. Data with different letters are significantly different ($p<0.05$). A) **IL-6 mRNA expression was higher at d21 compared to d7.** B) **TGF-β1 mRNA expression was higher at d21 compared to d7.**

A



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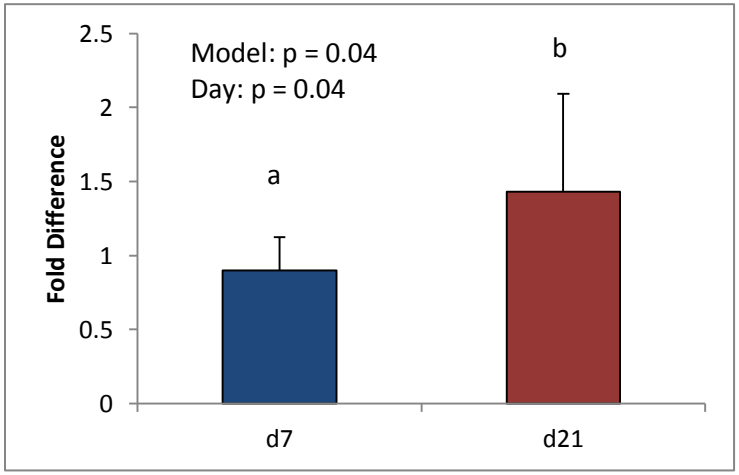


Figure 3.13: TNF-α and IFN-α mRNA expression in Lung. Piglets were sow-reared or formula-fed and euthanized on d7 or d21. Lung tissue were excised and snap frozen. Lung mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target gene were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for day 7 formula fed animals. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD of the fold difference relative to formula-fed animals. Data with different letters are significantly different ($p<0.05$). A) **TNF-α mRNA expression was higher on d21 compared to d7.** B) **INF-α mRNA expression was higher on d21 compared to d7.**

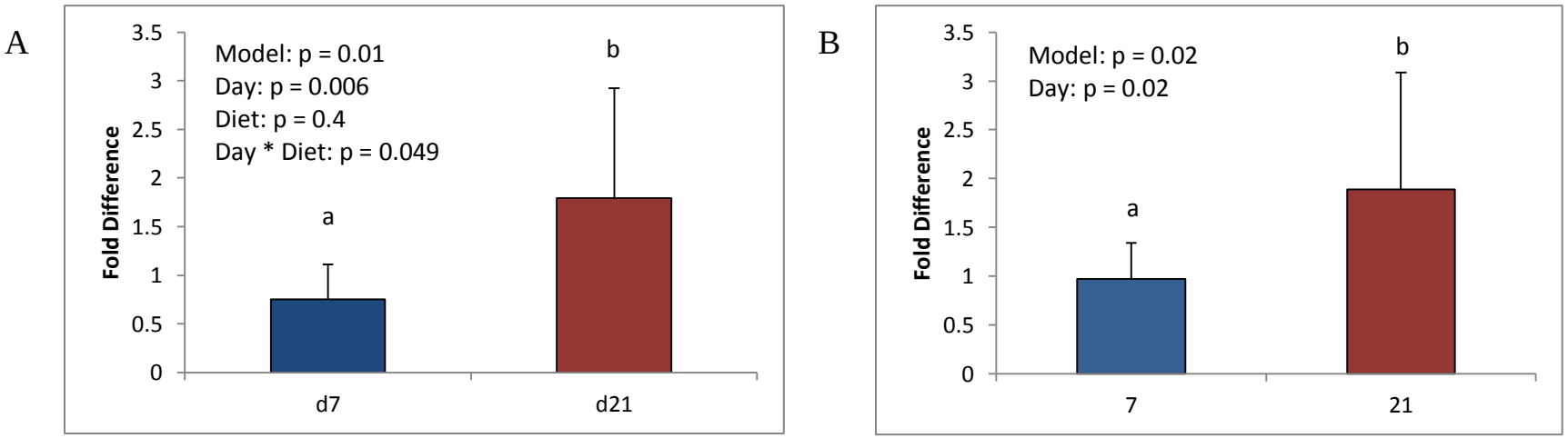


Table 3.3: Summary of cytokine/receptor results.

Tissue	Cytokine/Receptor	Day	Diet	Interaction
TLN	**IL-12**	**7>21**	**None**	**No**
TLN	**Dectin**	**7>21**	**None**	**Yes**
TLN	**IL-1β**	**None**	**FF>SR**	**No**
TLN	**TNF-α**	**None**	**FF>SR**	**No**
MSLN	**Dectin**	**None**	**FF>SR**	**No**
MSLN	**TGF-β2**	**None**	**FF>SR**	**No**
MSLN	**IFN-α**	**7>21**	**None**	**No**
Lung	**IL-6**	**7<21**	**None**	**No**
Lung	**TGF-β1**	**7<21**	**None**	**No**
Lung	**TNF-α**	**7<21**	**None**	**No**
Lung	**IFN-α**	**7<21**	**None**	**No**
Lung	**IFN-β**	**None**	**FF>SR**	**No**

Figure 3.14: IFN-β mRNA expression in lung. Piglets were sow-reared or formula-fed and euthanized on d7 or d21. Lung tissue sections were excised and snap frozen. Lung mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target gene were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for day 7 formula fed animals. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD of the fold difference relative to formula-fed animals. Data with different letters are significantly different ($p<0.05$). **IFN-β mRNA expression was higher in FF compared to SR piglets.**

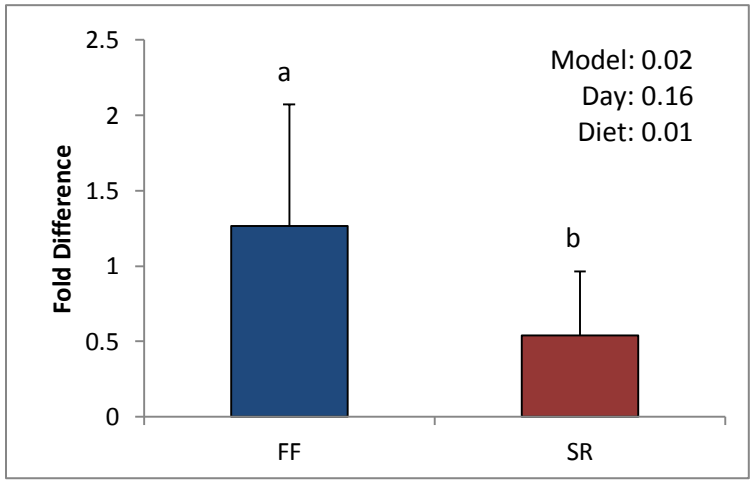


Figure 3.15: Serum IgG concentrations in SR and FF piglets. Sera were collected on day 7, 14, and 21**.** Data are expressed as mean ± SD. **Serum IgG concentrations were lower in the SR piglets when compared to the FF piglets**. **IgG concentrations on day 7 were higher than on day 14 and day 21.**

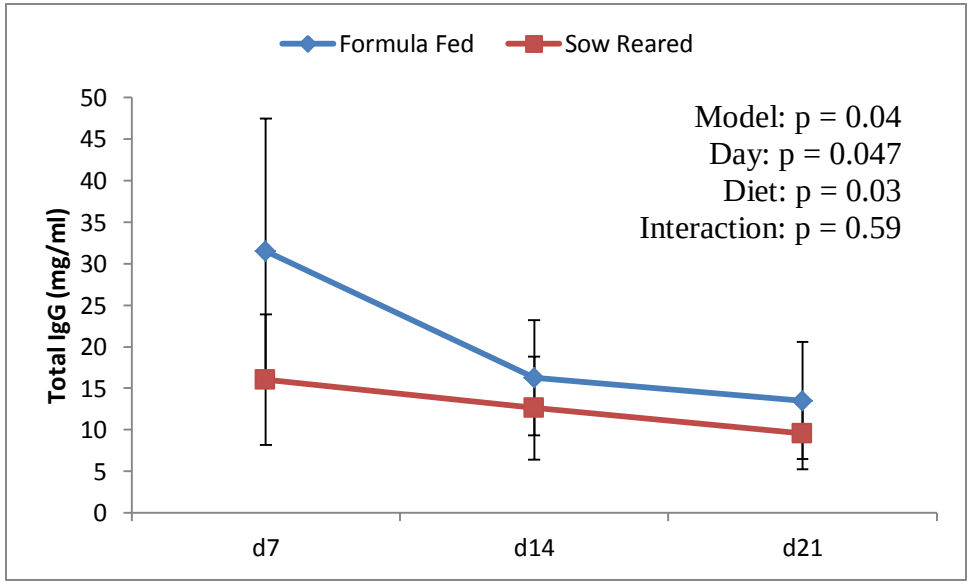
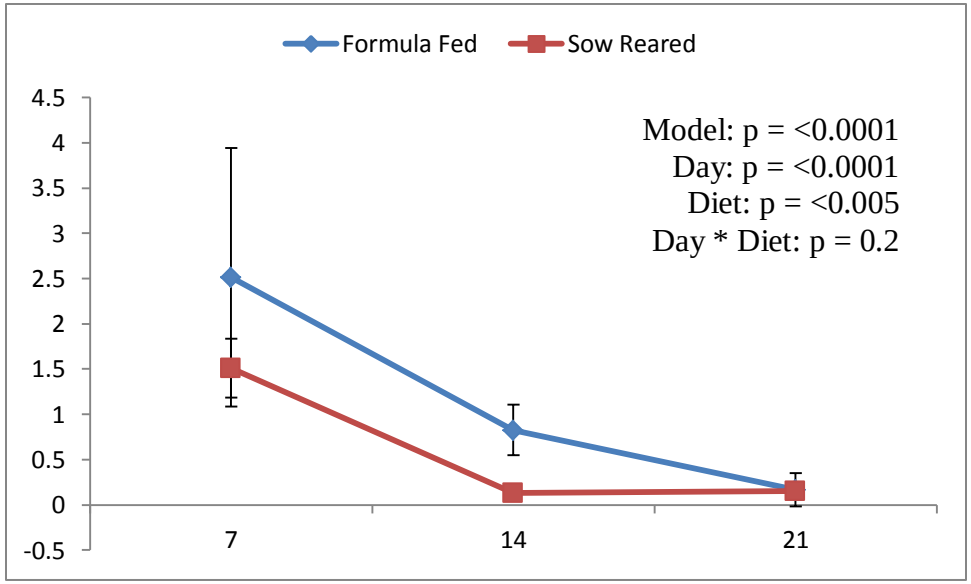


Figure 3.16: Serum IgA concentrations in SR and FF piglets. Sera were collected on day 7, 14, and 21. Data are expressed as mean ± SD. **Serum IgA concentrations were lower in the SR piglets when compared to the FF piglets**. **IgA concentrations on day 7 were higher than on day 14 and day 21.**



Chapter 4

Dietary Yeast β-glucan Minimally Impacts Development of Lung Immunity in the Neonatal Piglet

Abstract

Purified yeast (1,3/1,6)-β-D-glucan (WGP) has been shown to enhance immune responsiveness. Herein, the hypothesis that WGP would modulate the lung immune development and improve influenza vaccination response was tested. Piglets (n=5-6/group) were fed formula containing 0 (control), 5 (WGP5), 50 (WGP50), or 250 (WGP250) mg/L formula. Half of the piglets in each treatment were vaccinated (FV) by i.m. injection against influenza (Fluzone™, Sanofi Pasteur, Swiftwater, PA) on d7 and received a booster on d14. Piglets were euthanized on d7 and d21. Weight gain and formula intake were unaffected by diet or vaccination. Fluzone-specific serum IgG concentrations was measured by ELISA. FV piglets had higher ($p<0.0001$) fluzone-specific IgG titer at d14 and 21 than non-V piglets independent of diet. Vaccination response were unaffected by oral WGP supplementation. TNF-α, dectin, IL-1α, -2, -4, and -12 mRNA expression in lung were unaffected by age or dietary WGP. Lung TGFβ-1 mRNA expression was greater ($p<0.05$) at d21 than d7, and lung TGFβ-2 mRNA was lower ($p<0.01$) in all WGP diets compared to control. TNF-α, dectin, TGFβ-1, IL-2, -4, -6, or -12 mRNA in mediastinal lymph nodes (MSLN) were unaffected by age or dietary WGP. In MSLN, TGFβ-2 mRNA expression increased from d7 to d21 ($p<0.05$). TNF-α, TGFβ-1, TGFβ-2, IL-4, -6, or -12 mRNA in thorasic lymph nodes (TLN) were unaffected by age or dietary WGP. Dectin mRNA expression in TLN was lower at d21 compared to

d7 ($p<0.05$). T-cell phenotypes were examined in MSLN and TLN by flow cytometry. In MSLN and TLN, CD4+ T-cells decreased, while CD8+ T-cells increased between d7 and d21 piglets ($p<0.001$), but these developmental patterns were unaffected by dietary WGP. Total serum IgG, IgM and IgA concentrations were also analyzed via ELISA. Total serum IgG, IgM and IgA were unaffected by WGP but followed typical developmental patterns. Thus, with the exception of reducing TGFβ-2 mRNA in lung, dietary WGP did not affect cytokine expression, T-cell phenotypes or vaccination response in piglets.

Introduction

A continuing challenge in medicine today is finding the optimal diet for an infant who is unable to breastfeed. Thus, the identification and characterization of compounds that enhance the growth, development and health of those infants continues to be a goal for nutritional science. Newborns are transitioning into a world rich in foreign substances. They must rapidly develop immune protection to defend against infection while avoiding harmful inflammatory responses. Their immune systems develop in response not only to innate developmental signals, but also to signals from commensal bacteria and dietary components (Kelly and Coutts 2000). Herein, we explore the effects of one dietary component, yeast β-glucan (βG), on lung immune system development using the neonatal piglet as a model for human infants.

B-glucans are polysaccharides that occur as a principal component of cellular walls in yeast, fungi, seaweeds, mushrooms, and some cereals such as oats and barley. β-(1,3)-glucans belong to the group of Biological Response Modifiers (BRM) meaning that they do not have direct cytotoxic activities but are able to boost the natural defense mechanisms of the host. The immune system identifies βG by signaling through pattern-recognition receptors (PRR) present on immune cells. These PRRs include at least four receptors including dectin-1, complement receptor 3 (CR3; CD11b/CD18), lactosylceramide receptors, and scavenger receptors (Descroix *et al.* 2006). Through these receptors βG stimulate the innate immune system as well as modulate humoral and cellular immunity and may improve an individual's ability to fight infections (Mahauthaman *et al.* 1988; Saito *et al.* 1992; Adachi *et al.* 1994; Zhang and Petty 1994; Lebron *et al.* 2003).

The first point of contact for inhaled substances such as environmental pollutants, cigarette smoke, airborne allergens, and microorganisms is the epithelial lining of the upper airways and lungs (Diamond *et al.* 2000). Therefore, mucosal defense mechanisms are critical in preventing colonization of the respiratory tract by pathogens and penetration of antigens through the epithelial barrier. In recent years, it has become clear that airway epithelial cells not only function as a passive barrier, but also actively participate in innate immune responses (Diamond *et al.* 2000; Holgate *et al.* 2000). One of the ways by which is it posited they do this is through PRR signaling which in turn regulates the expression of a variety of genes including cytokines and chemokines. In fact, airway epithelial cells have been shown to secrete a large variety of molecules that are involved in inflammatory and immune processes including cytokines, chemokines, leukotrienes, calprotectin, β-defensins and other factors (Diamond *et al.* 2000; Holgate *et al.* 2000; Bals and Hiemstra 2004). Through the secretion of these substances, the airway epithelium is able to chemoattract and activate cells of the innate and adaptive immune system, to immobilize and kill microorganisms, to induce would healing and angiogenesis in response to injury and to orchestrate the initiation of and adaptive immune response (Bals and Hiemstra 2004).

β-D-Glucans have mixed effects on lung function and airway responsiveness. Generally, they appear to protect against microbial challenges. In one study, *Saccharmyces cerevisiae* βG fed orally (50 mg/day/pig) reduced the pulmonary lesion score and viral replication rate of pneumonia induced by swine influenza virus (SIV) in piglets (Jung *et al.* 2004). In another study, yeast β-(1,3)-glucan administered by subcutaneous injection increased survival rate, diminished bacterial load in the lungs and

increased the proportion of bacteria-free animals after infection with anthrax in mice (Kournikakis *et al.* 2003). Yeast βG also had an anti-viral effect against porcine reproductive and respiratory virus (PPRS) in weanling pigs and was associated with an up-regulation of the T_H1 cytokine IFN-gamma (Xiao *et al.* 2004). In contrast, pigs fed diets with 0.025 or 0.5% βG had decreased plasma haptoglobin and more pigs fed 0.025% βG died by d12 after *Streptococcus suis* challenge than control pigs despite increased average daily food intake (Dritz *et al.* 1995). B-glucans at a dose of 0.03% did not enhance the immune response to vaccination with PRRS (Hiss and Sauerwein 2003). It is unclear which factors affected the outcomes of these studies.

To date, minimal research has been done to explore the effects of βG on immune development of the lungs in very young animals or humans. The research presented herein investigates the effects of dietary βG on the development of lung-associated adaptive immunity in individuals less than three weeks of age. Immune characteristics analyzed include T cell phenotypes, cytokine secretion, and the antibody response to immunization with an intramuscular influenza vaccine.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Dietary Yeast β-Glucan

(1,3/1,6)-β-D-glucan (Wellmune WGP®) (see Appendix Figure B1**)** was obtained from Biothera, Inc. (Eagan, MN). This compound was extracted from *Saccharomyces cerevisiae*. The extraction process produces a whole glucan particle in which the outer surface of mannoprotein and inner cellular contents are removed (Babicek *et al.* 2007). WGP was provided at 1.8 mg/kg BW/day (WGP5), 18 mg/kg BW/day (WGP50) or 90 mg/kg BW/day (WGP250). The lowest dose provided an average WGP intake of 5 mg/day and is consistent with Biothera's recommendation of 2 mg/kg BW/day. The middle dose provided an average WGP intake of 50 mg/day. The highest dose provided an average WGP intake of 250 mg/d. These doses do not surpass the level generally recognized as safe by the United States FDA (200 mg/serving, GRN No. 239, www.FDA.gov accessed Oct 27, 2010). Furthermore, these concentrations are within the range that has been shown to result in no observed adverse effects (NOAEL) in toxicological testing (2-100 mg/kg BW/day) (Babicek *et al.* 2007).

Dietary Treatment and Animal Protocol

Piglets (n=68) were obtained at 48 h postpartum to allow for consumption of colostrum. The piglets were randomized to one of four dietary treatment groups: 1) a medicated sow milk replacer formula (Formula; Milk Specialties Global Animal Nutrition, Carpentersville, IL); 2) Formula + 5 mg/L (WGP5); 3) Formula + 50 mg/L (WGP50); or 4) Formula + 250 mg/L (WGP250) (see Appendix Figure B2)**.** Piglets were individually housed in environmentally controlled rooms (25°C) in cages, which maintain six piglets separated by Plexiglas partitions. Radiant heaters were attached to the tops of the cages to maintain an ambient temperature of 30°C. Formula was offered 14 times daily at a rate of 360 ml/kg BW/day. The piglets were monitored daily for normal growth and food intake, as well as the presence of fever, diarrhea or lethargy. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

Vaccination

On d7 postpartum, approximately half of the piglets in each treatment group (Formula n=5, WGP5 n=6, WGP50 n=5, WGP250 n=6) were vaccinated with a 0.25 ml i.m. injection of human influenza vaccine (Fluzone™, Sanofi Pasteur, Swiftwater, PA) (see Appendix Figure B3**)**. A blood sample was drawn from the jugular vein prior to administration of the vaccine. Vaccinated animals were boosted on d14 with the same dose of Fluzone™. Blood samples were collected longitudinally from all piglets on d14 and d21 by jugular vein or following euthanasia, respectively.

Sample Collection

On day 7 (Formula n=5, WGP5 n=5, WGP50 n=5, WGP250 n=5) or day 21 (Formula n=12, WGP5 n=13, WGP50 n=11, WGP250 n=12) post-partum, piglets were sedated with an intramuscular injection of Telazol (tiletamine HCl and zolazepam HCl, 3.5 mg/kg BW each, Pfizer Animal Health, Fort Dodge, IA). After sedation, blood was collected by cardiac puncture into non-coated vacutainer tubes (BD Biosciences, Franklin Lakes, NJ) for serum isolation. Piglets were then euthanized by an intravenous injection of sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, Michigan; 72mg/kg body weight) and a thoracotomy was performed. The lungs, mediastinal lymph nodes (MSLN) and thoracic lymph nodes (TLN) were quickly excised. Sections (3-4 cm) of the lung were snap frozen in liquid nitrogen or fixed in Bouin's solution. A portion of the MSLN and TLN were snap frozen in liquid nitrogen. The remaining MSLN and TLN were collected for isolation of mononuclear cells.

Lung histomorphology and T cell Immunohistochemistry

Bouin's-fixed lung samples were embedded in paraffin, sliced to approximately 5μm with a microtome, and mounted on glass microscope slides. Sections were then stained with hematoxylin and eosin or anti-human CD3 polyclonal antibody at the University of Illinois Veterinary Diagnostic Laboratory. Slides were scanned in the Institute of Genomic Biology at the University of Illinois using the NanoZoomer Digital Pathology System (Hamamatsu Corporation, Bridgewater, NJ). Images were then

analyzed by a board certified veterinary pathologist at the University of Illinois School of Veterinary Medicine.

Isolation and Phenotypic Identification of Mononuclear Cells from Tissues

Mononuclear cells from TLN and MSLN were obtained by cutting tissues into pieces and dissociating using a GentleMACS Dissociator (Miltenyi Biotec, Auburn, CA). Cells were then sequentially passed through 100um and 40um cell strainers (BD Biosciences, Bedford, MA) to form single cell suspensions. Cells were counted using a Countess automated cell counter (Invitrogen, Carlsbad, CA). The number of viable cells was assessed by trypan blue (Invitrogen Gibco) exclusion. The phenotypes of T lymphocyte subpopulations from MSLN and TLN were monitored by flow cytometry using a panel of fluorescently labeled mAbs. Lymphocytes were identified by anti-swine CD45 (Clone K252-1E4, AbD Serotec, Raleigh, NC). Anti-CD45 was conjugated to Alexa 647 with a Zenon Mouse Antibody Labeling Kit (Invitrogen Molecular Probes, Eugene, OR). T lymphocytes were identified by mouse anti-pig CD3:biotin (Clone BB23-8E6, Southern Biotech, Birmingham, AL) which was visualized with streptavidin:PE-Cy7 (Southern Biotech). To further differentiate T cell populations, cells were stained with mouse anti-pig CD4:FITC (Clone 74-12-4, Southern Biotech) and mouse anti-pig CD8:PE (Clone 76-2-11, Southern Biotech). All staining procedures took place on ice and care was taken to prevent unnecessary exposure to light. Briefly, one million cells per well were blocked with a mixture of 5% mouse serum (Southern Biotech) and 200ug/ml purified mouse IgG (Invitrogen) for 5min. Anti-CD3 was added to the wells, incubated for 20 min, centrifuged, and then fluid was aspirated. Cells were

then incubated for 20 min in a total volume of 40µl (10µl each: diluted Anti-CD45, Strep-PECy7, anti-CD4 and anti-CD8). Cells were washed twice with PBS/1% BSA/0.1% sodium azide, and then fixed with 2% paraformaldehyde. Staining was assessed using an LSRII flow cytometer (BD™, Biosciences, San Jose, CA). The relative number of T cell subpopulations was determined using FlowJo 7.0 software (FlowJo, Ashland, OR). CD45+CD3+ events were considered T cells. CD45+CD3+CD4+ events were considered T helper cells. CD45+CD3+CD8+ event were considered cytotoxic T cells. Gating procedure is included in Appendix B, Figure B4.

Mitogenic Cell Stimulation

A total of 2 x 10^5 cells/ml per well TLN and MSLN were plated in 96-well plates in a final volume of 200 µl complete culture medium (RPMI 1620 (Invitrogen Gibco), 20% fetal calf serum (Invitrogen Gibco), 2 mM L-glutamine (Invitrogen Gibco), 50 ug/ml gentamicin (Invitrogen Gibco), 100 µg/ml penicillin, and 100 µg/ml streptomycin) for 96 h at 37° C under 5% CO_2. 20 µl of LPS (0.5% 20 ug/ml), 20 µl ConA (25 µg/ml), or 20 µl BSA 0.075 g per 1ml was added on d0 (n=3 wells per sample per stimulant). Plates were incubated for 72 hours, then pulsed with ^{3}H-thymidine (1 µCi/ml; Perkin Elmer, Boston, MA) and incubated overnight. Plates were stored at -80°C until analyzed. The cells were then harvested (TomTech, Harvester 96 Mach III M, Hamden, CT) onto 1.5 µm glass fiber filter paper (Skatron Instruments, Sterling, VA) and placed into scintillation vials with either 7ml Ultima Gold™ F scintillation fluid (Perkin Elmer, Boston, MA) or Econo-Safe (Research Products International Corporation, Mt. Prospect, IL). Samples were counted on a Beckman Coulter, LS 6500 Scintillation System (Brea,

CA). Data are expressed as a change in counts per minute (Δcpm) which was obtained by subtracting counts from unstimulated control wells from counts for wells with mitogens. Samples were analyzed in triplicate. Data analysis was done on log transformed ΔCPM.

Fluzone™ Preparation for Ex Vivo Analyses

Prior to use in ex vivo assays, Fluzone™ was dialyzed to remove additives that inhibit cell proliferation. The Fluzone™ solution was placed into Spectra/Por® 4 dialysis membranes (Spectrum Laboratories, Rancho Dominguez, CA) and submerged in PBS for 24 hours at 4°C. A Bradford Assay (Bio-Rad Quick Start Bradford, Bio-Rad, Hercules, CA) was done to compare pre-dialyzed and dialyzed Fluzone™ protein levels. Both samples contained 130 μg protein/mL.

Assessment of Cell-Mediated Response

The cell-mediated immune (CMI) response of the piglets was monitored by stimulating TLN immune cells with dialyzed Fluzone™ *ex vivo* as had previously been used to assess CMI in response to the influenza vaccine in human subjects (Keylock *et al.* 2007). Cells (2×10^5) were added to each well of a round bottom microtiter plates in 150 μL of complete cell culture media (see above). 50 μL of Fluzone™ at concentrations of 5 μg/ml, 2.5 μg/ml, 1.25 μg/ml, 0.625 μg/ml or 0 μg/ml was added to each well (n=3 wells per sample per stimulant). On d4, ^{3}H-thymidine (Perkin Elmer, Boston, MA) was added

at a concentration of 1 μCi per well and plates were incubated overnight. Plates of cells were treated and analyzed as they were for mitogenic stimulation above.

Assessment of Antibody Response

An enzyme-linked immunosorbent assay (ELISA) was used to detect swine IgG specific for influenza antigens. Flat-bottomed ELISA plates (Nunc, Rochester, NY) were coated with dialyzed Fluzone™ vaccine at a 1:80 dilution in coating buffer [0.5M Carb/Bicarb Buffer, pH 9.6] and incubated overnight at 4°C. Following incubation, 200μL of PBS/10% FBS was added to each well to block non-specific binding. Following incubation (1 hour at 4°C), the plate was washed three times with PBS/0.05%Tween 20. 50 μL of diluted sera (1:100 PBS/10% FBS) was added to each well and plates were incubated for 1 hour at 37°C. Plates were washed three times with PBS/Tween. 50 μL of goat anti-pig IgG conjugated to peroxidase was added to each well at a dilution of 1:400 in PBS/10%FBS. Following a1 hour incubation at 37°C, the plate was washed three times with PBS/0.05%Tween. 50 μL of TMB (BD Biosciences, San Diego, CA) was added. The plate was allowed to develop at room temperature for 20 min and the enzymatic reactions were stopped by addition of 1M sulfuric acid. The plate was then analyzed on a microplate spectrophotometer (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) at 450 nm. Samples were run in duplicate. Positive stock serum was run on each plate in dilutions ranging from 1:100-1:1600. A standard curve was made using these dilutions. Fluzone-specific IgG was expressed in arbitrary units calculated from the linear portion of the standard curve.

Cytokine Expression

Total RNA was isolated and purified from snap frozen lung, TLN, and MSLN samples with TRIzol reagent (Invitrogen). RNA was quantified by spectrophotometry using a Nanodrop 1000 (Thermo Scientific, Rockford, IL) at 260 nm. RNA concentration was adjusted to 0.25 µg/l using RNase free water (Invitrogen). RNA quality was analyzed by a bioanalyzer (2100 Bioanalyzer, Agilent Technologies, Inc., Santa Clara, CA). All samples had an RNA integrity number (RIN) greater than 6.0. Reverse transcription (RT) was performed on 3 µg of total RNA in a reaction involving 100 mM deoxyribonucleotide triphosphate (dNTP), 10X RT Buffer, 10X RT Random Primers, MultiScribe Reverse Transciptase, RNAse inhibitor (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA), and DEPC water (Invitrogen). The final RT product was quantified by spectrophotometry as above. The final RT product was adjusted to a concentration of 1:150 or 1:200 using RNase free water. Quantitative real-time PCR was conducted using SYBR-Green (Roche Diagnostics GmbH, Mannheim, Germany) and fluorescence intensity was collected using the Taqman ABI 7900 machine (Applied Biosystems Inc., Foster City, CA). A total of 40 PCR cycles were run. The primers used are listed in Table 4.1 and final primer concentrations were 300 nM. Beta-actin was used as an internal standard reporter gene. Results are expressed using the Relative Standard Curve Method. In short, a dilution of standard curve from a stock of pooled porcine spleen cDNA was made and run on each plate. Each sample was run with primers to assess the target gene and beta-actin. Normalized values for each target were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target

values by the normalized calibrator sample (in this case the day 21, control diet, non-vaccinated group average). All samples that were statistically compared to each other were run on the same plate.

Serum Immunoglobulin Levels

Total serum immunoglobulin levels were detected by ELISA using assays specific for porcine IgG, IgM and IgA Quantification Sets (Bethyl Laboratories, Montgomery, TX, USA). A 96-well, flat-bottomed ELISA plate (Nunc, Rochester, NY) was coated with 100 µl coating antibody (µg coating antibody as suggested by the manufacturer diluted in 0.05M carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. The antibody solution was poured off, and the plate was washed three times with PBS/0.05% Tween20. The plates were blocked with 300 µl of 3% BSA/PBS for 1 h at room temperature (RT). The plates were washed as before. Serum samples were serially diluted in 0.05% gelatin/PBS, added to the wells in duplicate (100 µl per well), and plates were incubated for 1 h at RT. Samples for standard curves were included with the quantification sets and were used as directed. Plates were washed as before, and 100 µl HRP-conjugated detection antibody (concentration as recommended by the manufacturer) in 0.05% gelatin/PBS was added to each well. Plates were protected from light and incubated for 1 h at RT. Plates were washed four times with PBS-Tween 20. 100 µl TMB reagent solution (OptEIA, BD Biosciences, San Diego, CA) was added to each well and allowed to develop protected from light at RT for the time recommended by the manufacturer. The reaction was stopped with 100 µl 2N H_2SO_4 per well. The absorbance was then read at 450 nm with 570 nm correction using a microplate reader

(SpectraMax M2[e], Molecular Devices, Sunnyvale, CA). Total immunoglobulin values were determined based on a standard curve that was run on each plate.

Statistical Analysis

Analyses were performed using the PROC GLM (generalized linear model) procedure within SAS (Version 9.2, SAS Institute, Cary, NC). The initial model was day, diet, vaccination, day x diet, vaccination x diet. If the model was not significant vaccination was not significant, vaccinated and non-vaccinated were combined. If the model was still not significant, d7 and d21 data were combined. It diet was not significant, diet was excluded and all diet groups at d7 were combined and all diet groups at d21 were combined. Statistical significance was defined as $p<0.05$, with trends reported as $p<0.10$. All data are expressed as means ± SD.

Results

Formula intake, Body Weight

Formula intake and body weight (see Appendix Figure B5**)** were measured daily to determine if WGP affected acceptance of diet or growth. Formula intake and body weight were similar across all treatment groups. The piglets in all treatment groups ate most of the diet provided (95-99%). An average formula intake of 808±167 ml/day was consumed for the first 7 days and 1488 ± 129 ml/day over the course of 21 days. The increase in body weight during the 7 or 21 day period did not differ between groups. On day 7, each pig weighed an average of 2.3±0.5 kg. On day 21, each pig weighed an average of 4.4±0.8 kg at day 21. Therefore, inclusion of WGP had no effect on weight gain or dietary acceptance.

T lymphocyte Populations

Flow cytometry was used to analyze T cell phenotype. Neither WGP nor vaccination affected T cell phenotypes. However, MSLN and TLN CD45+CD3+CD4+ T cells decreased, while CD45+CD3+CD8+ T cells increased between d7 and d21 ($p<0.05$) (**Figure 4.1)**. Double positive (CD4+CD8+) T cells did not show the expected increase from day 7 to day 21 in either the MSLN or the TLN (see **Appendix Figure B6)**. In MSLN, 7.1%±0.03 of CD45+CD3+ T Cells were double positive on d7 while 8.3%±0.03 of CD45+CD3+ T Cells were double positive on day 21 ($p=0.31$). In TLN, 7.2%±0.02 of CD45+CD3+ T Cells were double positive on d7 while 9.1%±0.04 of CD45+CD3+ T Cells were double positive on day 21 in TLN ($p=0.24$). Therefore,

neither vaccination nor dietary WGP affected the expected temporal changes in the transition from the overwhelming CD4+ helper cell predominance to an increased CD8+ cytotoxic T cell presence as the animals aged. Although the expected increase in double-positive T cells was not observed, this seemed to be independent of dietary or immunization treatment, and thus an artifact of our experimental system.

Assessment of Antibody Response

To determine if fluzone-specific IgG was up-regulated in response to dietary βG treatment, a fluzone-specific ELISA was developed and serum IgG concentrations were evaluated (see **Appendix Figure B7**). Vaccinated piglets had a significantly higher ($p<0.0001$) IgG titer at d14 and d21 than non-vaccinated animals ($p<0.0001$). Day 21 piglets had a greater vaccine response compared to day 14 piglets ($p<0.0001$). Vaccinated piglets had a 5.9-fold increase in serum IgG of 5.9 ± 2.2 from d7 to d21, while non-vaccinated piglets had increase (0.49-fold) in serum IgG from d7 to d21. While vaccination increased fluzone-specific serum IgG production, dietary WGP did not enhance the ability of piglets to produce fluzone-specific IgG.

Cytokines

Despite an absence of dietary effects on serum IgG production, we investigated mRNA expression of various genes in whole tissue samples from the lung, TLN, and MSLN. Day, diet, and vaccination did not affect lung dectin, IL-1α, IL-2, IL-4, IL-12, or TNF-α mRNA expression (See **Appendix Table B1**). However, day and diet affected

lung TGF-β1 and TGF-β2, respectively (**Figure 4.2**). Although lung TGF-β1 expression was unaffected by WGP or vaccination, lung TGFβ-1 mRNA expression increased between d7 and d21 ($p<0.05$). WGP affected lung TGFβ-2 mRNA expression. On both d7 and d21, lung TGFβ-2 mRNA expression was lower in all animals fed WGP compared to those fed formula alone ($p<0.01$). IL-6 mRNA expression was not evaluated in the lung. Day, diet nor vaccination affected TLN IL-4, IL-6, IL-12, TGFβ-1, TGFβ-2 or TNF-α mRNA expression (See **Appendix Table B2**). Dectin mRNA expression was lower in TLN at d21 compared to d7 ($p<0.05$) (**Figure 4.3).** IL-2 and IL-1α were not analyzed in TLN because their expression was low in TLN. Neither WGP, vaccination. nor day affected MSLN mRNA expression of dectin, IL-2, IL-4, IL-6, IL-12, TFGβ-1 or TNF-α (See **Appendix Table B3)**. MSLN TGFβ-2 mRNA expression increased from d7 to d21 ($p<0.05$) (**Figure 4.4**). IL-1α mRNA expression could not be evaluated in MSLN because expression was below the detection limits of our assay. The only effect of dietary treatment with WGP was decreased TGF-β2 expression in whole lung tissue. No consistent effects of vaccination or dietary WGP treatment could be seen on tissue-wide expression of inflammatory (IL-2, IL-12, IL-6, IL-1α, TNF-α) or anti-inflammatory (IL-4, TGF-β1, TGF-β2) cytokine mRNA.

Mitogenic Cell Stimulation and Cell-mediated response

To determine whether cell proliferation was increased by dietary WGP or vaccination, cells were stimulated with Fluzone™, LPS, BSA, or ConA. In both MSLN and TLN, cells stimulated with ConA proliferated significantly ($p<0.05$) more than those treated with LPS, or BSA. The average log ΔCPM was 3.4 ± 1.7 in MSLN ConA

stimulated cells, 1.9 ± 1.4 in MSLN LPS treated cells, and 1.3 ± 1.3 in MSLN BSA treated cells. The average log ΔCPM was 4.2 ± 0.85 in TLN ConA stimulated cells, 2.0 ± 1.2 in MSLN LPS treated cells, and 1.3 ± 1.1 in MSLN BSA treated cells (see **Appendix Table B4**). Dietary WGP did not enhance proliferation of MSLN or TLN cells. Even in vaccinated animals, TLN cells treated with different concentrations (0.625, 1.25, 2.5, and 5 μg/mL) of dialyzed Fluzone™ did not proliferate more than untreated cells (see **Appendix Table B5**). Neither diet nor vaccination significantly affected *ex vivo* cellular proliferation.

Histomorphology and Immunohistochemistry

In order to evaluate the effects of WGP and vaccination on morphology, lung sections were excised, embedded in paraffin, sliced, mounted on slides, and stained with H&E (see **Appendix Figure B8**) or anti-human CD3 polyclonal antibody (see **Appendix Figure B9**). Slides were analyzed by a board certified veterinary pathologist at the University of Illinois School of Veterinary Medicine who determined that there was no effect of WGP®, vaccination or day on CD3+ T-lymphocytes numbers or lung morphology.

Serum Immunoglobulin Levels

In order to determine if circulating immunoglobulin concentrations differed between formula and WGP250, sera from each both groups were analyzed for IgG, IgM, and IgA concentrations at d7, d14 and d21. The serum IgG concentrations were higher

($p<0.05$) at d7 than at d14 and d21 (**Figure 4.5**). Serum IgG concentrations were unaffected by diet and vaccination. The complete statistical model for serum IgM concentrations was not significant; therefore vaccinated animals were combined with non-vaccinated animals. Formula piglets had higher ($p=0.04$) concentrations compared to WGP250 piglets (**Figure 4.6**). IgM concentrations were unaffected by vaccination and day. Serum IgA concentrations were higher ($p<0.05$) at d7 than at d14 and d21 (**Figure 4.7**). Serum IgA concentrations were unaffected by vaccination and diet.

Discussion

B-glucans are polysaccharides that occur as a principal component of cellular walls. βG have been shown to affect a variety of biological responses including activation of neutrophils (Zhang and Petty 1994), macrophages (Adachi *et al.* 1994; Lebron *et al.* 2003) complement (Saito *et al.* 1992) and possibly eosinophils (Mahauthaman *et al.* 1988). In addition, βG have been shown to exhibit hypocholesterolemic (Shin *et al.* 2005; Zekovic *et al.* 2005; Bernardshaw *et al.* 2006), anticoagulant properties (Shin *et al.* 2005), and anti-tumorigenic effects *in vivo* (Chihara *et al.* 1969; Di Luzio *et al.* 1979). This study utilized a neonatal piglet model to further explore the effects of dietary WGP on lung immunity. Overall, lung immune development in neonatal piglets was minimally affected by treatment with dietary WGP at 5, 50 or 250 mg/day. Additionally, WGP was found to have no negative impacts on growth, dietary acceptance or the immune characterisitcs analyzed.

One primary difference between the pig and the human involves the transfer of passive immunity. The pig is born with virtually no circulating immunoglobulins due to the lack of placental transfer (Gaskins and Kelley 1995). Pigs, therefore, depend on colostrum and milk consumption for immunoglobulin immunity (Bourne 1976). βG added to milk may contribute to the immune development of piglets. Cytokines are one immune factor that help protect neonates from pathogens. The cytokine TGF-β1 is of particular interest because of the immunoregulatory role it plays during pregnancy and birth in humans as well as its role in the Th2 bias of neonatal immune responses (Laouar *et al.* 2005). In vitro piglet studies have demonstrated that high concentrations (10 ng/ml-about the amount in piglet serum in the early suckling period) of TGF-β1 suppress the B

cell responses, while lower concentrations (0.1 ng/ml) of TGF-β1 increased the number of IgM and IgA short chain in response to both LPS and rotavirus antigens (Nguyen *et al.* 2007). Similarly murine TGF-β1 (0.1-10 ng/ml) acts in both early and late phases of IgA production, yet it suppresses IL-5 induced IgA synthesis in culture (Chen and Qing 1990). TGF-β1 has also been shown to down-regulate immune activation of intestinal epithelial cells and lamina propria immune cells (Mennechet *et al.* 2004) which is likely to allow for initial colonization of the intestine (Nguyen *et al.* 2007). It has also been shown that TGF-β helps regulate innate IFN-γ which is a signal that controls T_H1 development (Laouar *et al.* 2005). Laouar and collegues (2005) also showed that with blockage of TGF-β signaling in NK cells, which are the main source of innate IFN-γ production, NK cells increased and, therefore, the production of innate IFN-γ also increased which is responsible for T_H1 differentiation. In this study, TGF-β1 mRNA expression in the lung increased between d7 and d21 ($p<0.05$). This is interesting because an increase in TGF-β1 is likely contributing to a shift from a T_H1 response to a T_H2 response counter to the expected T_H2 to T_H1 shift.

The pig immune system is unique from humans and some other species in that blood and lymphoid tissues express double positive CD4+CD8+ subpopulations. These subpopulations have been shown to increase with age and immunological experience (Zuckermann and Gaskins 1996). Even though there was a trend for the population of double positive T cells to increase in both the MSLN and TLN, this increase did not reach significance. However, the size of this population in our animals was in line with that published by others (between 8 and 64% of the circulating pool of T cells)

((Pescovitz *et al.* 1985; Pescovitz *et al.* 1994; Zuckermann and Husmann 1996; Zuckermann and Husmann 1996; Zuckermann 1999).

Similar to our results, previous studies have shown a higher percentage of CD4+ than CD8+ cells in PBMCs of piglets (CD4/CD8 ratio >1) until the 40th day of life (Borghetti *et al.* 2006). Borghetti et al. (2006) also observed that CD4+ cells decrease after about one week and eventually become lower than CD8+ cells. We observed a similar shift in T cell populations in the MSLN and TLN (**Figure 4.1**). Typical developmental changes in T lymphocyte populations were not enhanced by βG in this study.

Furthermore, we found that βG at varying doses did not enhance the immune response to vaccination with Fluzone in our 21 day old piglets. Vaccinated piglets had a higher amount of Fluzone-specific IgG at d14 and d21 than the non-vaccinated animals, which is to be expected, but contrary to our hypothesis, βG did not increase IgG production. Influenza vaccines have been known to produce a protective effect which is largely dependent on the vaccine's ability to stimulate circulating antibody to the hemagglutinin (HA) (Hobson *et al.* 1972). It is possible that the βG did not substantially interact with the vaccine to increase stimulation of the antibody since WGP was fed orally and the vaccine was administered intramuscularly. Previous studies examining the effect of dietary supplementation with βG on the response to systemic immunization have produced mixed results. In one study, piglets vaccinated with atrophic rhinitis vaccine produced significantly less antibody when fed βG (Hahn *et al.* 2006) while, in another study, pigs injected with ovalbumin and receiving βG at a dose of 0.005% mounted a higher antibody response (Li *et al.* 2005).

Previous studies have questioned the ability of βG to target sensitive immune cells through the oral route (Gallois *et al.* 2009). After absorption of the βG particles and uptake by the Peyer's Patches, the βG may remain in the GALT thus limiting exposure of other tissues to the βG, specifically lung tissue to the βG.

Toll Like Receptors (TLRs) and other innate receptors were not examined in this study. However, dectin-1 expression was measured. In part, dectin was chosen because the mechanism by which this beta-glucan receptor translates recognition into intracellular signaling, stimulates cellular responses, and participates in coordinating the adaptive immune response is well-characterized (Goodridge *et al.* 2009). It has been identified as one of the PRRs for β-glucan. It is expressed mainly on phagocytes, especially macrophages and dendritic cells. (Brown and Gordon 2001) suggest that dectin-1 signals alone are sufficient to trigger phagocytosis and (Dillon *et al.* 2006) suggests that when dectin-1 collaborates with TLR signaling cytokine production is regulated. The decrease in dectin-1 in TLN from d7 to d21, seen in this study, may be a developmental effect which may impact the amount of phagocytosis and regulate cytokine production in the TLN.

Evidence suggests that biological activity of βG are associated with their structure which, in turn, is dependent on the source and method of extraction and isolation. Processing (Bohn and BeMiller 1995; Douwes 2005; Mantovani *et al.* 2008), solubility, degree of polymerization (solubility increases as degree of polymerization increases) (Douwes 2005; Mantovani *et al.* 2008), high molecular weight (100-200 kDa) fractions being most active while fractions from the same source with lower molecular weights showed no activity (Fabre *et al.* 1984; Kojima *et al.* 1986; Blaschek *et al.* 1992), charge

of polymers and structure in aqueous media, and primary structure also contribute to functionality of βG. Several previous studies used Hunter and co-workers'. (Hunter *et al.* 2002) method of extraction with some modifications (Li *et al.* 2005) with positive biological results. In this study the βG was extracted from *Saccharromyces cerevisiae* using a proprietary technology, which produced a whole glucan particle in which the outer surface of mannoprotein and inner cellular contents are removed to expose the β 1,3/1,6 glucan, WGP. Perhaps this additional processing decreased recognition of the particle and therefore altered the absorption of the molecule. Also, this processing may have decreased the solubility of the particle leading to limited absorption or decreased distribution of the βG in the formula due to the tube feeding method that was utilized. It is also possible that, since the βG was compartmentalized in the gut, the βG could not impact the vaccination response which was localized in the muscle and could not impact the development of lung immunity which was localized in the chest (Holmgren and Czerkinsky 2005). It is likely that these factors also contributed to the lack of differences seen in lung histomorphology or immunohistochemistry.

To gain a perspective of systemic immune development of piglets in the formula and WGP250 were compared. Total serum IgG, IgM and IgA concentrations were measured. We found that the serum IgG concentrations were higher ($p<0.05$) at d7 than at d14 and d21**.** Our developmental study showed similar results as did Bourne's work (Bourne 1973). Serum IgM concentrations were higher ($p<0.05$) in formula piglets compared to WGP250 piglets. The overall developmental pattern was similar, however WGP250 decreased IgM concentrations, likely due to differences in colostrum received. IgA serum concentrations were higher on d7 than on d14 and d21 ($P<0.05$). Our

previous developmental study as well as Bourne's research suggests that this is a typically developmental pattern (Bourne 1973).

In conclusion, the effects of βG on lung immunity in the healthy neonate are inconclusive. While no negative effects of the dietary supplement were seen, further research, perhaps targeted to effects on innate immune mechanisms or acute microbial challenges are needed to increase our understanding of the ability of WGP to enhance the biological activity of the neonate's immune system.

Table 4.1: Primers Used for Quantitative Real Time-PCR.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Gene Bank Accession ID
β-actin	CACGCCATCCTGCGTCTGGA	AGCACCGTGTTGGCGTAGAG	DQ845171.1
Dectin	CTCTCACAACCTCACCAGGAGAT	CAGTAATGGGTCGCCAATAAGG	FJ386384.1
IL-2	TCAACTCCTGCCACAATGT	CTTGAAGTAGGTGCACCGT	EU139160.1
IL-12	CGTGCCTCGGGCAATTATAA	CAGGTGAGGTCGCTAGTTTGG	NM_213993.1
IL-4	CACAGCGAGAAAGAACTCGT	GTCCGCTCAGGAGGCTCTTC	NM214123.1
IL-6	CTGGCAGAAAACAACCTGAACC	TGATTCTCATCAAGCAGGTCTCC	AB194100.1
IL-1α	GTGCTCAAAACGAAGACGAACC	CATATTGCCATGCTTTTCCCAGAA	X52731.1
TNF-α	AACCTCAGATAAGCCCGTCG	ACCACCAGCTGGTTGTCTTT	EU682384.1
TGF-β1	CCTGCAAGACCATCGACATG	GCCGAAGCTTGGACAGAATC	AF461808.1
TGF-β2	TGTGTGCTGAGCGCTTTTCT	GAGCGTGCTGCAGGTAGACA	L08375.1

Figure 4.1: CD4:CD8 T cell ratios decrease in MSLN and TLN as piglets age. Piglets were euthanized on d7 or d21. MSLN and TLN were collected. Cells were isolated and stained with a panel of fluorescently labeled mAbs including CD45, CD3, CD4, and CD8. No diet or vaccination differences were detected therefore data from all day 7 or all day 21 animals were pooled. Data are expressed as mean ± SD. Different letter superscripts indicate significant differences at $p \leq 0.05$. A) MSLN CD45+CD3+CD4+ T cells decreased while CD45+CD3+CD8+ T cells increased between d7 and d21 (data shown as CD4+CD8+ ratio as percentage of CD45+CD3+ lymphocytes). B) TLN CD45+CD3+CD4+ T cells decreased while CD45+CD3+CD8+ T cells increased between d7 and d21.

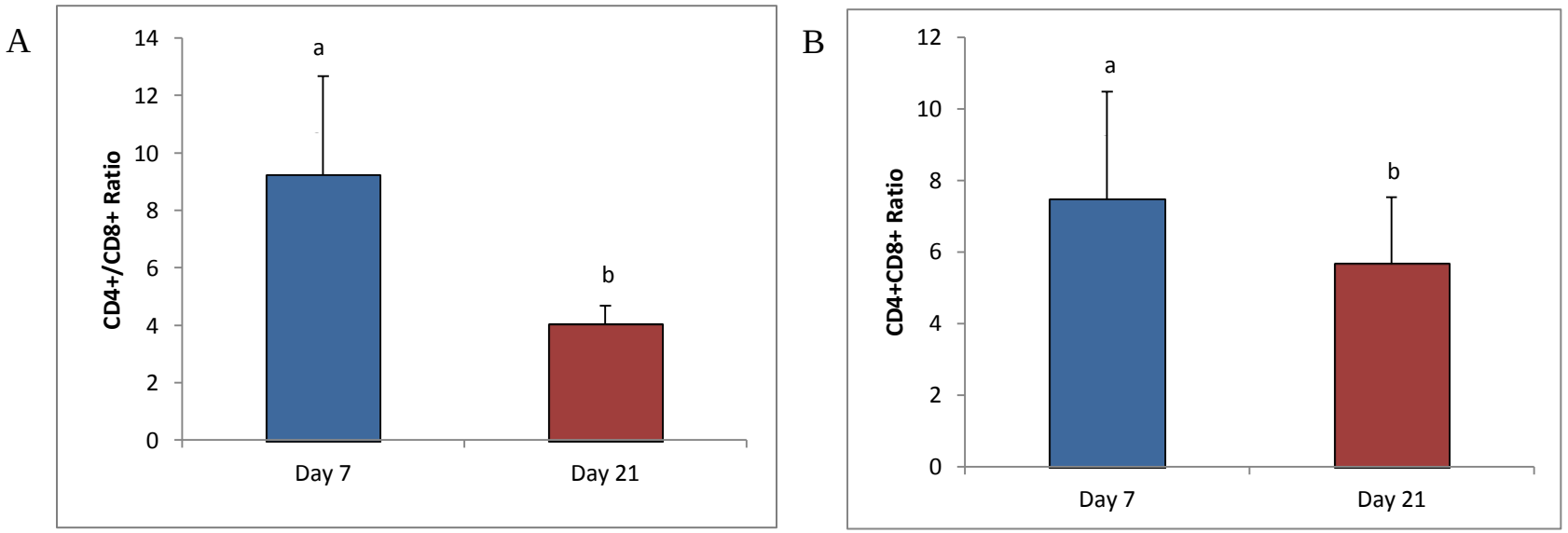


Figure 4.2: TGF-β mRNA expression in lung. Piglets were fed formula, WGP5, WGP50 or WGP250 and euthanized on d7 or d21. Lung tissue sections were excised and snap frozen. Lung mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target gene were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for non-vaccinated, 21 day old piglets fed the control diet. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD of the fold difference relative to day 21, formula-fed, non-vaccinated animals. Different letter superscripts indicate significant differences at p≤0.05.A) **TGF-β1 mRNA expression increased between d7 and d21.** No diet or vaccination differences were detected therefore data from all day 7 or all day 21 animals were pooled. B) **TGF-β2 expression in lung decreased with increasing dietary WGP.** There were no day or vaccination differences; therefore all days and all vaccinated and non-vaccinated groups were combined.

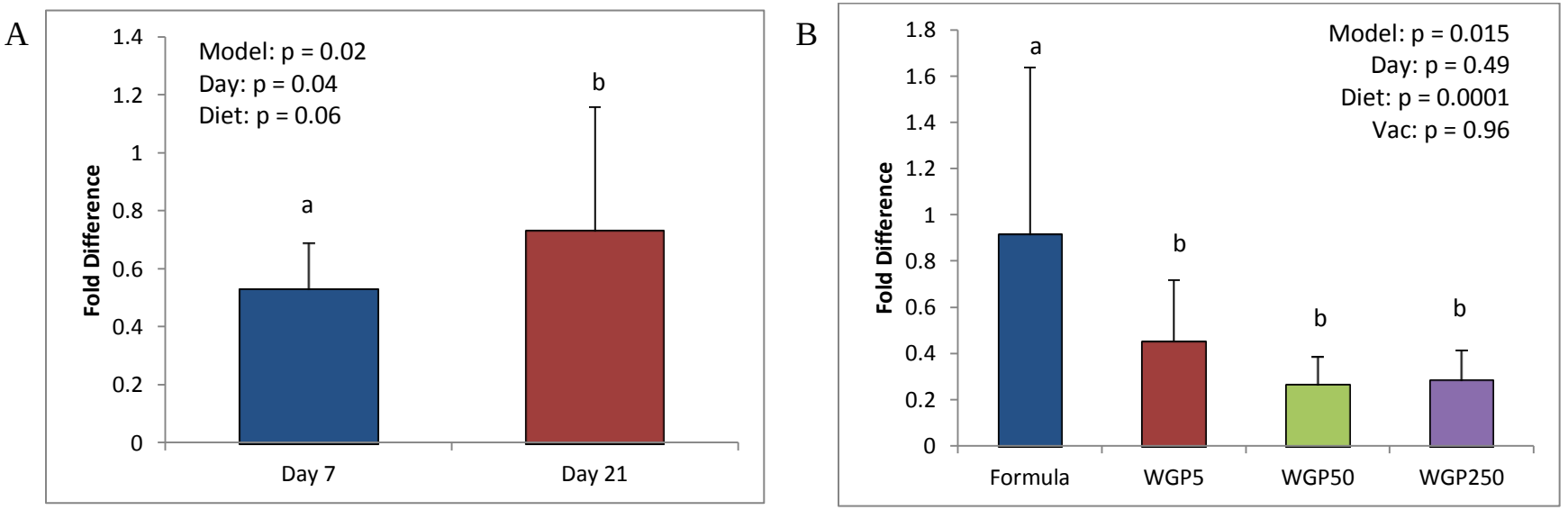


Figure 4.3: Dectin mRNA expression in TLN was lower at d21 compared to d7. Piglets were euthanized on d7 or d21. TLN were excised and snap frozen. mRNA was isolated, purified, and quantified by qRT-PCR. Normalized values for each target were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for non-vaccinated, 21 day old piglets fed the control diet. No diet or vaccination differences were detected therefore data from all day 7 or all day 21 animals were pooled.
All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD. Different letter superscripts indicate significant difference at $p \leq 0.05$.

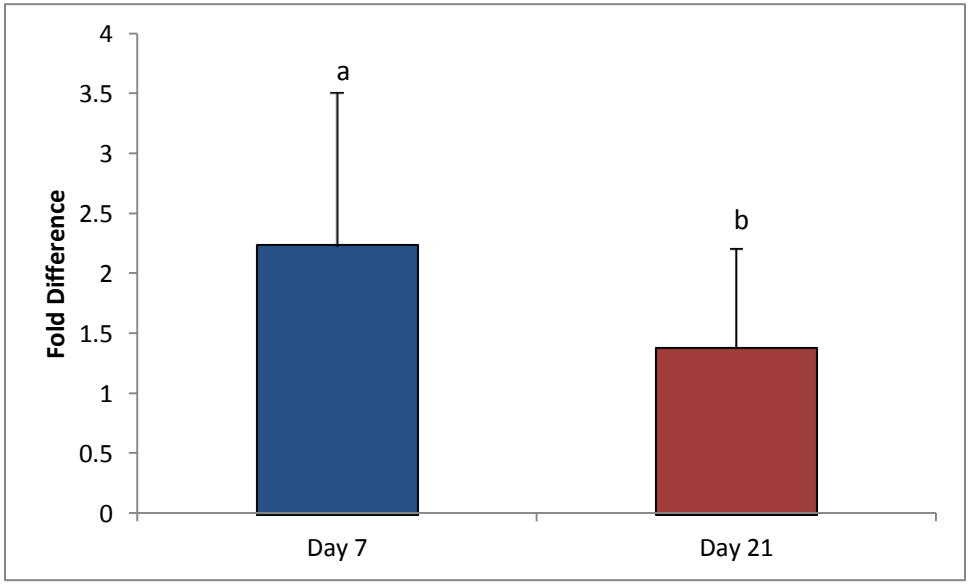


Figure 4.4: TGF-β2 mRNA expression in MSLN increased from day 7 to day 21 in all pigs. Piglets were euthanized on d7 or d21. TLN were excised and snap frozen. MSLN mRNA was isolated, purified, and quantified using qRT-PCR. Normalized values for each target were calculated by dividing the target quantity mean by the β-actin quantity mean. A fold difference was calculated for each measurement by dividing the normalized target values by the average normalized target value for non-vaccinated, 21 day old piglets fed the control diet. All samples that were statistically compared to each other were run on the same plate. No diet or vaccination differences were detected therefore data from all day 7 or all day 21 animals were pooled. All samples that were statistically compared to each other were run on the same plate. Data are expressed as mean ± SD. Different letter superscripts indicate significant difference at $p \leq 0.05$.

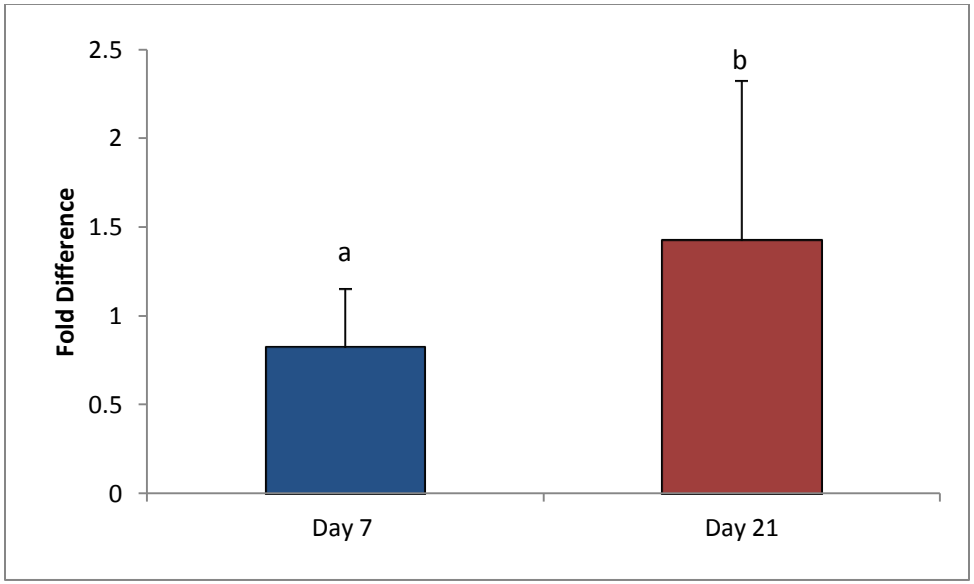


Figure 4.5: Serum IgG concentrations in formula and WGP250 piglets. Sera were collected on day 7, 14, and 21. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). **Serum IgG concentrations were higher on day 7 than on day 14 and day 21.**

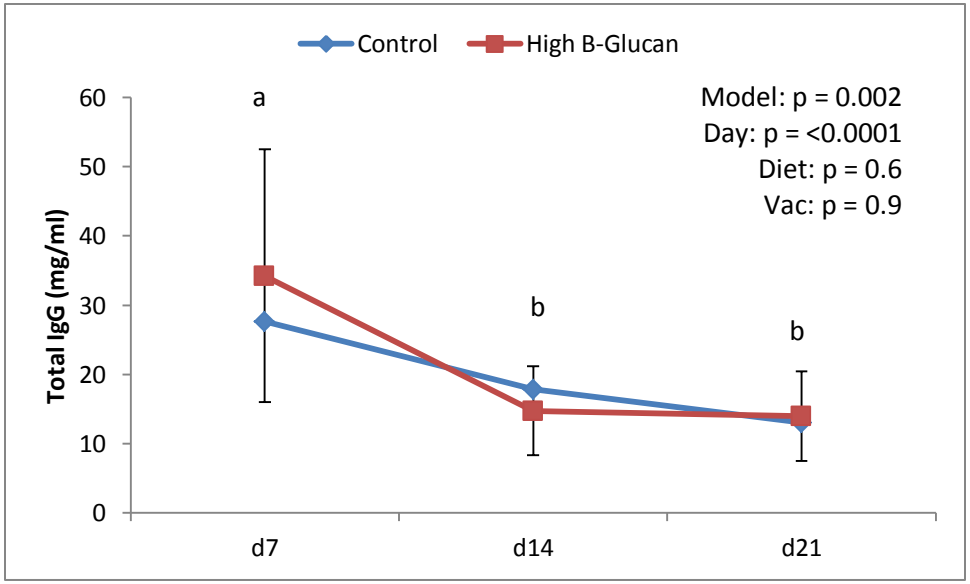


Figure 4.6: Serum IgM concentrations in formula and WGP250 piglets. Sera were collected on day 7, 14, and 21. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). Complete model was not significant, therefore, vaccinated and non-vaccinated data were pooled. **Serum IgM concentrations were higher in formula piglets than WGP250 piglets.**

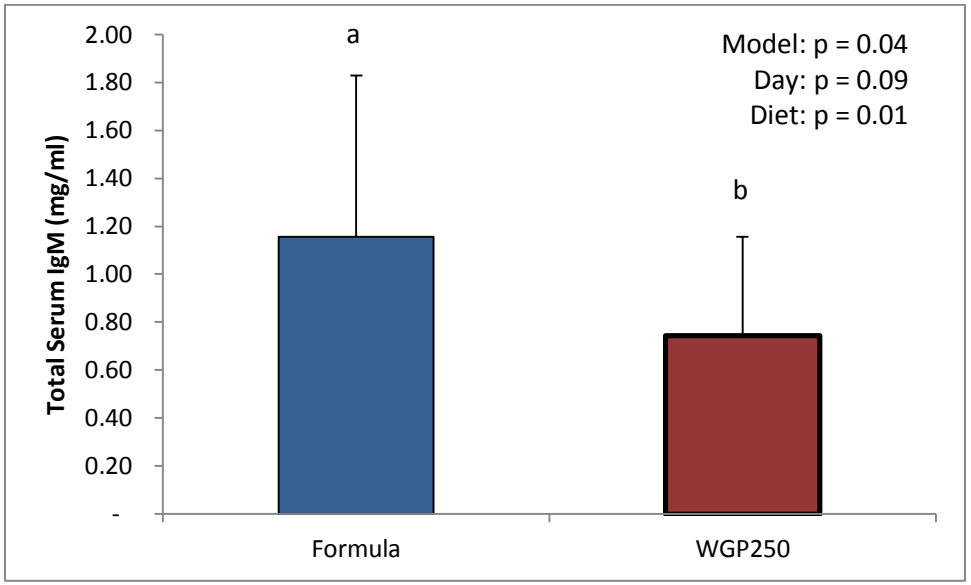
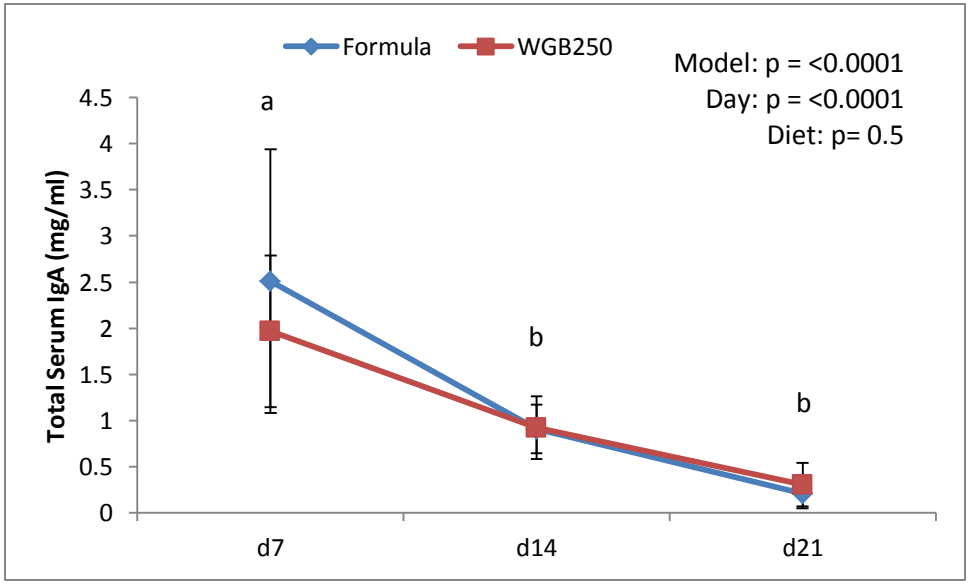


Figure 4.7: Serum IgA concentrations in formula and WGP250 piglets. Sera were collected on day 7, 14, and 21. Data are expressed as mean ± SD. Data with different letters are significantly different ($p<0.05$). **Serum IgA concentrations were higher at d7 than at d14 or d21.**



Chapter 5

Conclusions and Future Directions

These studies focused on understanding the effects of diet on the immune development of the lung, TLN, and MSLN in neonates. Our first objective was to identify differences in the BALT of SR versus FF piglets. It is important to identify differences in feeding modes to increase our understanding of how breastfeeding provides a protective effect for the neonates as compared to formula feeding. The implications of this research can be far reaching, particularly when they are applied to improving the immune system in neonates and, thereby, decreasing the ability of pathogenic microorganisms to attack the host. Overall, our results showed that immune development in lung, TLN, and MSLN of SR and FF piglets differ in T cell subpopulations and mRNA expression, as well as serum immunoglobulin levels.

Future work could investigate various respiratory infection models in order to increase understanding of whether immunological differences in lung, TLN and MSLN affect the neonate's ability to protect against the respiratory infection and/or how the neonate recovers from the infection. This would help identify which specific immune characteristics increase the neonate's ability to cope with infection and what if any deter it. It would also be interesting to look into various lobes of the lung to compare immune expression in order to further understand the interaction between immune parameters throughout the lung. It would be informative to carry out a longer study to see if the immune differences occurring in the early stages of life continue through the transition from formula to feed and beyond and if any benefits/impairments are recognized from the early immune developmental variances.

In the second study, our objective was to investigate the effect of yeast β-glucan supplemented to formula on the development of mucosal immunity in the lung and also investigate the immune response to immunization with an influenza vaccine. Overall, the results showed that yeast β-glucan supplemented to formula did very little to alter the development of mucosal immunity in the lung. Despite the lack of impact yeast β-glucan had on immunological development in the lung in this study, it would be interesting to study the effects of yeast βG supplementation in an infection model where the neonate was challenged. This may enhance any immunological differences the yeast βG supplementation provides to the neonate. It would also be interesting to trace the WGP through the immune system similar to the studies Beier and Gebert (1998) completed with yeast βG in order to better understand the uptake and processing of the WGP particle. This work would help to further our understanding of the impact of yeast βG supplementation on immunological development of the neonate.

References

Adachi, Y., M. Okazaki, N. Ohno and T. Yadomae (1994). "Enhancement of cytokine production by macrophages stimulated with (1- 3)-b-D-glucan, grifolan (GRN), isolated from Grifola frondosa." Biological & Pharmaceutical Bulletin **17**(12): 1554-1560.

Adjei, A. A., J. T. Jones, F. J. Enriquez and S. Yamamoto (1999). "Dietary nucleosides and nucleotides reduce *Cryptosporidium parvum* infection in dexamethasone immunosuppressed adult mice." Experimental Parasitology **92**(3): 199-208.

Babicek, K., I. Cechova, R. R. Simon, M. Harwood and D. J. Cox (2007). "Toxicological assessment of a particulate yeast (1,3/1,6)-β-D-glucan in rats." Food and Chemical Toxicology **45**(9): 1719-1730.

Bailey, M., K. Haverson, C. Inman, C. Harris, P. Jones, G. Corfield, B. Miller and C. Stokes (2005). "The development of the mucosal immune system pre- and post-weaning: Balancing regulatory and effector function." Proceedings of the Nutrition Society **64**(4): 451-457.

Bals, R. and P. S. Hiemstra (2004). "Innate immunity in the lung: How epithelial cells fight against respiratory pathogens." European Respiratory Journal **23**(2): 327-333.

Baran, J., D. J. Allendorf, F. Hong and G. D. Ross (2007). "Oral b-glucan adjuvant therapy converts nonprotective Th2 response to protective th1 cell-mediated immune response in mammary tumor-bearing mice." Folia Histochemica Et Cytobiologica **45**(2): 107-114.

Beier, R. and A. Gebert (1998). "Kinetics of particle uptake in the domes of Peyer's patches." American Journal of Physiology-Gastrointestinal and Liver Physiology **275**(1): G130-G137.

Bernardshaw, S., G. Hetland, B. Grinde and E. Johnson (2006). "An extract of the mushroom *Agaricus blazei Murill* protects against lethal septicemia in a mouse model of fecal peritonitis (vol 25, pg 420, 2006)." Shock **26**(5): 533-533.

Bessler, H., R. Straussberg, J. Hart, I. Notti and L. Sirota (1996). "Human colostrum stimulates cytokine production." Biology of the Neonate **69**(6): 376-382.

Blaschek, W., J. Kasbauer, J. Kraus and G. Franz (1992). "*Pythium-aphanidermatum* - culture, cell-wall composition, and isolation and structure of antitumor storage and solubilized cell-wall (1 - 3), (1 - 6)-β-D-glucans." Carbohydrate Research **231**: 293-307.

Bode, L. (2009). "Human milk oligosaccharides: Prebiotics and beyond." Nutrition Reviews **67**(11): S183-S191.

Boehm, G. and B. Stahl (2003). Oligosaccharides. Functional dairy products. M.-S. T. Cambridge, Woodhead Publishers**:** 203-243.

Boehm, G. and B. Stahl (2007). "Oligosaccharides from milk." Journal of Nutrition **137**(3): 847S-849S.

Boehm, G., B. Stahl, J. Jelinek, J. Knol, V. Miniello and G. E. Moro (2005). "Prebiotic carbohydrates in human milk and formulas." Acta Paediatrica **94**: 18-21.

Boeker, M., R. Pabst and H. J. Rothkotter (1999). "Quantification of B, T and null lymphocyte subpopulations in the blood and lymphoid organs of the pig." Immunobiology **201**(1): 74-87.

Bohn, J. A. and J. N. BeMiller (1995). "(1->3)-β-D-glucans as biological response modifiers: A review of structure-functional activity relationships." Carbohydrate Polymers **28**(1): 3-14.

Borghetti, P., E. De Angelis, R. Saleri, V. Cavalli, A. Cacchioli, A. Corradi, E. Mocchegiani and P. Martelli (2006). "Peripheral T lymphocyte changes in neonatal piglets: Relationship with growth hormone (GH), prolactin (PRL) and cortisol changes." Veterinary Immunology and Immunopathology **110**(1-2): 17-25.

Bourne, F. J. (1973). "Immunoglobulin system of suckling pig." Proceedings of the Nutrition Society **32**(3): 205-215.

Bourne, F. J. (1976). "Humoral immunity in pig." Veterinary Record **98**(25): 499-501.

Bourne, F. J. and J. Curtis (1973). "Transfer of immunoglobulins igg, iga and igm from serum to colostrum and milk in sow." Immunology **24**(1): 157-162.

Brandtzaeg, P. (1996). Development of the mucosal immune system in humans. Recent developments in infant nutrition. J. Bindels, A. Goedhart and H. Visser. London, Kluwer Academic Publishers**:** 349-376.

Brown, G. D. and S. Gordon (2001). "Immune recognition - a new receptor for β-glucans." Nature **413**(6851): 36-37.

Burrin, D. G., R. J. Shulman, P. J. Reeds, T. A. Davis and K. R. Gravitt (1992). "Porcine colostrum and milk stimulate visceral organ and skeletal-muscle protein-synthesis in neonatal piglets." Journal of Nutrition **122**(6): 1205-1213.

Butler, J. E., K. M. Lager, I. Splichal, D. Francis, I. Kacskovics, M. Sinkora, N. Wertz, J. Sun, Y. Zhao, W. R. Brown, R. DeWald, S. Dierks, S. Muyldermans, J. K. Lunney, P. B. McCray, C. S. Rogers, M. J. Welsh, P. Navarro, F. Klobasa, F. Habe and J. Ramsoondar (2009). "The piglet as a model for B cell and immune system development." Veterinary Immunology and Immunopathology **128**(1/3): 147-170.

Calder, P. C., S. Krauss-Etschmann, E. C. de Jong, C. Dupont, J. S. Frick, H. Frokiaer, J. Heinrich, H. Garn, S. Koletzko, G. Lack, G. Mattelio, H. Renz, P. T. Sangild, J. Schrezenmeir, T. M. Stulnig, T. Thymann, A. E. Wold and B. Koletzko (2006). "Early nutrition and immunity - progress and perspectives." British Journal of Nutrition **96**(4): 774-790.

Carver, J. D. (1994). "Dietary nucleotides - cellular immune, intestinal and hepatic system effects." Journal of Nutrition **124**(1): S144-S148.

Carver, J. D., W. I. Cox and L. A. Barness (1990). "Dietary nucleotide effects upon murine natural-killer-cell activity and macrophage activation." Journal of Parenteral and Enteral Nutrition **14**(1): 18-22.

CDC. (2010, October 5, 2010). "Breastfeeding report card, united states: Outcome indicators." Retrieved February 12, 2011.

Chantry, C. J., C. R. Howard and P. Auinger (2006). "Full breastfeeding duration and associated decrease in respiratory tract infection in us children." Pediatrics **117**(2): 425-432.

Chen, S. S. and L. Qing (1990). "Transforming growth factor-beta-1 (TGF-B1) is a bifunctional immune regulator for mucosal IgA responses." Cellular Immunology **128**(2): 353-361.

Chihara, G., Y. Maeda, J. Hamuro, T. Sasaki and F. Fukuoka (1969). "Inhibition of mouse sarcoma 180 by poly saccharides from *Lentinus-edodes*." Nature (London) **222**(5194): 687-688.

Crociani, F., A. Allesandrini, M. M. B. Mucci and B. Biavati (1994). "Degradation of complex carbohydrates by *bifidobacterium spp*." International Journal of Food Microbiology **24**(1-2): 199-210.

Descroix, K., V. Ferrieres, F. Jamois, J. C. Yvin and D. Plusquellec (2006). "Recent progress in the field of β-(1,3)-glucans and new applications." Mini-Reviews in Medicinal Chemistry **6**(12): 1341-1349.

Di Luzio, N. R., D. L. Williams, R. B. McNamee, B. F. Edwards and A. Kitahama (1979). "Comparative tumor inhibitory and anti-bacterial activity of soluble and particulate glucan." International Journal of Cancer **24**(6): 773-779.

Diamond, G., D. Legarda and L. K. Ryan (2000). "The innate immune response of the respiratory epithelium." Immunological Reviews **173**: 27-38.

Dillon, S., S. Agrawal, K. Banerjee, J. Letterio, T. L. Denning, K. Oswald-Richter, D. J. Kasprowicz, K. Kellar, J. Pare, T. van Dyke, S. Ziegler, D. Unutmaz and B. Pulendran (2006). "Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance." Journal of Clinical Investigation **116**(4): 916-928.

Dorea, J. G. (2009). "Breastfeeding is an essential complement to vaccination." Acta Paediatrica **98**(8): 1244-1250.

Douwes, J. (2005). "(1 -> 3)-β-D-glucans and respiratory health: A review of the scientific evidence." Indoor Air **15**(3): 160-169.

Drew, M. D. and B. D. Owen (1988). "The provision of passive-immunity to colostrum-deprived piglets by bovine or porcine serum immunoglobulins." Canadian Journal of Animal Science **68**(4): 1277-1284.

Dritz, S. S., J. Shi, T. L. Kielian, R. D. Goodband, J. L. Nelssen, M. D. Tokach, M. M. Chengappa, J. E. Smith and F. Blecha (1995). "Influence of dietary β-glucan on growth-performance, nonspecific immunity, and resistance to *Streptococcus suis* infection in weanling pigs." Journal of Animal Science **73**(11): 3341-3350.

Duijts, L., V. W. V. Jaddoe, A. Hofman and H. A. Moll (2010). "Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy." Pediatrics **126**(1): E18-E25.

Englund, J. A., E. Walter, S. Black, M. Blatter, J. Nyberg, F. L. Ruben, M. D. Decker and G. R. C. S. Team (2010). "Safety and immunogenicity of trivalent inactivated influenza vaccine in infants a randomized double-blind placebo-controlled study." Pediatric Infectious Disease Journal **29**(2): 105-110.

Fabre, I., M. Bruneteau, P. Ricci and G. Michel (1984). "Isolation and structural study of the glucans of *Phytophthora parasitica*." European Journal of Biochemistry **142**(1): 99-103.

Fiore, A. E. (2010). "Prevention and control of influenza with vaccines: Recommendations of the advisory committee on immunization practices (acip) " Morbidity and Mortality Weekly Report **59**(31): 993.

Fiore, A. E., D. K. Shay, K. Broder, J. K. Iskander, T. M. Uyeki, G. Mootrey, J. S. Bresee and N. J. Cox (2008). "Prevention and control of influenza - recommendations of the advisory committee on immunization practices (acip), 2008." Morbidity and Mortality Weekly Report **57**(RR7, Suppl. S): 1-60.

Firth, M. A., P. E. Shewen and D. C. Hodgins (2005). "Passive and active components of neonatal innate immune defenses." Animal Health Research Reviews **6**(2): 143-158.

Gallois, M., H. J. Rothkotter, M. Bailey, C. R. Stokes and I. P. Oswald (2009). "Natural alternatives to in-feed antibiotics in pig production: Can immunomodulators play a role?" Animal **3**(12): 1644-1661.

Gaskins, H. (1998). Immunological development and mucosal defense in the pig intestine. Progess in pig science. J. Wiseman, M. Varley and J. Chadwick. Nottingham, Nottingham University Press**:** 81-102.

Gaskins, H. and K. Kelley (1995). Immunology and neonatal mortality. The neonatal pig : Development and survival. M. Varley. Wallingford, CAB International**:** 39-58.

German, J., S. Freeman, C. Lebrilla and D. Mills (2008). "Human milk oligosaccharides: Evolution, structures and bioselectivity as substrates for intestinal bacteria." Nestle Nutr Workshop Ser Pediatr Program **62**: 205-222.

Gil, A. (2002). "Modulation of the immune response mediated by dietary nucleotides." European Journal of Clinical Nutrition **56**: S1-S4.

Golding, J., P. M. Emmett and I. S. Rogers (1997). "Gastroenteritis, diarrhoea and breast feeding." Early Human Development **49**: S83-S103.

Goldman, A. S. and R. M. Goldblum (1997). Transfer of maternal leukocytes to the infant by human milk. Reproductive immunology. **222:** 205-213.

Gonzalez-Chavez, S. A., S. Arevalo-Gallegos and Q. Rascon-Cruz (2009). "Lactoferrin: Structure, function and applications." International Journal of Antimicrobial Agents **33**(4): 301-306.

Goodridge, H. S., A. J. Wolf and D. M. Underhill (2009). "B-glucan recognition by the innate immune system." Immunological Reviews **230**: 38-50.

Grimble, G. K. and O. M. Westwood (2001). "Nucleotides as immunomodulators in clinical nutrition." Current Opinion in Clinical Nutrition and Metabolic Care **4**(1): 57-64.

Haesebrouck, F., F. Pasmans, K. Chiers, D. Maes, R. Ducatelle and A. Decostere (2004). "Efficacy of vaccines against bacterial diseases in swine: What can we expect?" Veterinary Microbiology **100**(3-4): 255-268.

Haesebrouck, F. and M. B. Pensaert (1986). "Effect of intratracheal challenge of fattening pigs previously immunized with an inactivated influenza h1n1 vaccine." Veterinary Microbiology **11**(3): 239-249.

Hahn, T. W., J. D. Lohakare, S. L. Lee, W. K. Moon and B. J. Chae (2006). "Effects of supplementation of β-glucans on growth performance, nutrient digestibility, and immunity in weanling pigs." Journal of Animal Science **84**(6): 1422-1428.

Hawkes, J. S., M. A. Neumann and R. A. Gibson (1999). "The effect of breast feeding on lymphocyte subpopulations in healthy term infants at 6 months of age." Pediatric Research **45**(5): 648-651.

Hiss, S. and H. Sauerwein (2003). "Influence of dietary β-glucan on growth performance, lymphocyte proliferation, specific immune response and haptoglobin plasma concentrations in pigs." Journal of Animal Physiology and Animal Nutrition **87**(1-2): 2-11.

Hobson, D., R. L. Curry, A. S. Beare and Wardgard.A (1972). "Role of serum hemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses." Journal of Hygiene **70**(4): 767-777.

Hodgins, D. C., S. Y. Kang, L. deArriba, V. Parreno, L. A. Ward, L. Yuan, T. To and L. J. Saif (1999). "Effects of maternal antibodies on protection and development of antibody responses to human rotavirus in gnotobiotic pigs." Journal of Virology **73**(1): 186-197.

Holgate, S. T., P. Lackie, S. Wilson, W. Roche and D. Davies (2000). "Bronchial epithelium as a key regulator of airway allergen sensitization and remodeling in asthma." American Journal of Respiratory and Critical Care Medicine **162**(3): S113-S117.

Holmgren, J. and C. Czerkinsky (2005). "Mucosal immunity and vaccines." Nature Medicine **11**(4): S45-S53.

Hunter, K. W., R. A. Gault and M. D. Berner (2002). "Preparation of microparticulate beta-glucan from saccharomyces cerevisiae for use in immune potentiation." Letters in Applied Microbiology **35**(4): 267-271.

Ito, T., J. Couceiro, S. Kelm, L. G. Baum, S. Krauss, M. R. Castrucci, I. Donatelli, H. Kida, J. C. Paulson, R. G. Webster and Y. Kawaoka (1998). "Molecular basis for the generation in pigs of influenza a viruses with pandemic potential." Journal of Virology **72**(9): 7367-7373.

Iwasa, Y., M. Iwasa, Y. Ohmori, T. Fukutomi and S. Ogoshi (2000). "The effect of the administration of nucleosides and nucleotides for parenteral use." Nutrition **16**(7-8): 598-602.

Jenssen, H. and R. E. W. Hancock (2009). "Antimicrobial properties of lactoferrin." Biochimie **91**(1): 19-29.

Jung, K., Y. Ha, S. K. Ha, D. U. Han, D. W. Kim, W. K. Moon and C. Chae (2004). "Antiviral effect of *saccharomyces cerevisiae* β-glucan to swine influenza virus by increased production of IFN-γ and nitric oxide." Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health **51**(2): 72-76.

Kelly, D. and A. G. P. Coutts (2000). "Development of digestive and immunological function in neonates: Role of early nutrition." Livestock Production Science **66**(2): 161-167.

Kelly, D. and A. G. P. Coutts (2000). "Early nutrition and the development of immune function in the neonate." Proceedings of the Nutrition Society **59**(2): 177-185.

Keylock, K. T., T. Lowder, K. A. Leifheit, M. Cook, R. A. Mariani, K. Ross, K. Kim, K. Chapman-Novakofski, E. McAuley and J. A. Woods (2007). "Higher antibody, but not cell-mediated, responses to vaccination in high physically fit elderly." Journal of Applied Physiology **102**(3): 1090-1098.

Kojima, T., K. Tabata, W. Itoh and T. Yanaki (1986). "Molecular-weight dependence of the antitumor-activity of schizophyllan." Agricultural and Biological Chemistry **50**(1): 231-232.

Kournikakis, B., R. Mandeville, P. Brousseau and G. Ostroff (2003). "Anthrax-protective effects of yeast-β-1,3 glucans." MedGenMed **5**(1): 1.

Kruzel, M. L., J. K. Actor, I. Boldogh and M. Zimecki (2007). "Lactoferrin in health and disease." Postepy Hig Med Dosw (Online) **61**: 261-267.

Laouar, Y., F. S. Sutterwala, L. Gorelik and R. A. Flavell (2005). "TGF-β controls T helper type 1 cell development through regulation of natural killer cell IFN-γ." Nature Immunology **6**(6): 600-607.

Leahy, S. C., D. G. Higgins, G. F. Fitzgerald and D. van Sinderen (2005). "Getting better with bifidobacteria." Journal of Applied Microbiology **98**(6): 1303-1315.

Lebron, F., R. Vassallo, V. Puri and A. H. Limper (2003). "Pneumocystis carinii cell wall β-glucans initiate macrophage inflammatory responses through NF-κβ activation." Journal of Biological Chemistry **278**(27): 25001-25008.

Legrand, D. and J. Mazurier (2010). "A critical review of the roles of host lactoferrin in immunity." Biometals **23**(3): 365-376.

Li, B., D. Cramer, S. Wagner, R. Hansen, C. King, S. Kakar, C. L. Ding and J. Yan (2007). "Yeast glucan particles activate murine resident macrophages to secrete proinflammatory cytokines via myd88- and SYK kinase-dependent pathways." Clinical Immunology **124**(2): 170-181.

Li, J., J. J. Xing, D. F. Li, X. Wang, L. D. Zhao, S. Lv and D. S. Huang (2005). "Effects of β-glucan extracted from *saccharomyces cerevisiae* on humoral and cellular immunity in weaned piglets." Archives of Animal Nutrition **59**(5): 303-312.

Liang, J. S., D. Melican, L. Cafro, G. Palace, L. Fisette, R. Armstrong and M. L. Patchen (1998). "Enhanced clearance of a multiple antibiotic resistant *Staphylococcus aureus* in rats treated with PGG-glucan is associated with increased leukocyte counts and increased neutrophil oxidative burst activity." International Journal of Immunopharmacology **20**(11): 595-614.

Lonnerdal, B. (2009). "Nutritional roles of lactoferrin." Current Opinion in Clinical Nutrition and Metabolic Care **12**(3): 293-297.

Macfarlane, G. T., H. Steed and S. Macfarlane (2008). "Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics." Journal of Applied Microbiology **104**(2): 305-344.

Mahauthaman, R., C. J. Howell, B. W. Spur, L. J. F. Youlten, T. J. H. Clark, M. H. Lessof and T. H. Lee (1988). "The generation and cellular-distribution of leukotriene-C4 in human eosinophils stimulated by unopsonized zymosan and glucan particles." Journal of Allergy and Clinical Immunology **81**(4): 696-705.

Mantovani, M. S., M. F. Bellini, J. P. F. Angeli, R. J. Oliveira, A. F. Silva and L. R. Ribeiro (2008). "B-glucans in promoting health: Prevention against mutation and cancer." Mutation Research-Reviews in Mutation Research **658**(3): 154-161.

Marodi, L. and L. D. Notarangelo (2007). "Immunological and genetic bases of new primary immunodeficiencies." Nature Reviews Immunology **7**: 851-861.

McCarthy, M. W. and D. R. Kockler (2004). "Trivalent intranasal influenza vaccine, live." Annals of Pharmacotherapy **38**(12): 2086-2093.

Mennechet, F. J. D., L. H. Kasper, N. Rachinel, L. A. Minns, S. Luangsay, A. Vandewalle and D. Buzoni-Gatel (2004). "Intestinal intraepithelial lymphocytes prevent pathogen-driven inflammation and regulate the SMAD/T-bet pathway of lamina propria CD4(+) T cells." European Journal of Immunology **34**(4): 1059-1067.

Mestecky, J. and J. R. McGhee (1987). "Immunoglobulin-A (IgA) - molecular and cellular interactions involved in IgA biosynthesis and immune-response." Advances in Immunology **40**: 153-245.

Murphy, K., P. Travers and M. Walport (2008). Janeway's immunobiology New York, Garland Science.

Newburg, D. S. (1997). "Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria?" Journal of Nutrition **127**: S980-S984.

Newburg, D. S., G. M. Ruiz-Palacios and A. L. Morrow (2005). "Human milk glycans protect infants against enteric pathogens." Annual Review of Nutrition **25**: 37-58.

Nguyen, T. V., L. J. Yuan, M. S. P. Azevedo, K. I. Jeong, A. M. Gonzalez and L. J. Saif (2007). "Transfer of maternal cytokines to suckling piglets: *In vivo* and *in vitro* models with implications for immunomodulation of neonatal immunity." Veterinary Immunology and Immunopathology **117**(3-4): 236-248.

Nicholson, K. G., J. M. Wood and M. Zambon (2003). "Influenza." Lancet **362**(9397): 1733-1745.

Ninonuevo, M. R., Y. Park, H. F. Yin, J. H. Zhang, R. E. Ward, B. H. Clowers, J. B. German, S. L. Freeman, K. Killeen, R. Grimm and C. B. Lebrilla (2006). "A strategy for annotating the human milk glycome." Journal of Agricultural and Food Chemistry **54**(20): 7471-7480.

Ogawa, J., A. Sasahara, T. Yoshida, M. M. Sira, T. Futatani, H. Kanegane and T. Miyawaki (2004). "Role of transforming growth factor-beta in breast milk for initiation of IgA production in newborn infants." Early Human Development **77**(1-2): 67-75.

Oguchi, S., W. A. Walker and I. R. Sanderson (1995). "Iran saturation alters the effect of lactoferrin on the proliferation and differentiation of human enterocytes (caco-2 cells)." Biology of the Neonate **67**(5): 330-339.

Pescovitz, M. D., J. K. Lunney and D. H. Sachs (1985). "Murine anti-swine T4 and T8 monoclonal-antibodies - distribution and effects on proliferative and cyto-toxic T-cells." Journal of Immunology **134**(1): 37-44.

Pescovitz, M. D., A. G. Sakopoulos, J. A. Gaddy, R. J. Husmann and F. A. Zuckermann (1994). "Porcine peripheral-blood CD4(+) CD8(+) dual expressing T-cells." Veterinary Immunology and Immunopathology **43**(1-3): 53-62.

Puddu, P., P. Valenti and S. Gessani (2009). "Immunomodulatory effects of lactoferrin on antigen presenting cells." Biochimie **91**(1): 11-18.

Rennels, M. B. (1996). "Influence of breast-feeding and oral poliovirus vaccine on the immunogenicity and efficacy of rotavirus vaccines." Journal of Infectious Diseases **174**: S107-S111.

Rooke, J. A. and I. M. Bland (2002). "The acquisition of passive immunity in the new-born piglet." Livestock Production Science **78**(1): 13-23.

Rooke, J. A., C. Carranca, I. M. Bland, A. G. Sinclair, M. Ewen, V. C. Bland and S. A. Edwards (2003). "Relationships between passive absorption of immunoglobulin G by the piglet and plasma concentrations of immunoglobulin G at weaning." Livestock Production Science **81**(2-3): 223-234.

Saito, K., M. Nishijima, N. Ohno, N. Nagi, T. Yadomae and T. Miyazaki (1992). "Activation of complement and limulus coagulation systems by an alkali-soluble glucan isolated from *Omphalia lapidescens* and its less-branched derivatives - (studies on fungal polysaccharide .39.)." Chemical & Pharmaceutical Bulletin **40**(5): 1227-1230.

Salmon, H., M. Berri, V. Gerdts and F. Meurens (2009). "Humoral and cellular factors of maternal immunity in swine." Developmental and Comparative Immunology **33**(3): 384-393.

Sangild, P. T. (2006). "Gut responses to enteral nutrition in preterm infants and animals." Experimental Biology and Medicine **231**(11): 1695-1711.

Shin, M. S., S. Lee, K. Y. Lee and H. G. Lee (2005). "Structural and biological characterization of aminated-derivatized oat β-glucan." Journal of Agricultural and Food Chemistry **53**(14): 5554-5558.

Strobel, S. and A. M. Mowat (1998). "Immune responses to dietary antigens: Oral tolerance." Immunology Today **19**(4): 173-181.

Thornton, B. P., V. Vetvicka, M. Pitman, R. C. Goldman and G. D. Ross (1996). "Analysis of the sugar specificity and molecular location of the β-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18)." Journal of Immunology **156**(3): 1235-1246.

Uauy, R., G. Stringel, R. Thomas and R. Quan (1990). "Effect of dietary nucleosides on growth and maturation of the developing gut in the rat." Journal of Pediatric Gastroenterology and Nutrition **10**(4): 497-503.

Van Reeth, K., S. Van Gucht and M. Pensaert (2002). "Correlations between lung proinflammatory cytokine levels, virus replication, and disease after swine influenza virus challenge of vaccination-immune pigs." Viral Immunology **15**(4): 583-594.

Vancikova, Z. (2002). "Mucosal immunity: Basic principles, ontogeny, cystic fibrosis and mucosal vaccination." Current Drug Targets - Immune Endocrine and Metabolic Disorders **2**(1): 83-95.

Volman, J. J., J. D. Ramakers and J. Plat (2008). "Dietary modulation of immune function by β-glucans." Physiology & Behavior **94**(2): 276-284.

Wang, T. and R. J. Xu (1996). "Effects of colostrum feeding on intestinal development in newborn pigs." Biology of the Neonate **70**(6): 339-348.

Ward, R. E., M. Ninonuevo, D. A. Mills, C. B. Lebrilla and J. B. German (2006). "In vitro fermentation of breast milk oligosaccharides by *Bifidobacterium infantis* and *Lactobacillus gasseri*." Applied and Environmental Microbiology **72**(6): 4497-4499.

Willment, J. and G. D. Brown. (2010). "Dectin-1." UCSD Molecule Pages 19 Jan 2010. Retrieved 31 March, 2011.

Wong, J. P., M. E. Christopher, S. Viswanathan, G. Schnell, X. Dai, D. Van Loon and E. R. Stephen (2010). "Aerosol and nasal delivery of vaccines and antiviral drugs against seasonal and pandemic influenza." Expert Rev Respir Med **4**(2): 171-177.

Xanthou, M. (1997). "Human milk cells." Acta Paediatrica **86**(12): 1288-1290.

Xiao, Z. G., C. A. Trincado and M. P. Murtaugh (2004). "B-glucan enhancement of T cell IFN-γ response in swine." Veterinary Immunology and Immunopathology **102**(3): 315-320.

Yu, V. Y. H. (2002). "Scientific rationale and benefits of nucleotide supplementation of infant formula." Journal of Paediatrics and Child Health **38**(6): 543-549.

Zekovic, D. B., S. Kwiatkowski, M. M. Vrvic, D. Jakovljevic and C. A. Moran (2005). "Natural and modified (1 -> 3)-β-D-glucans in health promotion and disease alleviation." Critical Reviews in Biotechnology **25**(4): 205-230.

Zhang, K. and H. R. Petty (1994). "Influence of polysaccharides on neutrophil function - specific antagonists suggest a model for cooperative saccharide-associated inhibition of immune complex-triggered superoxide production." Journal of Cellular Biochemistry **56**(2): 225-235.

Zuckermann, F. A. (1999). "Extrathymic CD4/CD8 double positive T cells." Veterinary Immunology and Immunopathology **72**(1-2): 55-66.

Zuckermann, F. A. and H. R. Gaskins (1996). "Distribution of porcine CD4/CD8 double-positive T lymphocytes in mucosa-associated lymphoid tissues." Immunology **87**(3): 493-499.

Zuckermann, F. A. and R. J. Husmann (1996). "Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells." Immunology **87**(3): 500-512.

Zuckermann, F. A. and R. J. Husmann (1996). "The human and swine CD4/CD8 double positive T lymphocyte." Advances in Swine in Biomedical Research, Vols 1 and 2: 331-343.

Appendix A

Supplemental Information: SR versus FF Study

The first point of contact for inhaled substances such as environmental pollutants, cigarette smoke, airborne allergens and microorganisms are the epithelial lining of the upper airways and lungs (Diamond *et al.* 2000). The lungs and upper airways are mucosal surfaces that have two purposes. They protect the organism from invasion of foreign antigens and bacteria, while allowing for the exchange of materials with the environment. Due to the high exposure of the lungs and upper airways to airborne pathogens, they are common sites for infection. Immune factors such as IgA are secreted in the lungs which helps protect against respiratory infections. The goal of this research was to better understand the development of immune cells in the lungs in sow-reared pigs compared to those that are formula-fed. In order to increase understanding of the development of immune cells in the lungs, MSLN, and TLN, T lymphocyte subpopulations were identified, cytokine mRNA expression was evaluated and total IgG, IgM, and IgA expression was analyzed.

In this study, 11, colostrum fed piglets received formula medicated sow milk replacer formula beginning at 48 hours of life and 12 piglets received sow milk through the duration of the study (**Figure A1**). On d7, approximately half of the piglets in each group were euthanized and blood and tissue samples were collected. On d21 the other half of the piglets were euthanized and blood and tissue samples were collected.

This study investigated the effects of diet on the development of lung immune development. Flow cytometry was used to identify T cell subpopulations. CD4:CD8 T cell ratios calculated and were not significantly different in PBMC at d7 or d 21 (**Figure**

A2). Relative abundance of mRNA expression in lung (**Table A1**), TLN (**Table A2**), and MSLN (**Table A3**) was also evaluated. Although several age and diet effects were seen in mRNA expression Lung, TLN, and MSLN (see Chapter 3), no differences were identified in mRNA expression of IL-1β, dectin, IL-12, and TGFβ-2 in lung, TGFβ-1, TGFβ-2, IL-6, IFN-α, and IFN-β in TLN, and IL-1β, IL-12, IL-6, TGFβ-1, and TNF-α in MSLN. Total serum immunoglobulin ELISAs were analyzed to better appreciate the immune system. Sera was collected on day 7, 14, and 21. Serum IgM levels in SR compared to FF piglets did not differ at $p<0.05$ (**Figure A3**).

In conclusion, there were several immunological developmental differences between sow-reared and formula fed neonatal piglets.

Figure A1: Experimental Design. Piglets (n=23) were assigned to one of two dietary treatment groups: sow-reared or formula-fed. Piglets assigned to formula group received colostrum for 48 hours prior to transition to formula.

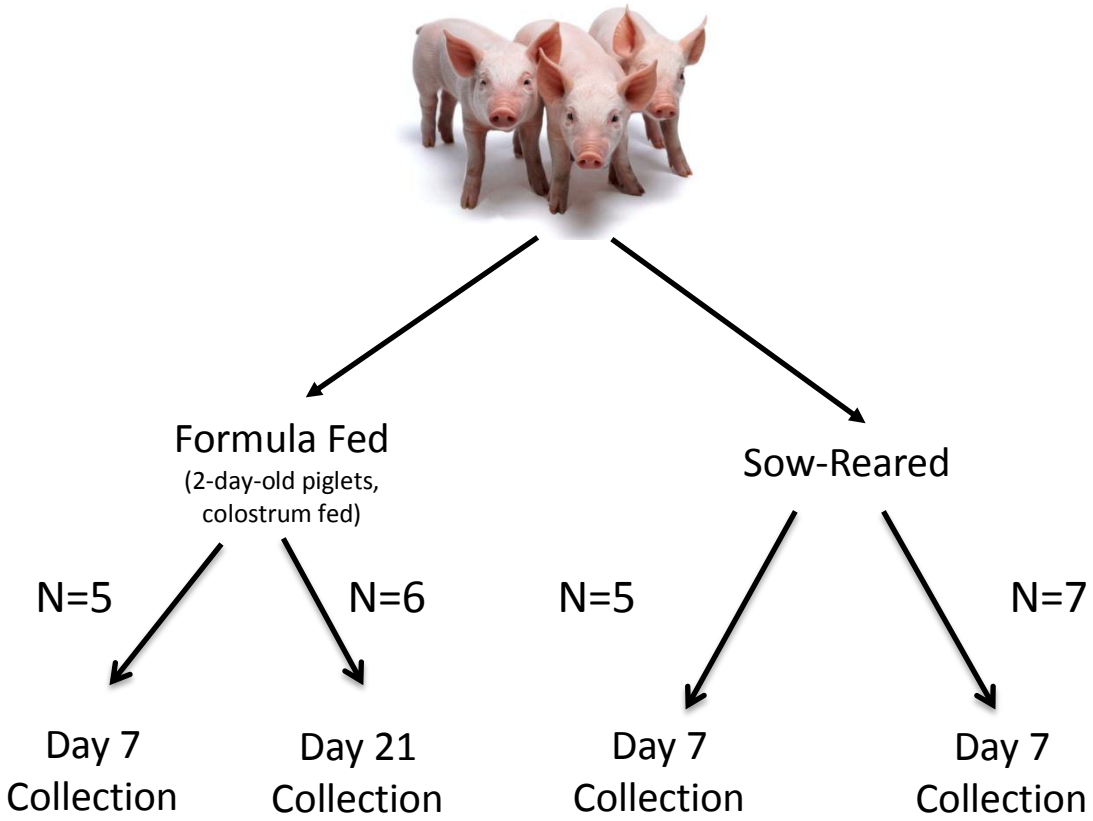
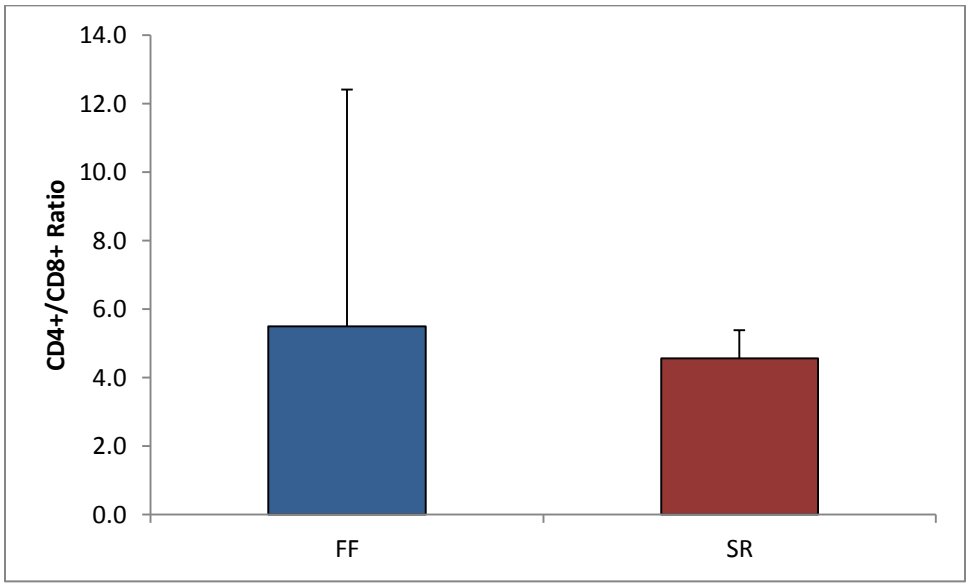


Figure A2: CD4:CD8 T cell ratios are not significantly different in d7 or d21 PBMC. Piglets were euthanized on d7 or d21. PBMC were collected. Cells were isolated and stained with a panel of fluorescently labeled mAb including CD45, CD3, CD4, and CD8. Data are expressed as mean ± SD. Statistical significance was set at a p-value of $p \leq 0.05$. A) PBMC CD4:CD8 T cell ratios were not significant at d7. B) PBMC CD4:CD8 T cell ratios were not significant at d21.

A



B

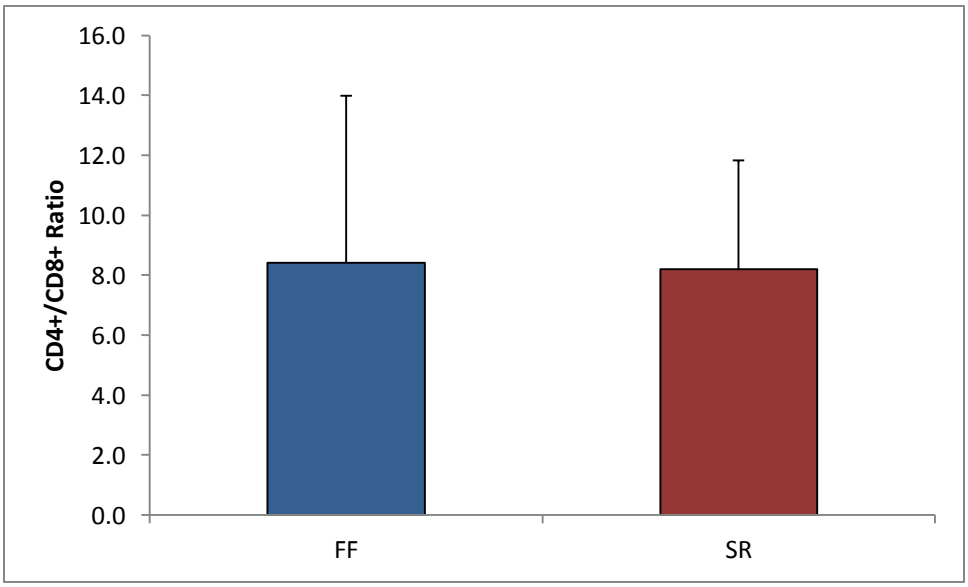


Table A1: Relative abundance of mRNA expression in lung. Data are expressed as mean ± SD of the fold difference relative to day 7, formula-fed piglets. Statistical significance was set at a p-value of $p \leq 0.05$.

	d7		d21		P-values
	FF	SR	FF	SR	
IL-β	1.0±0.72	0.71±0.28	0.66±0.40	1.87±1.45	0.1191
Dectin	1.0±0.45	1.39±0.53	1.65±0.40	1.36±0.47	0.7897
IL-12	1.0±1.0	0.32±0.85	1.1±0.94	1.75±1.77	0.2266
TGFβ-2	1.0±0.48	1.12±0.41	1.51±1.14	1.30±0.35	0.6035

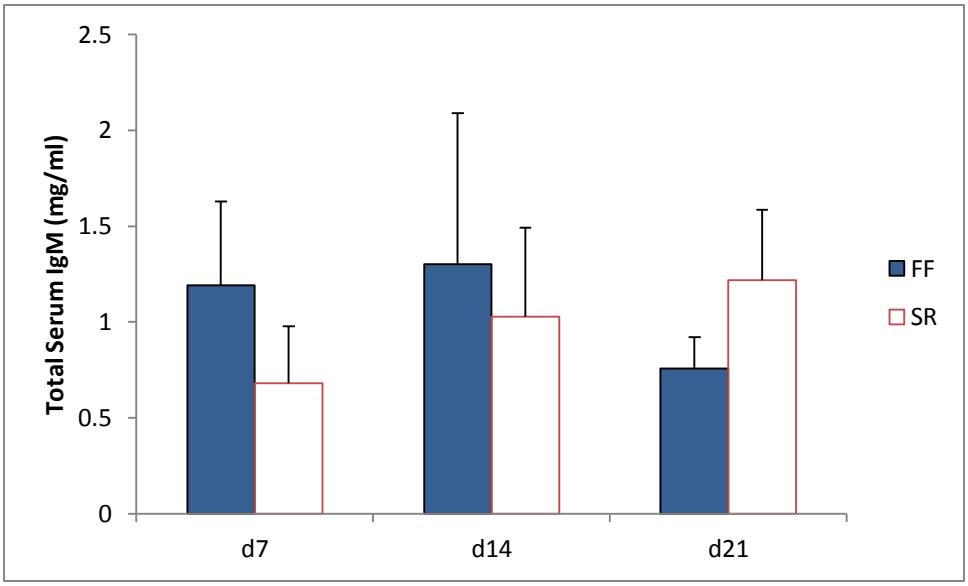
Table A2: Relative abundance of mRNA expression in TLN. Data are expressed as mean ± SD of the fold difference relative to day 7, formula-fed piglets. Statistical significance was set at a p-value of $p \leq 0.05$.

	d7		d21		P-values
	FF	SR	FF	SR	
TGFβ-1	1.0±0.58	0.66±0.13	0.60±0.44	0.65±0.27	0.3494
TGFβ-2	1.0±1.0	0.22±0.11	0.21±0.20	0.29±0.15	0.0546
IL-6	1.0±0.39	0.41±0.09	0.59±0.08	0.32±0.11	0.0997
IFN-α	1.0±1.7	0.02±0.001	0.18±0.12	0.02±0.001	0.1891
IFN-β	1.0±1.77	0.001±0.0002	0.16±0.20	0.02±0.02	0.468

Table A3: Relative abundance of mRNA expression in MSLN. Data are expressed as mean ± SD of the fold difference relative to day 7, formula-fed piglets. Statistical significance was set at a p-value of $p \leq 0.05$.

	d7		d21		P-values
	FF	SR	FF	SR	
IL-1β	1.0±0.63	0.29±0.17	0.92±0.61	0.82±0.49	0.1569
TNF-α	1.0±0.39	0.37±0.06	0.94±0.34	1.09±0.98	0.2479
IL-12	1.0±0.62	0.16±0.03	0.56±0.21	0.62±0.56	0.0595
TGFβ-1	1.0±0.35	0.43±0.02	1.02±0.44	0.87±0.60	0.1539
IL-6	1.0±0.37	0.51±0.24	1.03±0.47	1.02±0.76	0.348

Figure A3: Serum IgM levels in SR and FF piglets. Sera collected on day 7, 14, and 21. Data are expressed as mean ± SD. Statistical significance was set at a p-value of $p \leq 0.05$.



Appendix B

Supplemental Information: β-Glucan Study

Influenza is a major cause of morbidity and mortality in children worldwide, accounting for about 36,000 American deaths every year (CDC 2010). It is generally believed that breast-fed infants are less susceptible to respiratory infections compared to formula-fed infants; however, a large percentage of infants are fed formula at some point during their first year of life. Several studies have looked at the effects of adding various bioactive components to formula to enhance formula and the immunological protection it provides (Carver *et al.* 1990; Uauy *et al.* 1990; Newburg *et al.* 2005; Boehm and Stahl 2007). The goal of this research was to investigate the effect of yeast β-Glucan supplemented to infant formula on the development of mucosal immunity in the lung and explore the immune response to immunization with an influenza vaccine. To better understand the effects of yeast β-Glucan supplemented to infant formula, fluzone specific serum IgG was assessed, histomorphology and immunohistochemistry in lung was analyzed, mRNA expression in MSLN, TLN, and Lung was evaluated, T cell populations were investigated using flow cytommetry, and mitogenic and fluzone cell stimulated were performed.

In this study, 68 2-day-old piglets were divided into 4 groups with each group receiving formula with a differing amount of WGP® 1/3-1/6 β-D-glucan (**Figure B1**) ((1) a medicated sow milk replacer formula (Formula; Milk Specialties Global Animal Nutrition, Carpentersville, IL); 2) Formula + 1.8 mg WGP/kg BW/day (WGP5); 3) Formula + 18 mg WGP/kg BW/day (WGP50); or 4) Formula + 90 mg WGP/kg BW/day (WGP250)) (**Figure B2**). On d7 approximately one third of the piglets in each group

were euthanized and blood and tissue samples were collected. Approximately half of the remaining piglets in each treatment group (Formula n=5, WGP5 n=6, WGP50 n=5, WGP250 n=6) were vaccinated with a 0.25ml i.m. injection of human influenza vaccine (Fluzone™, Sanofi Pasteur, Swiftwater, PA) (**Figure B3**). A blood sample was drawn from the jugular vein prior to administration of the vaccine. Vaccinated animals were boosted on d14 with the same dose of Fluzone™. Blood samples were collected longitudinally from all piglets on d14 and d21 by jugular vein or following euthanasia, respectively. The piglets were monitored daily for normal growth and food intake, as well as the presence of fever, diarrhea or lethargy, no differences were found across treatments groups for growth or food intake (**Figure B5**). Formula was offered 14 times daily at a rate of 360 ml/kg BW/day.

This study investigated the effects of dietary βG on the development of lung-associated adaptive immunity. One of these immune parameters was relative abundance of mRNA expression in Lung (**Table B1**), TLN (**Table B2**), and MSLN (**Table B3).** Although some age and diet effects were seen in mRNA expression in Lung, TLN, and MSLN (see Chapter 4), no differences were identified in mRNA expression of dectin, IL-1α, IL-2, IL-4, IL-12, TNF-α in lung, IL-6, IL-12, TGFβ-1, TGFβ-2, and TNF-α in TLN, and dectin, IL-2, IL-4, IL-6, IL-12, TGFβ-1, and TNF-α in MSLN.

Expression of T cell subpopulations was another immune parameter that was analyzed in TLN and MSLN. Expression of double positive CD45+CD3+CD4+CD8+ T cells as a percentage of CD3+ lymphocytes were not different in respect to age or diet group (**Figure B6**). **Figure B4** exhibits the flow cytommetry gating procedure that was used to identify CD4+, CD8+, CD4+CD8+, and CD4-CD8- subpopulations. First the

lymphocyte population was selected on the side scatter vs. forward scatter plot. Stained CD45+ cells were selected after comparing an unstained sample to a stained sample. The region of positive CD45+ cells was identified by the area in the unstained sample graph that had <0.1% of the unstained cells in it. That region was then mimicked in the stained sample graph. Stained CD3+ cells were selected after comparing the stained and unstained populations in a similar fashion. Then CD8+, CD4+, and CD4+CD8+ cells were identified by comparing stained set to unstained set.

Influenza specific serum IgG (**Figure B7**) was also analyzed. While vaccination did increase influenza-specific serum IgG production, dietary WGP did not enhance the ability of piglets to produce fluzone-specific IgG. Vaccinated piglets had a significantly higher ($p<0.0001$) IgG titer at d14 and d21 than non-vaccinated animals ($p<0.0001$). Day 21 piglets had a greater vaccine response compared to day 14 piglets ($p<0.0001$). Vaccinated piglets had a fold increase in serum IgG of 5.9 ± 2.2 from d7 to d21 while non-vaccinated piglets had a fold increase in serum IgG of 0.49 ± 0.05 from d7 to d21.

Mitogenic (**Table B4**) and Fluzone™ cell stimulation (**Table B5**) were completed to determine if WGP and/or vaccination enhanced cell proliferation. MSLN and TLN cells were stimulated with LPS, BSA, or ConA. It was found that neither WGP nor vaccination enhanced cell proliferation in MSLN or TLN. Cells stimulated with ConA proliferated significantly ($p<0.05$) more than those treated with LPS, or BSA. TLN cells were stimulated with either 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, or 0 µg/ml of dialyzed Fluzone™. It was found that neither WGP nor vaccination enhanced cell proliferation.

Lastly, histomorphology (**Figure B8**) and immunohistochemistry (**Figure B9**) in lung were two other immune parameters that were examined. Vaccination, age and dietary WGP seemed to have an impact on the histomorphology or immunohistochemistry of lung tissue in piglets. In conclusion, WGP only minimally impacted the neonatal piglet immune response.

Figure B1: Structure of WGP® 1/3-1/6 β-D-glucan. The β-glucan particulate is extracted from *S. cerevisiae* using acid and alkaline extraction techniques that results in stripping the outer mannoprotein sheath and loss of the inner cellular lipids and proteins, leaving the β-glucan shell (Babicek et al., 2007).

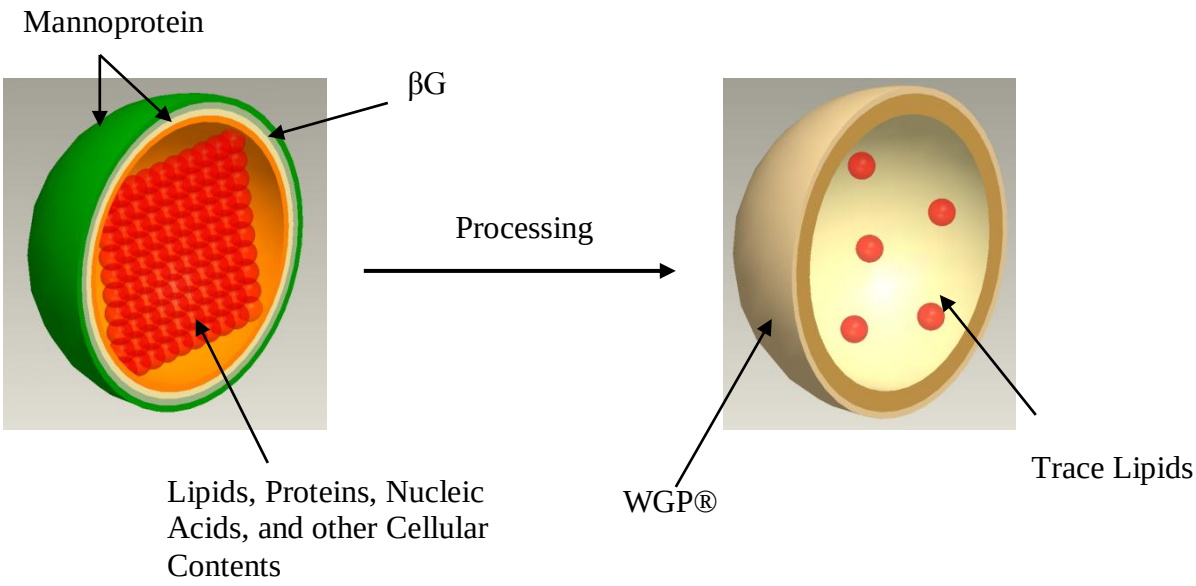


Figure B2: Experimental Design. Piglets (n=68) were randomized to one of four dietary treatment groups: a medicated sow milk replacer formula (Formula, Milk Specialties Company, Carpentersville, IL); Formula + 5 mg/L WGP® 1/3-1/6 β-D-glucan (WGP5); Formula + 50 mg/L WGP® 1/3-1/6 β-D-glucan (WGP50); or Formula + 250 mg/L WGP® 1/3-1/6 β-D-glucan (WGP250).

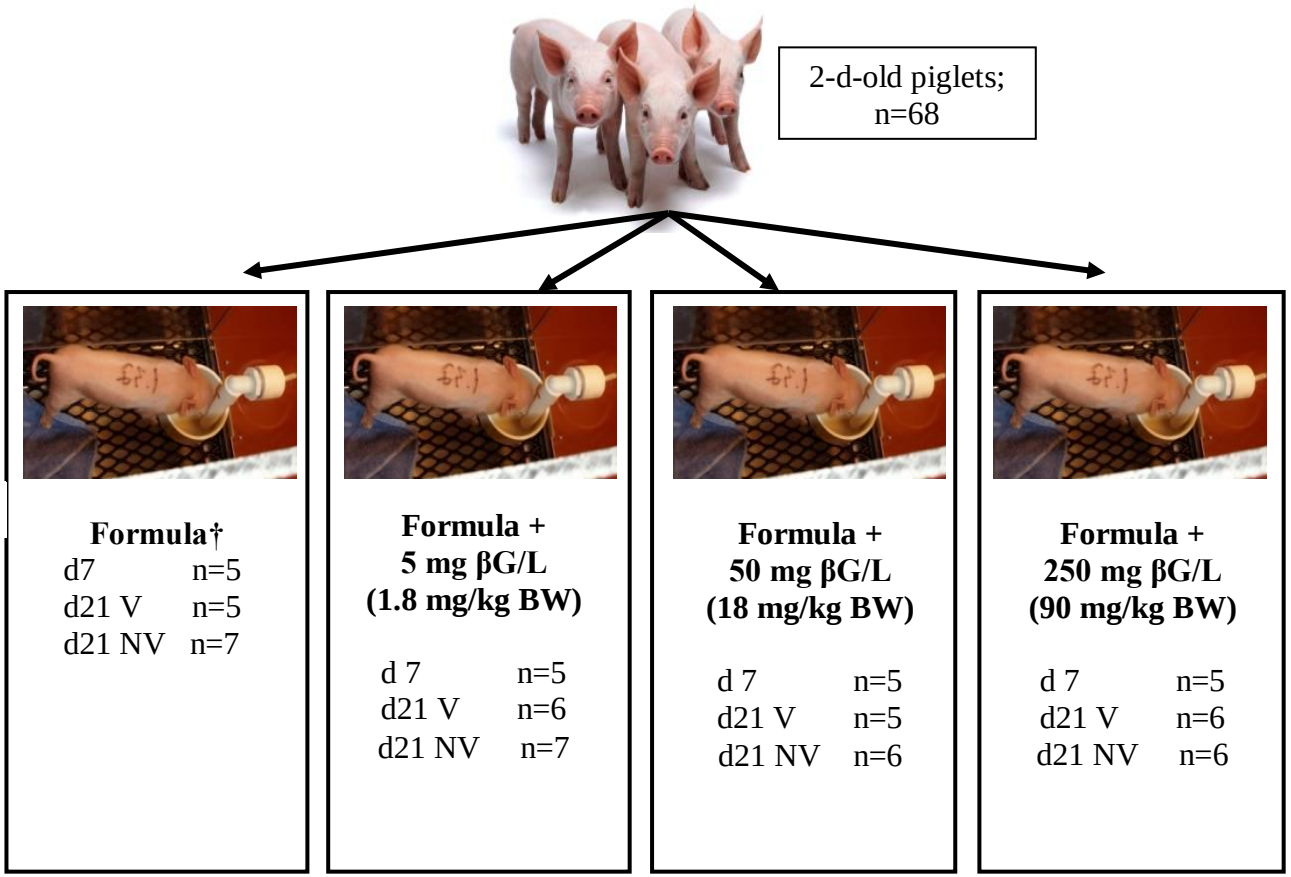


Figure B3: Vaccination and sampling design. Piglets were euthanized on day 7 or day 21. Blood samples were taken on day 7 prior to vaccination, in animals being vaccinated, and day 14 in all animals. Half of the animals in each diet group were vaccinated on day 7. These animals were boosted, with same dose, on day 14.

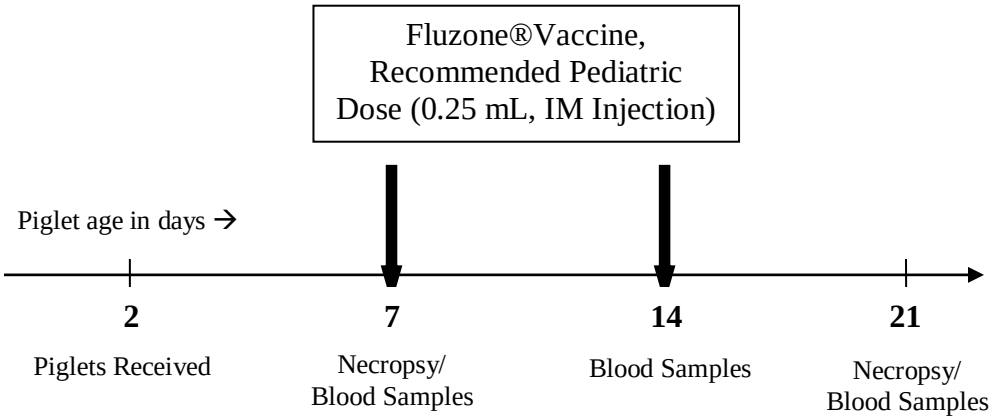


Figure B4: Flow cytometry gating procedure. The lymphocyte population was selected on the side scatter vs forward scatter plot. Stained CD45+ cells were selected after comparing stained lymphocyte population to unstained lymphocyte population. Stained CD3+ cells were selected after comparing the stained and unstained populations. Then CD8+, CD4+, and CD4+CD8+ cells were identified by comparing stained set to unstained set.

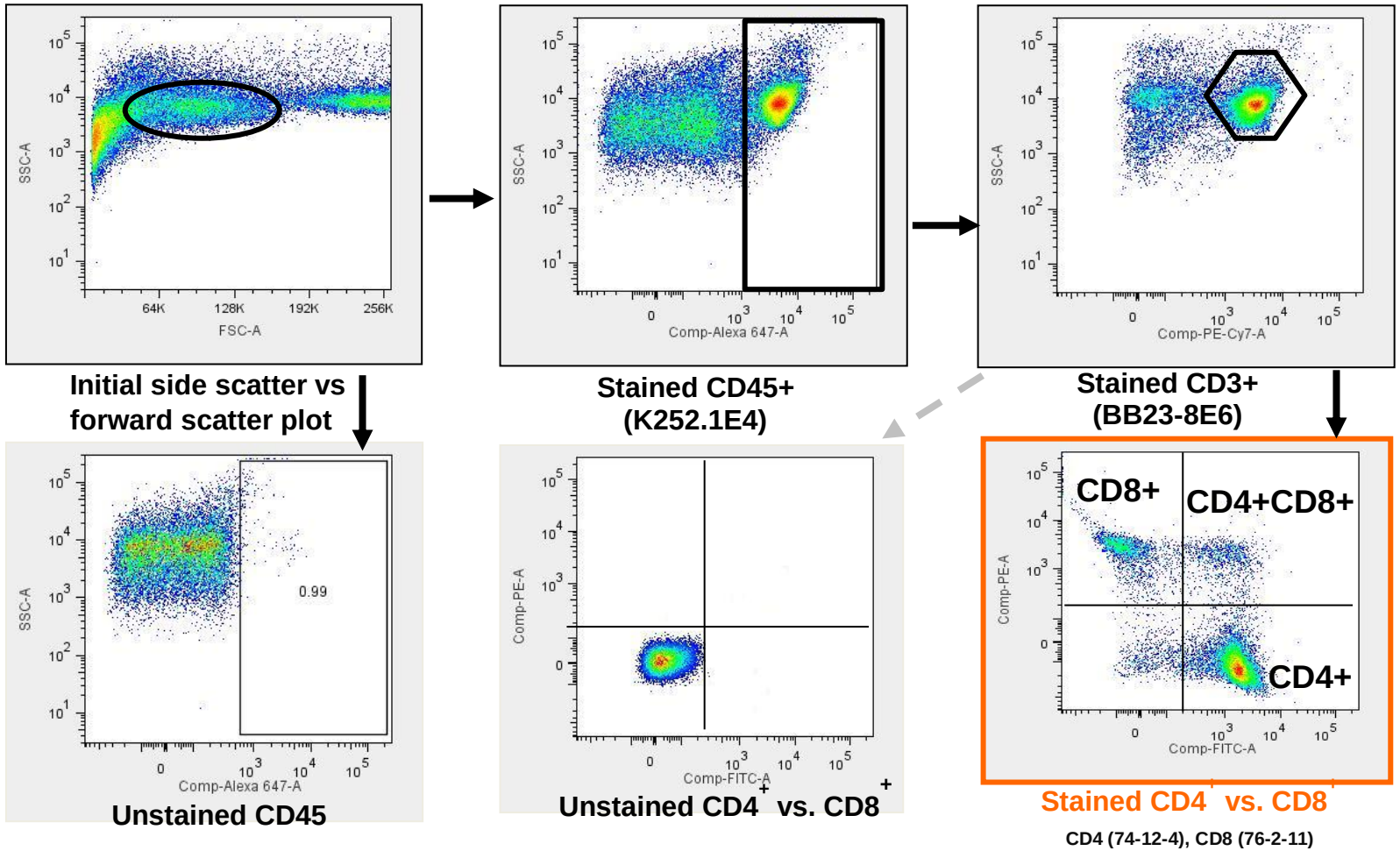
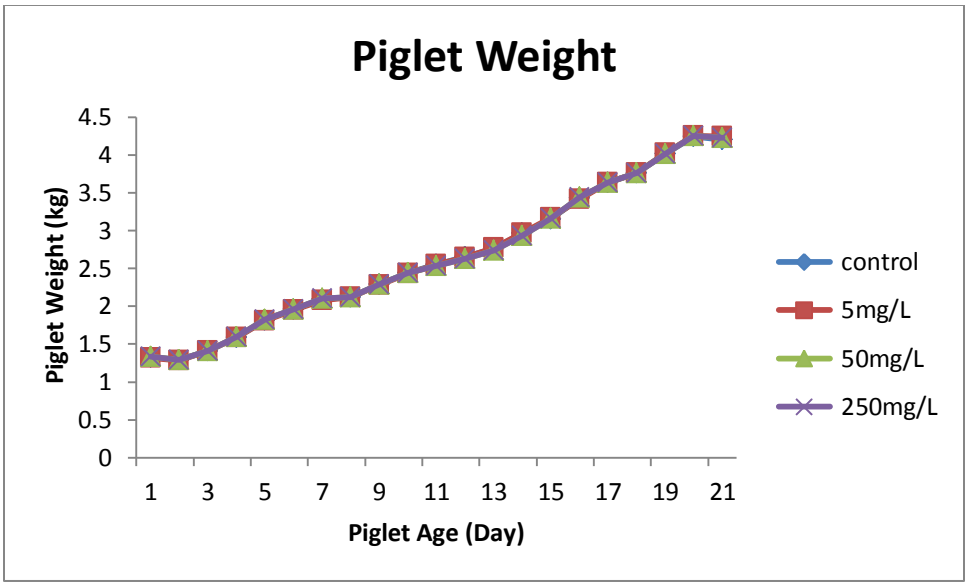


Figure B5: Piglet weight and formula intake. Data are expressed as mean ± SD. Statistical significance was set at $p \leq 0.05$. The increase in body weight during the 7 or 21 day period did not differ between groups. On day 7, each pig weighed an average of 2.3±0.5 kg. On day 21, each pig weighed an average of 4.4±0.8 kg at day 21. Formula intake and body weight were similar across all treatment groups. An average formula intake of 808±167 ml/day was consumed for the first 7 days and 1488±129 over the course of 21 days. Formula was offered 14 times daily at a rate of 360 ml/kg BW/day.



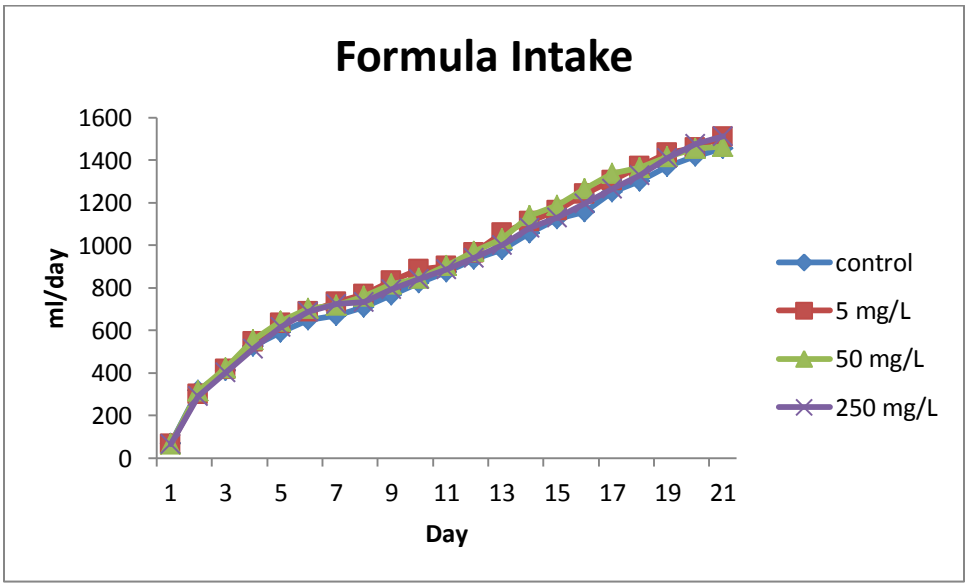


Figure B6: Expression of double positive CD45+CD3+CD4+CD8+ T cells as a percentage of CD3+ lymphocytes. Data are expressed as mean ± SD. Statistical significance was set at a p-value of $p \leq 0.05$. A) MSLN CD45+CD3+CD4+CD8+ T cells B) TLN CD45+CD3+CD4+CD8+ T cells.

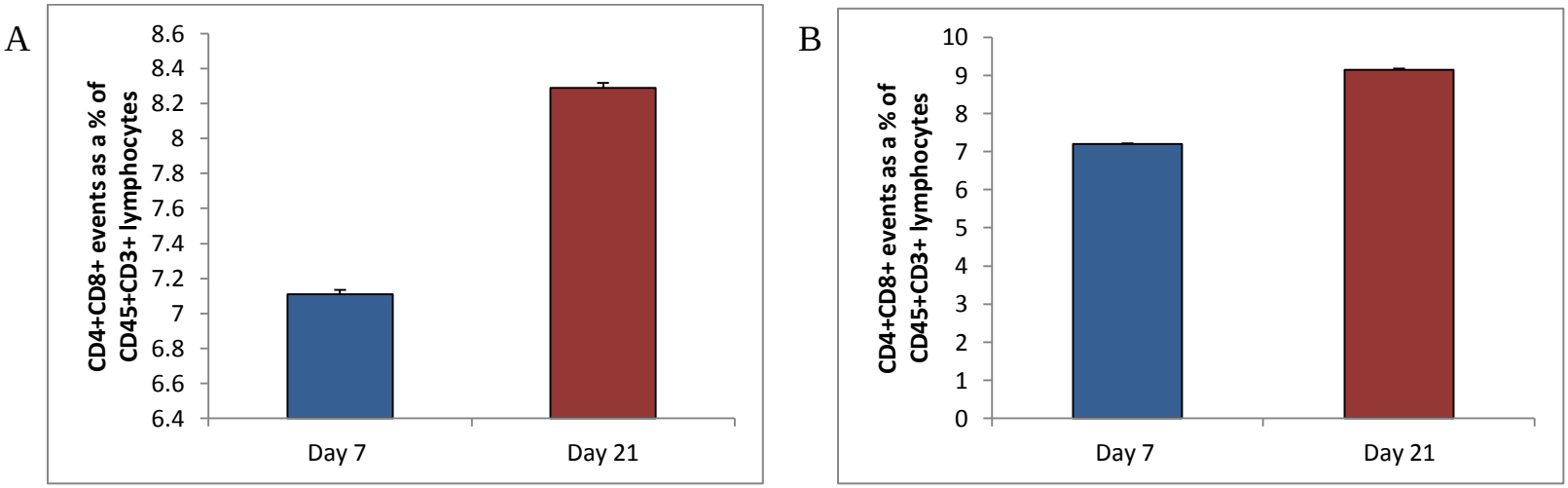


Figure B7: Influenza Specific Serum IgG. IgG was measured by ELISA. Data are expressed as mean ± SD. Statistical significance was set at $p \leq 0.05$. Vaccinated piglets had a significantly higher ($p<0.0001$) IgG titer at d14 and d21 than non-vaccinated animals ($p<0.0001$). Day 21 piglets had a greater vaccine response compared to day 14 piglets ($p<0.0001$). Vaccinated piglets had a fold increase in serum IgG of 5.9 ± 2.2 from d7 to d21 while non-vaccinated piglets had a fold increase in serum IgG of 0.49 ± 0.05 from d7 to d21. While vaccination did increase influenza-specific serum IgG production, dietary WGP did not enhance the ability of piglets to produce influenza-specific IgG.

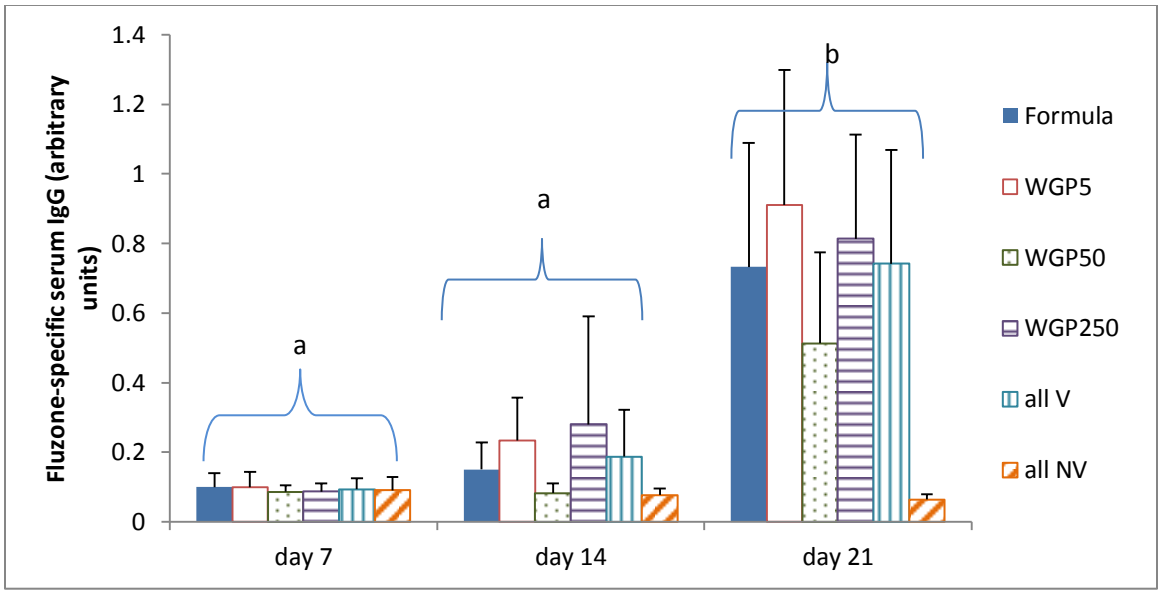


Figure B8: Histomorphology: Haematoxylin and Eosin Stained Lung Images.

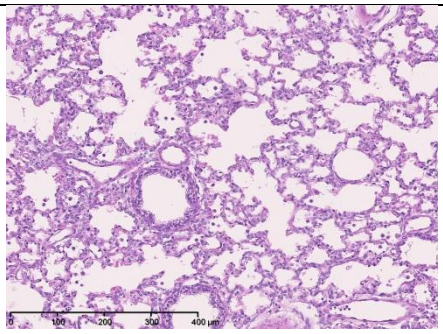
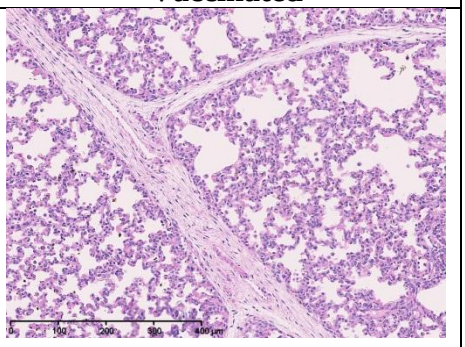
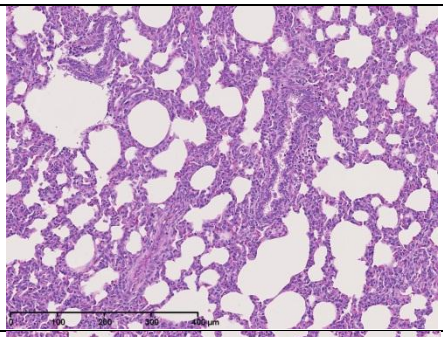
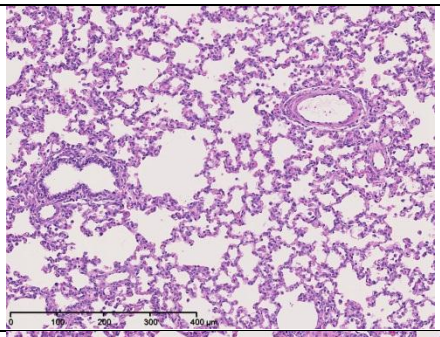
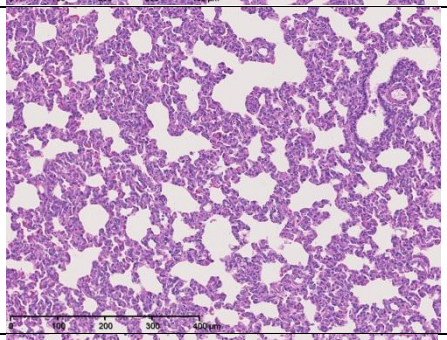
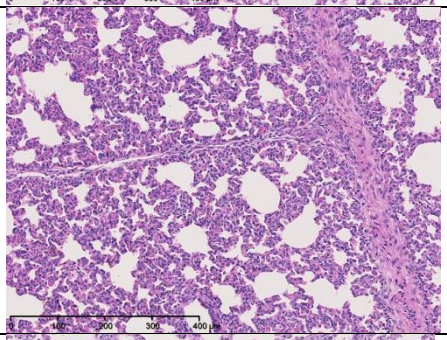
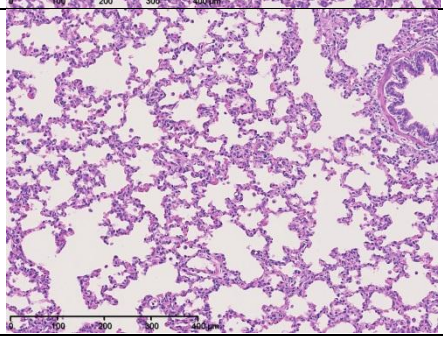
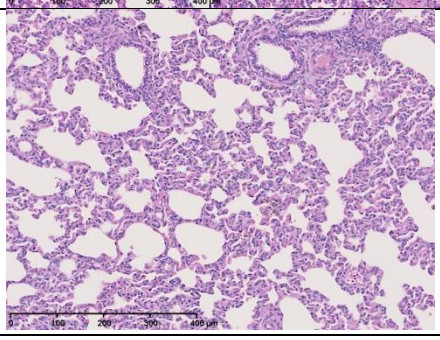
	Non-vaccinated	Vaccinated
Formula		
5mg/L		
50mg/L		
250mg/L		

Figure B9: Immunohistochemistry, CD3+ Stained Lung Images.

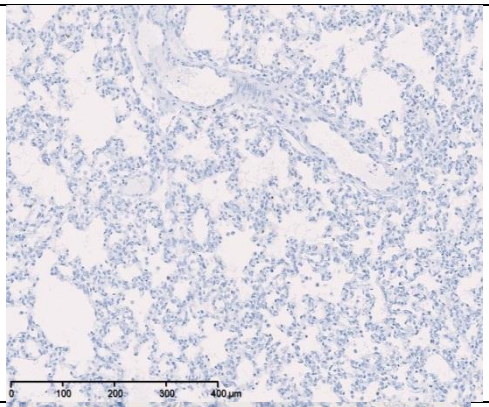
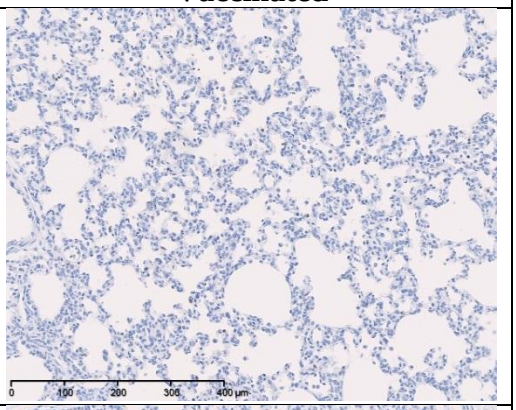
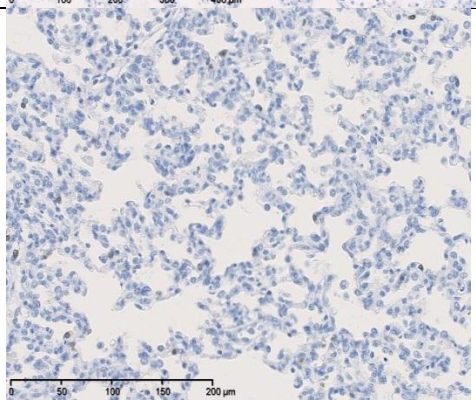
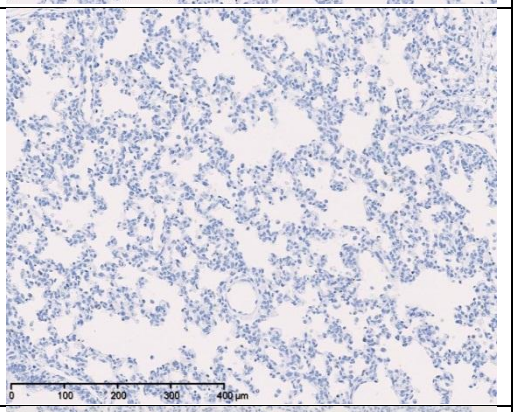
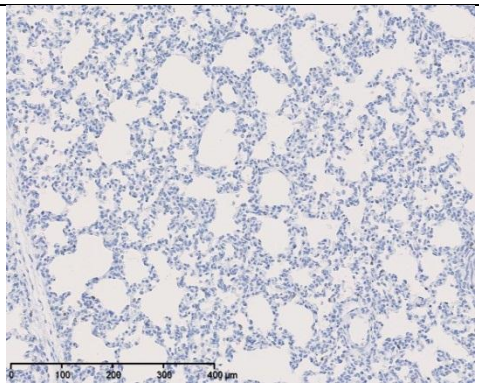
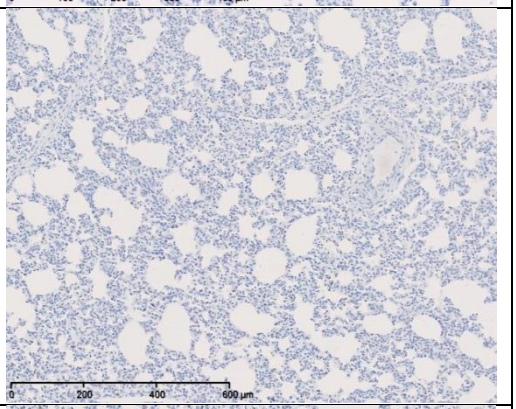
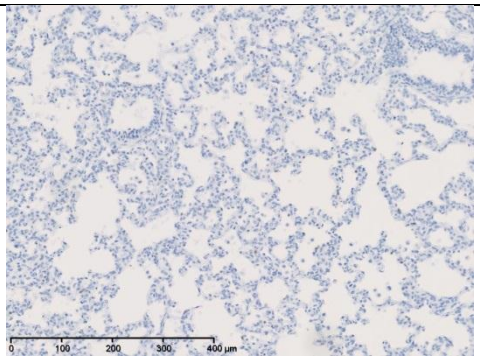
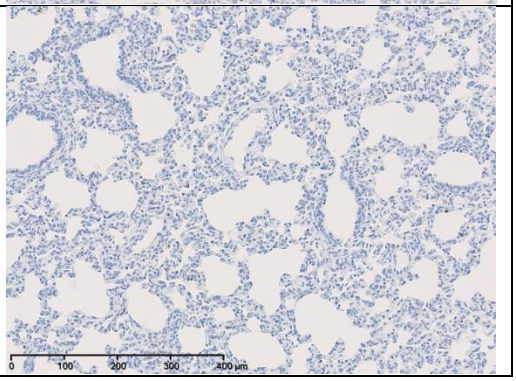
	Non-vaccinated	Vaccinated
Formula		
5mg/L		
50mg/L		
250mg/L		

Table B1: Relative abundance of mRNA expression in Lung. Data are expressed as mean ± SD of the fold difference relative to day 21, formula-fed, non-vaccinated. Statistical significance was set at $p \leq 0.05$.

	Day 7				Day 21				P-values
	Formula	WGP5	WGP50	WGP250	Formula	WGP5	WGP50	WGP250	Model
IL-1α	0.56±0.38	0.63±0.36	0.43±0.12	0.47±0.21	1.0±0.98	0.94±1.33	0.46±0.30	0.74±0.60	0.7502
Dectin	0.97±0.61	0.71±0.35	0.71±0.35	0.69±0.15	1.0±0.75	0.62±0.52	0.89±0.14	0.85±0.41	0.6488
IL-2	1.65±1.93	0.52±0.38	0.43±0.17	1.44±1.35	1.0±0.56	1.69±2.24	0.50±0.33	2.3±2.27	0.2178
TNF-α	0.89±0.36	0.97±0.36	0.63±0.45	0.92±0.63	1.0±0.48	1.38±1.81	1.34±0.9	1.62±0.70	0.7021
IL-4	0.85±0.40	0.70±0.38	0.40±0.07	0.53±0.17	1.0±1.0	0.91±0.87	0.81±0.78	0.93±0.70	0.7183
IL-12	1.41±1.62	0.92±0.54	0.34±0.17	1.34±1.25	1.0±0.64	2.07±2.31	0.85±0.56	2.23±2.05	0.1761

Table B2: Relative abundance of mRNA expression in TLN. Data are expressed as mean ± SD of the fold difference relative to day 21, formula-fed, non-vaccinated. Statistical significance was set at $p \leq 0.05$.

	Day 7				Day 21				P-values
	Formula	WGP5	WGP50	WGP250	Formula	WGP5	WGP50	WGP250	Model
IL-6	1.43±0.55	1.21±0.28	0.72±0.25	1.73±0.88	1.0±0.31	1.18±0.81	1.64±0.79	0.90±0.48	0.0786
IL-12	1.08±0.44	0.92±0.95	0.68±0.78	1.44±1.48	1.0±0.46	1.14±1.14	0.68±0.71	0.73±0.69	0.8443
TGFβ-1	1.31±0.76	1.14±0.18	0.46±0.27	0.83±0.39	1.0±0.60	1.32±1.02	1.46±1.13	0.582±0.13	0.1055
TGFβ-2	2.8±2.92	2.0±1.91	0.49±0.51	1.32±1.49	1.0±0.88	1.14±0.92	1.37±1.06	0.61±0.21	0.0739
TNF-α	2.7±2.89	0.57±0.27	0.82±0.25	0.83±0.35	1.0±0.48	1.10±1.36	1.5±1.07	0.59±0.36	0.0775

Table B3: Relative abundance of mRNA expression in MSLN. Data are expressed as mean ± SD of the fold difference relative to day 21, formula-fed, non-vaccinated. Statistical significance was set at $p \leq 0.05$.

	Day 7				Day 21				P-values
	Formula	WGP5	WGP50	WGP250	Formula	WGP5	WGP50	WGP250	Model
Dectin	1.65±0.7	1.42±1.3	0.97±0.6	1.14±0.7	1.0±0.5	1.75±1.6	1.27±0.8	2.03±1.6	0.5942
IL-12	2.83±1.8	0.97±1.3	0.26±0.1	0.32±0.3	1.0±0.8	1.03±2.2	1.03±2.2	0.15±0.07	0.0851
TNF-α	1.24±0.3	0.96±0.6	0.75±0.5	0.70±0.3	1.0±0.4	0.85±0.5	0.91±0.5	1.57±1.3	0.3444
IL-4	1.55±0.9	1.38±1.2	0.88±0.6	1.01±0.6	1.0±0.5	1.64±1.4	1.16±0.9	1.95±1.5	0.5738
IL-6	1.39±0.6	0.84±0.3	0.78±0.7	0.71±0.4	1.0±0.5	0.97±0.4	1.06±0.4	1.19±0.9	0.539
TGFβ-1	1.05±0.4	0.80±0.3	0.69±0.3	0.64±0.2	1.0±0.4	1.15±0.7	0.95±0.6	1.29±0.8	0.4093
IL-2	2.49±1.5	0.86±0.5	1.09±1.0	1.21±1.2	1.0±0.4	1.41±1.0	1.12±0.5	1.87±0.6	0.0749

Table B4: Mitogenic Cell Stimulation (log ΔCPM) of TLN cells isolated from 21-day-old animals. Data are expressed as mean ± SD. Statistical significance was set at $p \leq 0.05$. In both MSLN and TLN, cells stimulated with ConA proliferated significantly ($p < 0.05$) more than those treated with LPS, or BSA. Neither dietary WGP or vaccination enhanced proliferation were significant, therefore data from vaccinated and non-vaccinated animals and all diet groups were pooled. Data within the same row with different letters are significantly different.

	ConA	LPS	BSA
MSLN	3.4 ± 1.7^{a}	1.9 ± 1.4^{b}	1.3 ± 1.3^{b}
TLN	4.2 ± 0.85^{a}	2.0 ± 1.2^{b}	1.3 ± 1.1^{b}

Table B5: Fluzone Cell Stimulation (average non-log-transformed CPM) of TLN cells isolated from 21-day-old animals. Data were expressed are mean ± SD. Statistical significance was set at $p \leq 0.05$.

	Non-vaccinated				Vaccinated			
	Formula N=7	WGP5 N=7	WGP50 N=6	WGP250 N=6	Formula N=5	WGP5 N=6	WGP50 N=5	WGP250 N=6
Unstimulated	258.4±237.4	331.7±257.2	148.0±119.6	424.9±363.5	163.1±123.4	275.1±211.2	515.6±190.9	447.7±342.3
5 µg/mL	161.2±140.4	225.1±229.6	70.6±38.2	203.9±163.4	109.2±24.0	165.7±99.6	188.0±121.56	288.6±218.6
2.5 µg/mL	210.4±196.9	310.0±85.9	118.8±38.1	243.2±98.8	178.6±73.4	212.6±142.7	252.8±108.2	538.8±428.7
1.25 µg/mL	234.0±232.1	351.5±62.2	140.3±102.7	382.2±334.9	225.5±181.1	256.3±150.0	338±244.0	398.8±210.0
0.625 µg/mL	168.6±125.0	250.7±146.8	184.4±141.0	323.3±253.1	344.8±296.5	257.9±152.0	460.5±359.6	347.9±402.6

Curriculum Vitae

SHANNON THORUM
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EDUCATION

University of Illinois at Urbana-Champaign, Urbana, IL May 2011
- M.S. in Nutritional Sciences
- Thesis title: Impact of early nutrition on the development of lung immunity in the piglet.
- GPA-General 3.87/4.00

Brigham Young University, Provo, UT April 2008
- B.S. in Genetics and Biotechnology
- Minor in Physical Education and Coaching
- GPA-General 3.41/4.00

CERTIFICATIONS

Registered Dietitian Expected June 2011
Certified Food Safety Manager March 2010
CPR Certified March 2011

EXPERIENCE

University of Illinois at Urbana-Champaign, Urbana, IL August 2008–Present
Research Assistant
- Ran a series of animal studies to determine the effect of diet on lung immunological development of piglets
- Performed various lab experiments such as ELISA, Flow Cytometry, and PCR and analyzed data
- Communicated findings at various nutrition symposia and lectures

Missionary Training Center, Provo, UT June 2007–May 2008
Teacher
- Developed and taught lessons for young men and young women ages 19-22
- Worked with a variety of individuals in a group setting

BYU Catering, Provo, UT April 2007–August 2007
Wait Staff
- Arranged dining area
- Prepared, plated, and served customers

Lab of Dr. G. Burton, Department of Chemistry, BYU, Provo, UT
Lab Assistant August 2006–December 2006

- Performed various tests including western blot and PCR
- Assisted in formulation of buffer/media
- Presented research to a small group

University of Utah, in Association with Julio Facelli, University of Utah, Salt Lake City, UT
Research Assistant Summer 2004

- Performed literature review regarding aneurysms in the brain
- Explored various software for application to aneurysm study
- Reported weekly to the project manager about the status and results of the project

PUBLICATIONS

Abstracts

- **Cope SC**, Hester SN, Comstock SS, Monaco MH, and Donovan SM. Dietary yeast β-glucan does not improve the response to influenza vaccination in neonatal piglets. *FASEB J.* 2010 24:332.6.
- Hester SN, **Cope SC**, Comstock SS, Monaco MH, and Donovan SM. Effect of dietary yeast β-glucan on immune development in neonatal piglets. *FASEB J.* 2010 24.925.5.

Manuscripts

- **Thorum SC**, Hester SN, Comstock SS, Monaco MH, Pence BD, Wood JA, and Donovan SM. Dietary Yeast β-glucan minimally impacts development of lung immunity in the neonatal piglet. In preparation
- **Thorum SC**, Comstock SS, Hester SN, Shunk JM, Monaco MH, and Donovan SM. Impact of diet on development of lung immunity in the neonatal piglet. In preparation

AWARDS

Margin of Excellence Travel Award, 2010
University of Illinois, Urbana-Champaign, IL, Nutritional Sciences

SKILLS

- Technical writing
- Proficient in Microsoft Word, PowerPoint, and Excel
- Able to type 80+ words per minute
- Familiar with nutrition analysis programs such as Nutritionist Pro
- Skillful in Java and SAS