

EFFECT OF SUPPLEMENTING AN OMEGA-3 FATTY ACID-CONTAINING PRODUCT
TO PIG DIETS ON GROWTH AND IMMUNE RESPONSES OF WEANLING PIGS

By

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To all the people who ever helped me

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The objective of this study was to examine the effect of feeding an *n*-3 PUFA-enriched diet on growth and immune response of weanling piglets. Newly weaned pigs (averaging 27 ± 2 days of age and 8.1 ± 0.7 kg of body weight) were assigned randomly to receive a control (3% vegetable oil, $n = 20$) or *n*-3 PUFA-supplemented (3% Omega, $n = 20$) diet for 28 days post-weaning.

A diet \times gender \times week interaction was detected ($P < 0.04$) for body weight. Female pigs consuming the *n*-3 PUFA-enriched diet were lighter ($P < 0.01$) at week 4 post-weaning than their counterparts. Newly weaned pigs gained more weight ($P < 0.01$), consumed more feed ($P < 0.01$) and had better feed efficiency ($P < 0.01$) between day 14 and 28 post-weaning. In piglets consuming the vegetable oil-enriched diet, plasma TNF- α concentration increased ($P < 0.04$) from 33.7 ± 16.9 pg/mL to 92.7 ± 14.1 pg/mL between days 0 and 14 post-weaning and remained elevated through out the remaining weeks of the study. The weaning-induced increase in serum TNF- α was completely abolished by adding *n*-3 PUFA to the piglet's diet. Fecal consistency scores

improved ($P < 0.01$) with increasing weeks after weaning, but were similar between the two dietary treatments.

Results showed female pigs consuming the *n*-3 PUFA-supplemented diet were lighter at week 4 post-weaning compared with control group. Dietary *n*-3 PUFA may improve the immune status of weanling pigs, as reflected by considerably lower plasma TNF- α in pigs consuming *n*-3 PUFA.

CHAPTER 1 INTRODUCTION

The original efforts to use early weaning and segregation technologies to obtain pathogen-free piglets were attempted in the early 1980s (Alexander *et al.*, 1980). The early weaning technique has since been developed and widely utilized in the United States swine industry (Fangman and Tubbs, 1997). This strategy improves the productivity of the sow herd, breaks the cycle of disease transmission from the facilities, but exasperates the weanling challenges of piglets (Corl *et al.*, 2008). Nutritional, environmental and immune challenges associated with weaning may lead to substantial economic losses to pork producers. This period is generally characterized by decreased voluntary feed intake, altered gut integrity and increased production of inflammatory cytokines (Le Dividich and Seve, 2000; Pie *et al.*, 2004; Montagne *et al.*, 2007). These nutritional and physiological abnormalities often result in diarrhea and depression of growth in newly weaned piglets. Restrictions of antibiotic usage in swine have compelled the industry to find alternatives that offer both performance enhancement and protection from disease (Cromwell, 2002; Vondruskova *et al.*, 2010). In this regard, Liu *et al.* (2003) reported that dietary fish oil reduced the release of pro-inflammatory cytokines in weaned pigs challenged with *E. coli* lipopolysaccharide, and a more recent study indicated that prenatal exposure to long-chain *n*-3 PUFA increased postnatal glucose absorption in piglets (Gabler *et al.*, 2009). Although exact mechanisms by which dietary *n*-3 PUFA modulate immune and metabolic functions in pigs are yet to be fully elucidated, the above study would indicate that dietary *n*-3 PUFA may help the piglets adapt quickly to the rapid change of diet at weaning.

Currently, there is very little information regarding the use of *n*-3 PUFA in the diets of pigs raised under minimal disease and stress conditions. To test the hypothesis that nutritional management strategies that attenuate intestinal inflammation may provide a physiological milieu that is conducive to optimal growth and lean tissue accretion, this study was designed to examine the effects of dietary *n*-3 PUFA on growth and immune response of weanling pigs raised without an added bacterial or environmental challenge.

Nutrition and Health

Sow Nutrition

Maintaining a good physical condition and achieving a high productive efficiency are always key factors of profitability for sow feeding and management. A good health status may prevent disease outbreak and extend the breeding age of a sow, which may have a shorter period of post-weaning anestrus and farrow a litter of heavier piglets. Besides the genotype, management and facilities, sow nutrition is one of the prerequisites that determine good health and productive efficiency. Gestation and lactation are two critical phases during the reproductive cycle of the sow and thus, optimal nutrition is needed to meet the requirements of macro- and micronutrients.

During pregnancy, energy is partitioned between maintenance, fetal growth and body tissue accretion. The priority is given to maintenance, fetal development and mammary gland development. If nutrients are below these requirements, body reserves will be mobilized. Conversely, if nutrient intake exceeds these requirements, the excess will contribute to the sow's body reserves (van Milgen *et al.*, 2008). Energy requirements for pregnancy vary according to BW, housing conditions, and the amount of maternal gain and lactation BW loss desired by the producer (Noblet *et al.*, 1990).

The National Research Council (NRC, 2012) suggested that the daily maintenance energy requirement was 105 kcal ME/kg metabolic body size ($BW^{0.75}$), but the factorial approach is used to precisely determine feeding requirements of individual sows. As fetal growth and mammary development occur mainly in late gestation, the 2-phase feeding strategy may be more appropriate than the constant diet single-phase feeding (Ji *et al.*, 2005; Mcpherson *et al.*, 2004). In addition, insufficient energy intake may allow successful lactation but will increase the weight loss of the sow at weaning and delay post-weaning estrus (Trottier and Johnston, 2001). Excess feed intake during gestation increases the growth of the fetus and the deposition of body fat and protein. But it reduces energy intake and increases weight loss during lactation. So, when increasing feed intake during the last 30 days to support the rapid fetal growth, it is still necessary to limit energy intake and avoid excess weight gain by the sow for the entire gestation (NRC, 2012).

During lactation, energy is partitioned between maintenance, milk production and lipid and protein mobilization from maternal body reserves (van Milgen *et al.*, 2008). The lactating sow requires 5-10% greater energy than during pregnancy (NRC 2012) and increased amounts of nutrients for milk production which is estimated to be at least 78% of the net energy (NE) required for the modern prolific sow (Boyd and Kensingen, 1995). The sow's feed intake directly affects the growth and development of the neonatal piglets via the milk produced, and also influences the reproductive efficiency of the sow. The energy needed daily is the sum of energy for maintenance and energy for lactation, and the energy amount associated with milk production can be calculated as following:

$$\text{Milk Energy (GE, kcal/day)} = (4.92 \times \text{ADG}) - (90 \times \text{LS}) \quad (1-1)$$

where GE means gross energy, ADG = average daily gain of litter (g), and LS = number of pigs per litter (NRC, 2012). Improvements in energy intake may be achieved by supplementing diets with fat, which may improve litter weight gain and subsequent reproductive performance of the sows (Rosero *et al.*, 2011). In addition, increasing the level of soluble fiber in the diet improves energy digestibility of the sow (Renteria-Flores *et al.*, 2007).

Dietary amino acid requirements of the pregnant sow vary depending on the rate of protein accretion and are influenced by their requirements for maintenance and protein deposition in maternal and fetal tissues. The factorial approach is useful to define amino acid requirements of sows (Trottier and Guan, 2000). Sows fed extremely low protein levels during gestation could farrow a litter with normal size and weight, but it had a dramatic negative effect on milk production during the subsequent lactation and on the return to estrus. During gestation, sows fed a high dietary protein level gained more lean tissue and less fat than those fed lower-protein diets (Pettigrew and Yang, 1997). Also, prolific sows nursing large litters need high levels of dietary protein to maximize milk production, prevent excessive BW loss during even a relatively short lactation period, and prevent delayed return to estrus following weaning of litters (Cromwell, 2009). Consequently, high protein diets are recommended for lactating sows.

Lysine is considered to be the first limiting amino acid for establishing an ideal amino acid balance for both gestation and lactation. The daily requirement for lysine is the sum of the requirements for maintenance and protein accretion during gestation, or

the sum of the requirements for maintenance, protein accretion and milk production during lactation. The daily maintenance requirement for true ileal digestible lysine is considered to be $34.79 \text{ mg/kg BW}^{0.75}$ of gestation sow and $46.26 \text{ mg/kg BW}^{0.75}$ for lactating sow (NRC, 2012). The requirement for protein accretion is thought to be 0.129 g of true ileal digestible lysine above maintenance per g of accreted protein. The requirement for milk production is suggested to be 22 g of apparent ileal digestible lysine/kg of litter weight gain (NRC, 2012). The lysine requirement seems to be greater during late than early gestation and the lysine requirement of heavy sows is less than that of light sows at a similar ME intake (Pettigrew and Yang, 1997).

Requirements for the essential amino acids other than lysine are also considered to consist of separate components for maintenance and protein deposition. Calculations are based on the ideal protein system in which requirements for each of the other amino acids throughout gestation result in overfeeding amino acids in early gestation and underfeeding amino acids in late gestation. So, phase feeding sows in gestation will more closely meet the demands for nutrients (Levesque *et al.*, 2011). The relative ideal ratios for Lys : Thr : Val : Leu are 100:79:65:88 for day 0 to 60 of gestation, and changed to 100:71:66:95 from day 60 to day 114 of gestation (Kim *et al.*, 2009). Dietary supplementation with 1% Glutamine between d 90 and 114 of gestation improves fetal growth in gilts, reduces preweaning mortality of piglets and enhances milk production by lactating sows (Wu *et al.*, 2010).

Sows vary greatly in their mineral and vitamin requirements, which largely reflect the nutritional demands during different phases of their reproductive cycle. Minerals and vitamins are needed for conceptus formation, mammary secretion and growth and

maintenance, and therefore, the sow's mineral and vitamin requirements are higher during late gestation (Mahan, 1990). Feeding sows organic trace minerals may improve their reproductive performance (Peters and Mahan, 2008). Dietary addition of excess levels of vitamins A and D has been demonstrated to have toxic effects in swine. In contrast, very few toxicity signs have been reported for the vitamin E, K and vitamin B group. Massive riboflavin supplementation of the sow's diet during early pregnancy may increase the number of farrowings, but does not increase the litter size (Pettigrew *et al.*, 1996).

Neonatal Piglet Nutrition

Body condition and health status influence the survival rate of piglets, and determine the growth potential and carcass characteristics of growing-finishing pigs (Gondret *et al.*, 2005). Piglets heavier at birth have higher post-natal survival rates than lighter piglets, and keep their weight superiority after weaning (Smith *et al.*, 2007). The growth of low-birth weight piglets can be enhanced by feeding a high protein diet but the provision of a high-protein diet that exceeds the protein requirement does not further increase protein synthesis or the expression of translation initiation factor activation (Frank *et al.*, 2005). So, the appropriate feeding and management of neonatal piglets are critical to maintaining efficient weight gain and good health condition. Also, heavier and healthier piglets tend to have stronger immune system that protects them from pathogens and prevents disease outbreaks. Less disease and fast growth rate will allow the growing-finishing pig to reach the market weight rapidly.

Piglets are usually born indoors and the temperature is around 15-30°C. They are poorly insulated because of sparse hair covering and a lack of subcutaneous fat, thus, they need to increase their heat production to avoid hypothermia. Shivering

thermogenesis predominates as there is little or no brown adipose tissue in new-born piglets. Before suckling colostrum, the body glycogen and protein reserve is the only energy source for heat production (Noblet and Le Dividich, 1981). A new born piglet must get energy to meet its maintenance requirements, including thermoregulation, physical activity and growth, and the maintenance requirements could be as high as 275 KJ/kg BW, it has been estimated that the extra energy required for thermoregulation averages 2 KJ/kg BW/h per degree centigrade below the lowest critical temperature. Thus, the energy and nutrient requirements of piglets are maximal at birth, which is estimated at 2.6 times higher than those at weaning (Le Dividich *et al.*, 1994). Standing costs 9.5 KJ/kg BW/h and the minimal energy needed for physical activity is about 105 KJ/kg BW during the first 24 h following birth (Le Dividich *et al.*, 1994). The average energy associated with BW gain at the first day of birth is 70 ± 67 g/kg BW (Le Dividich *et al.*, 2005). Under thermoneutral conditions, the lowest net energy required for survival of a 1.0 kg piglet is approximately 700 KJ during the first 24 h after birth, and could be as high as 900-950 KJ in a moderately cold surrounding. Based on recent studies (NRC, 2012), the total lysine requirements for neonatal piglets are estimated to be 1.10%, 1.10%, 1.35% and 1.30% for a 5, 10, 15 and 20 kg piglet. The other amino acids are calculated from lysine using the ratios established for maintenance and protein accretion on a true ileal digestible basis (NRC, 2012).

The primary source of energy for new born piglets is their body reserve. This energy is derived from protein, glycogen and fat oxidation. As body protein catabolism occurs at a very low rate at birth, glycogen reserves, estimated at 30 to 38 g/kg BW at birth, are depleted rapidly. Under normal environmental conditions, 75% of liver

glycogen and 41% of muscle glycogen are utilized by 12 hours postpartum (Elliot and Lodge, 1977). However, available energy derived from glycogen is still as low as 100.38 Kcal/kg BW. Because a large proportion of fat is structural fat and not available for mobilization, energy produced from these substrates does not meet the energy requirement for maintenance and physical activity during the first day of life (Mellor and Cockburn, 1986).

As first suckling occurs 10 to 30 min after birth, colostrum provides energy and maternal antibodies for piglet survival. Colostrum is the first 24-30 h secretion of the mammary gland. It is very digestible and both colostral energy and nitrogen (N) are retained with a very high efficiency. Compared with milk, colostrum has higher concentrations of dry matter and crude protein and lower concentrations of fat and lactose. The protein concentrations vary widely because of the variation in immunoglobulin concentration. Fats are the main source of energy, accounting for 40 to 60% of the total energy supplied by colostrum, and the content and the composition of fat are both largely dependent on the fat composition of the diet fed to the sow during late gestation. In contrast, lactose concentration of colostrum and milk is only marginally related to the diet of the sow. Amino acid composition of colostrum is relatively constant, but more threonine is contained in colostrum than milk. Colostrum contains less minerals than milk.

Colostrum and milk contain a variety of growth factors and hormones, including IGF-I, EGF, TGF- β and leptin (Estienne *et al.*, 2000). Both colostrum and milk are used remarkably well by piglets with ratios of ME to GE from 0.93 to 0.98, and a ratio of N retained to N intake between 0.88 and 0.91 (Le Dividich *et al.*, 1999). The true

digestibility of amino acids is about 92% and is lower than their apparent digestibilities (Mavromichalis *et al.*, 2001). The efficiency of ME utilization for total energy and for energy retained as protein are higher for colostrum than for milk (0.91:0.72; retention 0.91:0.56), and colostrum stimulates more muscle protein synthesis compared with mature milk. Because piglets have low immunity at birth, the most important function of colostrum is to provide immunoglobulins that transfer the passive immunity to the neonatal piglets. Colostrum contains more IgG than mature milk. Because gastric and pancreatic proteolytic enzymes do not digest immunoglobulins, these molecules are absorbed directly into the blood stream to build the piglet's immunity (Le Dividich *et al.*, 2005). Colostrum also plays an important role in the development of the gastrointestinal tract of piglets (Xu and Wang, 1996). The minimal enteral nutrient intake necessary to sustain normal mucosal proliferation and growth is 60% of the total nutrient intake (Burrin *et al.*, 2007).

As a high-quality colostrum results in greater growth rate of piglets, early and increased intake of good quality of colostrum is very important. The production and composition of colostrum and milk are influenced by the parity and genotype. The endocrine status of the sow also affects the process of colostrogenesis and changes in the sow's endocrine status can have an impact on the quantity and quality of colostrum produced. Nutrition is undoubtedly a major factor that could be used as a tool to alter colostrum and milk composition with fat content being the most affected by the diet (Farmer and Quesnel, 2008). In order to increase the production of colostrum and milk, the prenatal stress of the sow must be reduced before, during and after farrowing. The

appropriate diet should be fed and one must ensure that the sow has unrestricted access to fresh drinking water at all times.

Colostrum and milk consumption by individual piglets varies considerably. It is independent of birth order, but related positively to birth weight (increased by 26-37 g per 100 g increased in BW). Colostrum and milk consumption is negatively related to litter size. During the first postnatal day, ingestion of 160-170 g colostrum/kg BW is required to ensure survival of piglets (Le Dividich *et al.*, 2004), whereas 280 g/kg BW is recommended (Le Dividich *et al.*, 1994).

Milk arginine is usually insufficient for supporting the maximal growth of young pigs, and oral administration of N-carboamoylglutamate will enhance plasma arginine level and weight gain (Wu *et al.*, 2004). Piglets develop an iron deficiency anemia during the first weeks of life, which will lead to slow-growth and weakness. Because 21 mg Fe/kg BW are needed to maintain Fe status (Braude and Newport, 1977), neonatal pigs are usually injected with a chelated form of Fe within 3 d of age to prevent anemia (Kegley *et al.*, 2002).

Weanling piglet nutrition

Natural weaning occurs at 70 days or so of age without human intervention. The improvement of feeding and management technologies for the young pig has not only provided the opportunity for improvement of the productivity of the sow herd, but has also provided a means of breaking the cycle of disease transmission from the facilities. Although it is possible to successfully wean pigs at 14 to 21 days of age, it is more challenging to keep these young, newly weaned pigs healthy and growing than pigs weaned at older ages. Weaning can often be a time associated with a lag in performance that includes depressed gain and feed intake and increased disease

occurrence and mortality. Unless managed properly, this post-weaning lag will be more magnified in pigs weaned at younger ages, because their gastrointestinal tracts have not been fully developed to digest and absorb solid feeds. The competence of the digestive system of the suckled pig to handle a non-milk diet begins to develop between 14 and 28 days of age. Under conditions of gradual diet change from liquid to solid feed, growth is likely to be fully supportable, so feeding of a mixture of milk and externally sourced solid feed to the gut of the young pig is relevant to natural development.

Besides the diet change, piglets are exposed to a series of stressors that can lead to poor performance and increased mortality. Reduction of feed intake results in proportionate decline in metabolic body heat generation, making the pig more susceptible to chilling at the time of weaning. Gastrointestinal maladies are the most severe diseases because the requirements of the intestine are dramatically increased at weaning. Also the intestine serves as the first line of defense against both viral and bacterial pathogens that are ubiquitous in the postnatal environment (Corl *et al.*, 2008).

In addition to the stresses of nutrition and disease, the impact of a sub-optimal environment and disturbances to the previous social order must not be underestimated. The Prairie Swine Centre have demonstrated that about 30% of the variation in age to market can be attributed to the time it takes a pig to reach a body weight of 23 kg. So, it is very critical to understand and meet the nutritional requirements of weaned pigs.

The suckling pig partitions nutrients toward lipid deposition to reach 150-160 g per kg of BW at the time of weaning, and the ratio of lipid to protein in the body is around 1:1 at weaning. Post-weaning feed intake inadequacies, together with stress and disease challenges, cause a rapid loss of body lipid in support of maintenance and

protein synthesis. So, adequate water must be provided ad libitum. The young pig has a high potential for growth (Hodge, 1974). Among numerous factors which influence the extent to which this potential is expressed, energy intake is the most important. The energy requirements of the weaned pig include maintenance (MEm), protein and fat deposition (NRC, 2012). Metabolizable energy required to maintain a weaned pig is the same as that required to maintain the suckling pig ($112.33 \text{ kcal ME/kg BW}^{0.75}$ per day), with the energy for activity accounting for 15-30% of the total MEm (Halter *et al.*, 1980). Heat production (HP) increases to meet the energy requirement for thermoregulation when piglets are in an environment where temperature is below the lower critical temperature (LCT). The extra ME requirement for thermoregulation is about $3.8-4.3 \text{ kcal/kg BW}^{0.75}$ per $^{\circ}\text{C}$ at the time of weaning (Le Dividich *et al.*, 1998). Although energy intake is the first limiting factor, the choice of dietary ingredients for a weaned pig must fit its digestive capacity, maintain gut health and promote feed intake. Once adaptation to digestion of solid feed is complete, the piglet can be considered a growing pig with regard to the net energy system (NRC, 2012). The ability of the young pig to use diets of variable energy concentration is more closely related to its tolerance of the fiber used for the dilution of energy, and of the fat used to provide energy. Fat and fiber are known to decrease the rate of passage of the digesta in the upper part of the digestive tract, particularly at the stomach level, and, thus, cause a decrease in total energy intake (McConnell *et al.*, 1982). Therefore, it is important to add an appropriate ratio of fat while reducing the fiber quantity in the diet. Shields (2009) suggested that glycerol is a potential valuable and economical energy source for swine diets.

The protein requirement of the weaner pig has to be considered differently because raw materials used in diets designed for this age supply not only N and essential amino acids but also Immunoglobulins. In order to meet the amino acid requirements of the weaner pig, a two-stage approach is used to achieve the ideal protein level. Early additional protein supply is necessary to achieve the growth potential of the pig (Sèvre and Ballèvre, 1991), and the first-limiting amino acid requirements vary with genotype, sex and liveweight. van Lunen and Cole (1998) suggested that the requirement for hybrid pigs could be 1.2 g lysine/MJ, which is higher than the value of Agricultural Research Council (ARC, 1981). Lysine, methionine and threonine are commonly used as supplements in weaner diets to achieve the ideal balance of amino acid. Protein sources commonly used in weaner feeds include milk proteins, crystalline amino acids, vegetable proteins and animal protein sources. Porcine plasma would appear to be the beneficial protein supplement in simple diets containing raw cereals and soybean products (Cole and Sprent, 2001).

Vitamin B supplementation is necessary to maximize growth performance of early weaned pigs (James *et al.*, 2002), and it appears that adding a dietary supplement of B₆ by 50 mg/kg saturates the B₆ in red blood cell pool of weaned pigs. Additionally, vitamin B₆, and along with vitamin B₂, influences the glucose homeostasis through the entero-insular axis (Matte *et al.*, 2005).

Because weaned pigs are susceptible to vitamin E deficiency, feeding a high level of vitamin E has been suggested to improve the immune response of weanling pigs (Bonnette *et al.*, 1990). Carlson (1995) suggested that both early- and traditionally weaned pigs need to be fed pharmacological concentrations of Zn provided as ZnO for

a minimum of 2 wk immediately after weaning to enhance growth. Addition of 250 ppm Cu to the diet stimulates intestinal lipase and phospholipase A activities, leading to an improvement of dietary fat digestibility in weanling pigs (Luo and Dove, 1996).

Physiological Changes at Weaning

Rapid increases in intestinal dimensions of piglets occur during the early postnatal period, particularly the first 6 h of suckling (Zhang *et al.*, 1997). The onset of suckling stimulates a rapid growth of the neonatal intestine that is supported by a high rate of protein synthesis (Burrin *et al.*, 1992). The ingestion of colostrum promotes the maturation of the developing intestinal epithelium (Burrin *et al.*, 1992) and stimulates crypt cell proliferation (Zhang *et al.*, 1997). The non-nutritive colostral factors that elicit faster intestinal growth include both immunoglobulins and biologically active substances such as insulin-like growth factor-I (IGF-I) (Xu and Wang, 1996) that can enhance macromolecular absorption and increase circulating IGF-I levels in newborn pigs (Wester *et al.*, 1998). The enterocytes of the small intestine of newborn piglets possess complex apical endosomal systems comprising tubular endosomes and associated vesicles that are involved in the extensive uptake of maternal immunoglobulins and other colostral constituents (Murata and Namioka, 1977). An age-related decrease in ion and nutrient fluxes takes place in the immediate postnatal period (Sangild *et al.*, 1993), but the total nutrient transport capacity increases with age due to a large increase in intestinal mass (Pacha, 2000). Much of the decline in carrier-mediated nutrient absorption during the first 24 h of suckling is caused not by loss of transporters but instead by the rapid increase in the tissue mass that effectively ‘dilutes’ the transporters (Buddington *et al.*, 2001). The decline in carrier-mediated amino acid and glucose transport also coincides with the postnatal replacement of the fetal enterocytes,

leading to a redistribution of transport functions along the crypt villus axis (Buddington *et al.*, 2001). The detection of intestinal glucosidase (sucrase, maltase) in the suckling pig, albeit at low activities, suggests that pigs have a limited capacity to process carbohydrates other than lactose (Zhang *et al.*, 1997). The microbial colonization of the pig gut by non-pathogenic microorganisms greatly modifies the gut structure and function and their stimulation of the intestinal immune system results in a constitutive, low-level inflammation and epithelial changes that can have both negative and positive effects on nutrient and energy absorption in the young (Gaskins, 1997). Microbial colonization is a complex process of natural selection and ecological succession (Rolle, 1996) and is influenced by numerous regulatory factors of both bacterial and host origin, including bacterial antagonisms, animal genotype and physiology and, importantly, nutrition (Conway, 1999). The colonization pattern of the suckling pig is similar for most animals, with lactic acid bacteria, enterobacteria and streptococci appearing first, followed by obligate anaerobes (Conway, 1999). Piglets are immunodeficient at birth and are only able to generate limited T and B cell responses when challenged with pathogens, so they are highly dependent upon a supply of both specific and non-specific immune factors present in maternal colostrum and milk for passive immunity for protection, development and survival (Stokes *et al.*, 2004).

Weaning is a critical period in the pig's life. The pig must cope with separation from the sow, the transition from highly digestible milk to a less digestible and more complex solid feed, separation from littermates, and exposure to unfamiliar pigs. These stressors cause the reduction of growth during the first week and are associated with changes in the histology and biochemistry of the small intestine such as villous atrophy

and crypt hyperplasia. These changes result in decreased digestive and absorptive capacities and contribute to post -weaning diarrhea (Pluske *et al.*, 1997). Hampson (1986) reported that following weaning at 21 days of age, the villous height was reduced to around 75% of pre-weaning values within 24 hours (940 to 694 μm). Subsequent reductions in villous height were smaller but continued to occur until the fifth day after weaning, at which point the villous height at most sites along the gut was approximately 50% of initial values found at weaning. Five to eight days after weaning villous height began to increase. Crypt elongation occurs after two days post-weaning because the number of cells in the crypt increases steadily from the third to eleventh day after weaning (Hampson, 1986). As a result of these changes in villous height and crypt depth, the villous height : crypt depth ratio in weaned pigs is markedly reduced and is manifested in a change from the longer, finer-like villi seen in newborn and sucking pigs to wider leaf-like or tongue-like villi (Pluske *et al.*, 1997). The pre- and post-weaning periods are characterized by major developmental and diet-induced changes in the expression of intestinal brush-border enzymes. The specific activity (activity per unit mass of tissue) of lactase which hydrolyses milk lactose, reaches a peak in 3-week-old pigs but declines rapidly over the weaning period. Also, sucrase and isomaltase activities fall by at least 50% by five days and recover by 11 days after weaning (Miller *et al.*, 1986). Thus, the intestines of weaned pigs have high sucrase and maltase activities and are characterized by correspondingly high rates of glucose and fructose transport across the apical membranes of enterocytes (Puchal and Buddington, 1992). Carrier-mediated amino acid absorption declines significantly in the post-weaned pig intestine. This decline is consistent with a shift to an adult diet that is lower in protein

(Buddington *et al.*, 2001). The exception is the post-weaning increase in the rate of carrier-mediated absorption of lysine, the principal amino acid limiting the performance of early weaned pigs (Thacker, 1999). After weaning, the net absorption of fluid and electrolytes in the small intestine of pigs is temporarily decreased (Nabuurs *et al.*, 1994). The primary factor for the compromised villus to crypt ratio and the intestinal function at weaning is inadequate feed intake during the post-weaning period (Hampson *et al.*, 1986). A compromised mucosal barrier caused by acute fasting may allow the passage of nutritional or bacterial antigens into the lamina propria where activation of immune responses can occur. When feed intake patterns return to normal, intestinal inflammation subsides and epithelial morphology improves in the young pig (McCracken *et al.*, 1999).

During the postnatal development, alterations in the diet are believed to induce a succession of related changes in the gut microbial ecosystem (Conway, 1999). In the immediate post-weaning period, the balance between the development of healthy commensal microbiota and the establishment of a bacterial intestinal disease can be easily tipped towards disease expression (Hopwood and Hampson, 2003). The microbial colonization changes more in the ileum than in cecum or colon. The obligate anaerobic bacteria become numerically dominant, particularly in the hindgut of the pig, and high levels of such bacteria are also found in the ileum (Conway, 1997). Enterococci and Enterobacteriaceae dramatically decrease from the fifth to the eleventh day post-weaning. Yeast counts decrease five days post-weaning but recover by the eleventh day. The number of lactobacilli decreases on the first day post-weaning and recovers within five days to initial levels (Pieper *et al.*, 2006). These changes may

predispose the weaner pig to malabsorption, dehydration, diarrhea and enteric infections (McCracken *et al.*, 1999) because of the significant increase of potentially pathogenic coliforms such as Enterotoxigenic *E. Coli* (ETEC) and rotaviruses. ETEC produce heat-labile enterotoxins or heat-stable enterotoxins that cause the inflammation and diarrhea in weanling pigs (Nakazawa *et al.*, 1987). Intestinal ischaemia is a key predisposing factor to post-weaning diarrhea in weaned pigs, and it has been suggested that it may lead to intestinal acidosis and increase permeability to ETEC toxins (Nabuurs *et al.*, 1994).

Weaning is associated with changes in the regulation of the lymphoid cells in the mucosal immune system, and pro- and anti-inflammatory cytokines are increased due to intestinal inflammation (Stokes *et al.*, 2004). The newborn piglet has very few lymphocytes in its intestinal epithelium or lamina propria. Clusters of lymphocytes are present in the mucosa, in the areas that will subsequently develop into Peyer's patches (Pabst, 1988). In the first two weeks of life, the intestine rapidly becomes colonized with lymphoid cells. These cells express the CD2⁺ surface marker but do not co-express CD4⁺ or CD8⁺. The Peyer's patches begin to get organized during this period, reaching a relatively "adult architecture" by 10 to 15 days after birth (Stokes *et al.*, 2004). In piglets 2-4 weeks old the intestinal mucosa becomes colonized by CD4⁺ T cells, primarily in the lamina propria. CD8⁺ cells are still largely absent. Small numbers of B cells appear, preferentially expressing IgM. Early weaning at 3 weeks of age is associated with a transient reduction in the ability of intraepithelial lymphocytes to respond to mitogens and splenic T cells to secrete IL-2 (Bailey *et al.*, 2005), and 4 days after weaning at 3 weeks of age, there are increases in CD2⁺ and granulocyte cells in

proximal small intestinal villi (Vega-Lopez *et al.*, 1994). Thus, the young piglet is capable of active immune responses to live viruses and to dietary components by 3 weeks of age, but the mucosal immune system remains relatively immature throughout the weaning period (Stokes *et al.*, 2004). Also, weaning is associated with a transient increase of pro-inflammatory cytokines in the small intestine and the colon, including TNF- α , IL-1 β and IL-6. Increased mRNA expression of these cytokines are detected in the spleen, thymus, tonsil and mesenteric lymph nodes (Stokes *et al.*, 2004). These changes could reflect an inflammatory reaction occurring in the lymphatic organs and immune system in response to a pathogenic challenge and reduced feed intake.

Nutritional, psychological and environmental stressors associated with weaning contribute to the subsequent reduction in growth rate, and the nutritional status has a strong influence on the expression and secretion of a variety of growth-related hormones, including growth hormone (GH), insulin-like growth factor-I (IGF-I) and thyroid hormones. Serum concentration of GH sharply declines during the first 4 days of postnatal development, serum IGF-I concentrations are inversely correlated with levels of GH but positively correlated with average daily gain (Carroll *et al.*, 1998), while weaning does not affect IGF-I, IGF-2 or GH receptor mRNA levels in the liver (Matteri *et al.*, 2000).

In summary, the weaning process and the development of the gastrointestinal tract of the pig have a marked effect on feed digestion, nutrient absorption and protection from pathogenic challenges. Diets formulated for young pigs should take into account the changes that occur at weaning and utilize ingredients that the young pig can better absorb, thus, support intestinal health.

Immunity

Innate Immunity

Pigs are constantly exposed to infectious agents, but, in most cases, they are able to overcome these infections. It is their immune system that enables them to overcome infections. The immune system is composed of two major components: the innate or non-specific immune system and the adaptive or specific immune system. The innate immune system is the first line of defense against invading organisms, whereas the adaptive immune system acts as a second line of defense and also offers protection against re-exposure to the same pathogen. Each of the major subdivisions of the immune system has cellular and humoral components by which they carry out their protective function. In addition, the innate immune system also has anatomical features that function as barriers to infection. Although these two arms of the immune system have distinct functions, there are interactions between these systems (Male *et al.*, 2006). The elements of the innate (non-specific) immune system include anatomical barriers, secretory molecules and cellular components.

The infectious agent must overcome innate host defenses to establish a focus of infection (Janeway, 2005). The anatomical barriers consist of the skin, the internal epithelial layers, movement of the intestines and the oscillation of broncho-pulmonary cilia. Associated with these protective surfaces are chemical and biological agents. The desquamation of skin epithelium helps remove bacteria and other infectious agents that have adhered to the epithelial surfaces. Fatty acids in sweat inhibit the growth of bacteria. Lysozyme and phospholipase found in tears, saliva and nasal secretions can breakdown the cell wall of bacteria and destabilize bacterial membranes. The mucosal epithelium covering the orifices of the body is protected by mucous secretions and cilia

(Murray *et al.*, 2012). Movement of cilia and peristalsis help keep air passages and the gastrointestinal tract free of microorganisms. The trapping effect of the mucus that covers the respiratory and gastrointestinal tracts helps protect the lungs and digestive systems from infection. The low pH of sweat and gastric secretions prevents growth of bacteria. Defensins (low molecular weight proteins) found in the lung and gastrointestinal tract have antimicrobial activity. Surfactants in the lung act as opsonins (substances that promote phagocytosis of particles by phagocytic cells). The microflora of the skin and that of the gastrointestinal tract can prevent the colonization of pathogenic bacteria by secreting toxic substances or by competing with pathogenic bacteria for nutrients or attachment to cell surfaces (Male *et al.*, 2006).

If the microorganisms are not suppressed by nonpathogenic bacteria or chemical factors, they may cross the epithelial barrier and begin to replicate in the tissues of the host (Janeway, 2005), ultimately causing acute inflammation (Male *et al.*, 2006). Inflammation plays three essential roles in combating infection. The first is to deliver additional effector molecules and cells to the sites of infection to augment the killing of invading microorganisms by the front-line macrophages. The second role is to provide a physical barrier in the form of microvascular coagulation to prevent the spread of the infection in the bloodstream. The third role is to promote the repair of injured tissue (Janeway, 2005). Humoral immunity is the aspect of immunity that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins and certain antimicrobial peptides. Humoral factors play an important role in inflammation, which is characterized by edema and the recruitment of phagocytic cells. These humoral factors are found in serum or are formed at the site of infection. The

complement system is an alarm and a weapon against infection, especially bacterial infection. The complement system is activated directly by bacteria and bacterial products (alternate or properdin pathway), by lectin binding to sugars on the bacterial cell surface (mannose-binding protein), or by complexes of antibody and antigen (classical pathway). Activation by either pathway initiates a cascade of proteolytic events that cleave the proteins into “a” and “b” subunits. The “a” subunits (C3a, C5a) attract (chemotactic factors) phagocytic and inflammatory cells to the site of infection, allow access to soluble molecules and cells by increasing vascular permeability (anaphylactic C3a, C4a, C5a) and activate responses. The “b” subunits are bigger and bind to the invading agents to promote their phagocytosis by opsonization together with antibody, and build a molecular drill that can directly kill the infecting agent (Murray *et al.*, 2012).

Pathogens also can be recognized by the mononuclear phagocytes without the help of complement. Sites of infection are reinforced by the recruitment of large numbers of neutrophils which are attracted by the molecules secreted by mononuclear phagocytes. Both of these phagocytic cells have a key role in the innate immunity, because they can recognize, ingest and destroy many pathogens without the aid of an adaptive immune response. Interaction of many cell-surface receptors on phagocytic cells by pathogens leads to phagocytosis of the pathogen (Janeway, 2005). After attachment of a bacterium, the phagocyte begins to extend pseudopods around the bacterium. The pseudopods eventually surround the bacterium and engulf it, and the bacterium is enclosed in a phagosome. During phagocytosis, the granules or lysosomes of the phagocyte fuse with the phagosome and empty their contents. The result is a

bacterium engulfed in a phagolysosome which contains the contents of the granules or lysosomes. After activation of phagocytes, bacteria will be killed inside the phagocyte by hydrolytic enzymes and oxygen metabolites (Male, 2006). The most important phagocytes (macrophages) are formed from blood monocytes and contain opsonin receptors which promote phagocytosis of microbes, PAMP receptors which initiate activation and response, and cytokine receptors which promote their activation. Macrophages also express MHC II proteins for antigen presentation to CD4 T cells. Macrophages can be activated by IFN- γ (classical activation) produced by NK cells and CD4 and CD8 T cells as part of the TH1 response and are then able to kill phagocytosed bacteria. These are called M1 macrophages (Murray *et al.*, 2012). There is an increase in glucose and oxygen consumption within M1 macrophages, and this is referred to as the respiratory burst inside M1 macrophages. The function of the respiratory burst is to generate a number of oxygen-containing compounds which kill the bacteria being phagocytosed. This is referred to as oxygen-dependent intracellular killing. In addition, bacteria can be killed by pre-formed substances released from granules or lysosomes when they fuse with the phagosome. This is referred to as oxygen-independent intracellular killing (Male *et al.*, 2006). On the other hand, activated M1 macrophages produce cytokines, enzymes, and other molecules to promote antimicrobial action. They also reinforce local inflammatory reactions by producing various chemokines to attract neutrophils, immature dendritic cells (iDC), Natural Killer cells (NK), and activated T cells. Activation of the macrophages makes them more efficient killers of phagocytosed microbes, virally infected cells, and tumor cells.

Dendritic cells (DC) provide the bridge between the innate and the immune responses. The cytokines they produce determine the nature of the T-cell response. Monocytes and precursor myeloid DC circulate in the blood and then differentiate into immature dendritic cells (iDC) in tissue and lymphoid organs. Immature DC are phagocytic and, upon activation by danger signals, release an early cytokine-mediated warning system and then are converted to Mature DC. Mature DC are the ultimate antigen-presenting cells, one of the antigen-presenting cells that can initiate an antigen-specific T-cell response. These cells express different combinations of danger sensors that can detect tissue trauma (adenosine triphosphate, adenosine, reactive oxygen species, heat shock proteins (Murray *et al.*, 2012).

Natural killer (NK) cells are innate lymphoid cells that provide an early cellular response to a viral infection. They have antitumor activity, and amplify inflammatory reactions after bacterial infection. Natural killer cells are also responsible for antibody-dependent cellular cytotoxicity, in which they bind and kill antibody-coated cells. The NK cell sees every cell as a potential victim, especially those that appear in distress, unless it receives an inhibitory signal from the target cell by binding to carbohydrates and surface proteins on the cell surface. The interaction of a class I major histocompatibility complex (MHC I) molecule on the target cell with a killer-cell immunoglobulin-like (KIR) inhibitory receptor is like communicating a secret password indicating that all is normal, and this provides an inhibitory signal to prevent the killing of the target cell by NK cells. Virus-infected and tumor cells express “stress-related receptors” and are often deficient in MHC I molecules and become NK-cell targets. The NK cells neither recognize a specific antigen nor require presentation of antigen by MHC molecules. Additionally,

the NK system does not involve memory or require sensitization, and, therefore, cannot be enhanced by specific immunization (Murray *et al.*, 2012).

Natural killer T (NKT) cells and γ/δ T cells reside in tissue and in the blood and differ from other T cells because they have a limited repertoire of T-cell receptors. Unlike other T cells, NKT and γ/δ T cells sense non-peptide antigens, including bacterial glycolipids (mycobacteria) and phosphorylated amine metabolites from some bacteria (*Escherichia coli*, mycobacteria) but not others (streptococci, staphylococci). These T and NK cells produce IFN- γ , which activate macrophages and DC to enforce a protective TH1 cycle of cytokines and local cellular inflammatory reactions. The NKT cells also express NK-cell receptors and large amount of TH2 cytokines (Murray *et al.*, 2012).

In general, as the first line of defense against pathogenic infection, the innate immune system uses several components and mechanisms to recognize and respond to pathogens. Some of these components directly stimulate phagocytosis, other molecules are secreted and promote the phagocytosis of pathogens by opsonization and intensify inflammation, while other components can initiate the adaptive immune system later on (Janeway, 2005).

Acquired Immunity

Acquired immunity is induced after pathogens multiply within the host, and is mediated by B and T lymphocytes which have specific surface receptors for the antigens. Thus, acquired immunity is also called antigen-specific immunity and extends the hosts protection provided by innate immunity. The B and T cells are specific immune cells that distinguish and fight specific antigens. The memory capacity of

lymphocytes allows them to respond more rapidly and effectively to re-exposure of the same antigen (Male *et al.*, 2006).

The acquired immunity includes humoral and cell-mediated immunity. During the first exposure to the pathogens, the antigen is recognized by and binds to the receptors on the antigen presenting cells (APCs). These APCs then move into the secondary lymphoid organs where they encounter with T cells. After processing of the antigen within the APC, the specific class I and II major histocompatibility complex (MHC I and MHC II) appears on the surface of APCs and engages the T cells through T cell receptor. Development of an antigen-specific immune response progresses from the innate responses through dendritic cells (DC), and results in activation of the T which trigger the necessary responses. Dendritic cells provide the bridge between the innate and the acquired immune responses, and the cytokines they produce determine the nature of the T-cell response. The DC have octopus -like arms with large surface area (dendrites), produce cytokines, and have an MHC-rich cell surface to present antigen to the T cells. Macrophages and B cells also can present an antigen to the T cells, and activate a naive T cell to initiate a new immune response. The activated helper T cells (CD4) extend and control immune and inflammatory responses by specific cell-to-cell interactions and by releasing cytokines (soluble messengers). The repertoire of cytokines secreted by a specific CD4 T cell in response to an antigenic challenge defines its lineage. Initially, T helper 0 (TH0) cells produce cytokines to promote expansion of the cellular response and develop towards either a T helper 1 and T helper 2 cell lineage that express different cytokines. The TH0 cells are converted to helper 1 (TH1) cells by interleukin-12 (IL-12). The TH1 produce interferon- γ (IFN- γ) to activate

macrophages and DC and promote responses that are especially important for controlling intracellular (mycobacterial and viral) and fungal infections and for promoting certain types of IgG production. The T helper 2 (TH2) cells are formed in the absence of IL-12 and promote antibody responses. The T helper 17 (TH17) cells are a separate lineage that secrete interleukin (IL)-17 to activate neutrophils and promote antibacterial and antifungal responses and inflammation. A separate class of CD4 T cells are immune regulatory T (Treg) cells that express CD4, CD25 and Foxp3 to prevent excessive activation of T cells, and control the immune response. The cytokines produced by each of these T cell types reinforce their own production but may antagonize other responses (Murray *et al.*, 2012).

Upon conversion from TH0 cell, TH2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, which activate naive B cells to secrete antibody, enhance IgG production and promote antibody switch to IgE or IgA. The TH2 response also promotes terminal differentiation of B cells to plasma-cell antibody factories. Antibodies are the primary protection against extracellular bacteria and reinfection and prevent the spread of bacteria in the blood. The antibody promotes complement activation, opsonizes bacteria for phagocytosis, blocks bacterial adhesion, and neutralizes exotoxins and other cytotoxic proteins produced by bacteria. Immunoglobulin M (IgM) is produced early in the antibacterial response, bind to bacteria and trigger the classical complement cascade, promoting both the direct killing of gram-negative bacteria and the inflammatory responses. Immunoglobulin M is usually the only antibody produced against capsular carbohydrates. The large size of IgM limits its ability to spread into the tissue. Later in the immune response, the T-cell promotes the differentiation of the B

cells and immunoglobulin class switching to produce Immunoglobulin G (IgG), which is the predominant antibody, especially on rechallenge. Immunoglobulin G fixes complement and promotes phagocytic uptake of the bacteria through Fc receptors on macrophages. The production of Immunoglobulin A (IgA) requires TH2 cytokines and other factors. Immunoglobulin A is the primary secretory antibody and is important for protecting mucosal membranes. Secretory IgA acquires the secretory component that promotes interaction and passage of IgA through mucosal epithelial cells. Immunoglobulin A prevents the spreading of bacteria and neutralizes their toxins at epithelial cell surfaces (Murray *et al.*, 2012).

The CD8 T cells include cytotoxic T lymphocytes (CTLs) and suppressor cells. The CTL are important for eliminating virally infected cells and tumor cells. The CTL response is initiated when naive CD8 T cells in the lymph node are activated by antigen-presenting DC and cytokines produced by TH1 CD4 T cells. Class I MHC molecules are found on all nucleated cells and are the major marker for CD8 to distinguish normal from abnormal cells. They display antigen to CD8 T cells which can divide and differentiate into mature cytotoxic T lymphocytes (CTL). They bind through interactions of the T cell receptor (TCR) with antigen-bearing class I MHC proteins and adhesion molecules on both cells. Granule-containing toxic molecules, granzymes (esterases), and a pore-forming protein (perforin), move to the site of interaction and release their contents into the immune synapse formed between the T cell and target cell. Perforin generates holes in the target cell membrane to allow the granule contents to enter and induce apoptosis in the target cell. Apoptosis is characterized by degradation of the target cell DNA into discrete fragments of approximately 200 base

pairs and disruption of internal membranes. The cells shrink into apoptotic bodies, which are readily phagocytosed by macrophages and DC. Suppressor T cells provide the antigen-specific regulation of helper T-cell function through inhibitory cytokines and other means. Like CTLs, suppressor T cells interact with class I MHC molecules (Janeway, 2005).

The activated T helper cells also activate macrophages. Macrophages require two signals for activation: one of these is provided by interferon γ (IFN- γ), the other can be provided in different ways. It is also needed for the sensitization of the macrophage to respond to IFN- γ . The TH1 cells can deliver both signals. Interferon γ is the most important cytokine produced by TH1 cells upon interacting with their specific target cells, whereas the CD40 ligand expressed by the TH1 cell delivers the sensitizing signal by interacting with CD40 on the macrophage. The CD8 T cells are also an important source of IFN- γ and can activate macrophages to present antigens derived from cytosolic proteins. Macrophages can be made more sensitive to IFN- γ by very small amounts of bacterial lipopolysaccharide, and the latter pathway may be particularly important when CD8 T cells are the primary source of IFN- γ . It is also possible that membrane-associated TNF- α or TNF- β can substitute for CD40 ligand in macrophage activation. The TH2 cells are inefficient macrophage activators, because they produce IL-10, a cytokine that can deactivate macrophages. However, they do express CD40 ligand, and can deliver the contact-dependent signal required to sensitize macrophages to respond to IFN- γ (Janeway, 2005). After activation by the TH1 cells, macrophages have the capacity to kill the engulfed microbes and secrete cytokines to activate the acute-phase response (Janeway, 2005).

In addition to immediately eliminating the pathogens in the host, all activated T and B cells are converted to memory T and B cells which have longer half-lives and circulate in the whole body. Upon encountering the antigens, they react more rapidly and more efficiently to the second infection.

Inflammatory Mediators

Inflammation represents a protective defense reaction induced by external or internal trauma such as tissue injury, infection, or tumor growth. It is caused by several mediators released by fibroblasts, neurons, and immune cells. These mediators include neurotrophins, histamine, bradykinin, prostanoids, serotonin, protons, free radicals, chemokines and cytokines (Table 1; Dray, 1995). The mediators can have a direct action, stimulate the release of other chemicals, activate the immune system, facilitate vasodilatation and plasma exudation or sensitize the nociceptive system (Marnet *et al.*, 2002). It is generally accepted that the main inflammatory mediators induced by bacteria and their cell wall components are chemokines and cytokines (Wang *et al.*, 2000). Chemokines represent a sophisticated communication system used by all the cell types. Chemokine messages are decoded by specific receptors that initiate the signal transduction events leading to a multitude of cellular responses, leukocyte chemotaxis and adhesion at infection sites. Infectious microorganisms can directly stimulate inflammatory chemokine production by tissue dendritic cells and macrophages as well as by many parenchymal and stromal cells (Rot and von Andrian, 2004). Inflammatory chemokines function mainly as chemoattractants for leukocytes, monocytes, neutrophils and other effector cells.

Table 1-1. Inflammatory cytokines and chemokines (Murray *et al.*, 2012)

Factor	Source	Major Target	Function
Innate and acute-phase responses			
Interleukin 1 α , Interleukin 1 β	Macrophage, DC1, fibroblasts, epithelial cells	T cells, B cells, PMN2, tissue, central nervous system, liver	Promotion of inflammatory and acute-phase responses, fever, activation of T cells and macrophages
Tumor necrosis factor- α	Similar to interleukin 1	Macrophages, T cells, NK3 cells, epithelial	Similar to IL-1, and also antitumor, wasting functions, sepsis, endothelial activation
Interleukin 6	DC, Macrophages, T and B cells, fibroblasts, epithelial cells, endothelial cells	T and B cells, hepatocytes	Stimulation of acute-phase and inflammatory responses, T and B cell growth and development
Interleukin 12, Interleukin 23	DC, Macrophages	NK cells, TH1, TH17 cells	Activation of T-cell-mediated and inflammatory responses, IFN- γ production
Chemokines			
α -chemokines: CXC chemokines (IL-8, IP-10, GRO- α , GRO- β , GRO- γ)	Many cells	Neutrophils, T cells, macrophages	Chemotaxis, activation
β -Chemokines: CC chemokines (MCP-1, MIP- α , MIP- β , RANTES)	Many cells	T cells, macrophages, basophils	Chemotaxis, activation

1DCs, dendritic cells; 2PMN, polymorphonuclear leukocyte; 3NK, natural killer cell; TH, T helper cell; IP, interferon- α protein; RANTES, regulated on activation.

Some inflammatory chemokines activate cells to initiate an immune response or promote wound healing. Inflammatory chemokines include chemokine ligand 2 (CCL2), CCL3 and CCL5, interleukin 1 (IL-1 or CXCL1), IL-2 and IL-8. Interleukin 8 (IL-8) is produced by macrophages and other cell types such as epithelial cells, airway smooth

muscle cells and endothelial cells and is one of the most effective chemo-attractants for neutrophils in the innate immunity (Graham and Locati, 2013). Interleukin-8 has two primary functions: the first one is to induce chemotaxis of neutrophils and other granulocytes, causing them to migrate toward the site of infection, and the second function is to induce the phagocytosis of phagocytes. Interleukin-8 induces a series of physiological responses such as increased intracellular Ca^{2+} , exocytosis, and respiratory burst, which are required for migration and phagocytosis (Modi *et al.*, 2004).

Cytokines are proteins made by cells that affect the behavior of other cells. When secreted by lymphocytes, cytokines are called lymphokines or interleukins. They act via specific cell surface receptors (Janeway, 2005). The cytokines secreted by macrophages play important roles in innate responses. These include tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). The T helper 1 cells (TH1) not only activate macrophages to secrete interferon- γ (IFN- γ), but also sensitize them to respond to IFN- γ . After activation, macrophages begin to release cytokines in the liver, spleen and other systemic sites that have long-range effects that contribute to the host defense. Tumor necrosis factor- α , IL-1 β and IL-6 are referred to as endogenous pyrogens, because they cause fever and are derived from an endogenous source rather than from bacterial components. Fever is generally beneficial to the host defense. Most pathogens grow better at normal body temperatures and adaptive immune responses are more intense at elevated temperatures. The host cells are also protected from the deleterious effects of TNF- α at elevated temperatures. Tumor necrosis factor- α induces the migration of DC to the lymph nodes which initiate the acquired immune responses. Tumor necrosis factor- α also causes vasodilation, which

leads to a loss of blood pressure, increased vascular permeability and increased migration of IgG, complement and phagocytes to the tissue. However, increased vascular permeability can cause a loss of plasma volume and, thus, the septic shock of the host. In septic shock, a random intravascular coagulation can also be triggered by TNF- α , leading to the generation of clots in many small vessels. This condition frequently causes the failure of vital organs such as the kidneys, the liver, the heart, and the lungs, and often results in a very high mortality rate (Janeway, 2005). Interleukin-1 β triggers the responses of vascular endothelium and lymphocytes, increases tissue permeability, and allows more effector cells to access the infection site. Interleukin-1 β also induces the secretion of IL-6 which can activate the lymphocytes and increase antibody production. These cytokines also have other biological activities that help coordinate the body's responses to infection. They activate bone marrow endothelium to release neutrophils that intensify the phagocytosis at the infection site. They increase protein and energy mobilization in the muscle and fat tissues and allow a rise in body temperature and a decrease in viral and bacterial replication. They also increase the antigen processing and acquired immune response. One of the most important functions of the cytokines is the initiation of acute-phase response. This involves a shift in the proteins secreted by the liver. During the acute-phase response, concentrations of some proteins in plasma decrease, whereas levels of others increase markedly. The proteins whose synthesis is induced by the cytokines are called acute-phase proteins. Several of these proteins are of particular interest, because they mimic the action of antibodies, but, unlike antibodies, these proteins have a broader specificity for pathogen-associated molecular patterns (Janeway, 2005).

Prostaglandins (PG) are small-molecules derived from arachidonic acid (AA) by the action of cyclooxygenases (COX) and PG synthases. Prostaglandin E₂ (PGE2) has been shown to regulate multiple functions of immune cells (Phipps *et al.*, 1991). It mediates inflammation, promotes local vasodilatation, and causes local attraction and activation of neutrophils, macrophages, and mast cells at early stages of inflammation. The ability of PGE₂ to trigger the synthesis of IL-10 and to suppress the production of multiple pro-inflammatory cytokines allows it to limit nonspecific inflammation and to promote the immune suppression associated with chronic inflammation and cancer. Although PGE₂ can promote the activation, maturation, and migration of DC to the lymph nodes, it has been shown to suppress both innate and antigen-specific immunity at multiple molecular and cellular levels, earning PGE₂ the paradoxical status of a pro-inflammatory factor with immunosuppressive activity (Kalinski, 2012).

Although inflammatory mediators induce the innate and immune responses against infection at the early stage of inflammation, the excessive production of these molecules often will cause a malaise and damage the host tissue.

Nutrition and Immunity

It is generally accepted that nutrition is an important determinant of immune response. Deficiency of one or several nutrients can negatively affect the host's immune defense system. Incorporation of nutrients into the cell membrane affects the membrane structure and fluidity, which may have a major effect on transmembrane signaling. The production of antibodies or cytokines is altered by the availability of proteins or limiting amino acids. Additionally, deficiency of nutrients suppresses cell proliferation through its effect on DNA replication and cell-cycle regulation. The antimicrobial and antitumor functions of macrophages are known to be modified by

nutrients that promote the synthesis of reactive oxygen or nitrogen intermediates. Prostaglandin synthesis by phagocytes is related to the types and quantities of dietary fatty acids. These reports collectively indicate that nutrients can affect immune system at several levels (Kubena *et al.*, 1996).

Lymphoid atrophy is known to result from protein-energy malnutrition. Under these conditions, the size and weight of the thymus are reduced, and there is a loss of corticomedullary differentiation; there are fewer lymphoid cells and the Hassall bodies are enlarged, degenerated and occasionally calcified. These changes lead to a primary immune deficiency, such as DiGeorge syndrome (Roitt and Brostoff, 1991). Under condition of protein-energy deficiency, there is a significant reduction of lymphocytes in the lymph nodes. Poor nutrition leads to a marked decrease in the proportion of CD4+ on the surface of T helper cells. There also is a moderate reduction in the number of suppressor cytotoxic CD8+ cells. Thus, the ratio of CD4+ and CD8+ cells are significantly lower in malnourished subjects compared with well-nourished subjects. Moreover, co-culture experiments showed a reduction in the number of antibody-producing cells and in the amount of immunoglobulin secreted (Chandra, 1997). This may be due to decreased help provided by T lymphocytes. In undernourished subjects, lymphocyte proliferation and DNA synthesis are reduced, especially when an autologous plasma from a patient is used in cell cultures. This may be caused by inhibitory factors as well as deficiency of essential nutrients in the patient's plasma. Serum antibody responses are generally intact in protein-energy malnutrition. However, antibody affinity is decreased in patients who are malnourished. This may provide an explanation for a higher frequency of antigen-antibody complexes found in such

patients. Concentrations of secretory immunoglobulin A antibody also are lower after immunization with viral vaccines. Phagocytosis is also affected in protein-energy malnutrition. Because of the reduction in C3, C5, factor B and total hemolytic activity, there is a slight reduction in opsonic activity of plasma, and metabolic activation and intracellular destruction of bacteria are reduced. Malnutrition not only decreases the production of several cytokines, including interleukins 1 and 2 and interferon γ , but also alters the ability of T lymphocytes to respond appropriately to cytokines. Poor nutrition has negative effects on the integrity of physical barriers, the quality of mucus and several other innate immune responses, which ultimately leads to immune failure (Chandra, 1997).

Deficiencies of essential amino acids can also suppress the synthesis of proteins, including those which contribute uniquely to host defense. These proteins encompass all of the cytokines produced by lymphocytes, macrophages, and other cells. In addition, essential amino acid deficiencies decrease the production of complement proteins, numerous tissue enzymes and metalloenzymes that are activated during acute phase responses. All of the acute phase glycoproteins produced by the liver are also reduced by amino acid deficiencies (Beisel, 1996).

Fatty acids may influence the immune system in a number of ways. Alteration in membrane composition is known to result in changes in fluidity and transmembrane signal transduction in immune cells. Arachidonic acid metabolism may be altered by the quantity and quality of dietary fat, ultimately resulting in changes in highly immunomodulatory prostaglandin production (Calder and Newsholme, 1993).

Immunosuppression associated with fatty acids, particularly polyunsaturated fatty acids,

may be beneficial in conditions involving an overactive immune response. When vitamin E is added to the diet, the inhibition of lymphocyte proliferation by polyunsaturated fatty acids is reduced (Martinez-Alvarez *et al.*, 2005). On the contrary, excessive selenium combined with fat consumption can increase the inhibitory effect of polyunsaturated fatty acids on antibody circulation by polyunsaturated fatty acids (Harik-Khan *et al.*, 1993).

Adequate fiber ingestion is needed to support the growth of microbiota in the host. Short chain fatty acids (SCFA) are end products of microbial fermentation, and exert marked effects on host immune responses. Low levels of butyrate modify the cytokine produced by TH cells and promote intestinal epithelial barrier integrity, which in turn can help limit the exposure of the mucosal immune system to luminal microbes and prevent aberrant inflammatory responses. Production of another SCFA, acetate, by the microbiota promotes the resolution of intestinal inflammation by the G-protein-coupled receptor GPR43. Acetate production plays an important role in preventing infection with the enteropathogen *Escherichia coli* (0157:H7). This effect is linked to the ability of acetate to maintain gut epithelial barrier function. Also, intestinal microbiota can synthesize several vitamins involved in various aspects of microbial and host metabolism. These include cobalamin (vitamin B12), pyridoxal phosphate (the active form of vitamin B6), pantothenic acid (vitamin B5), niacin (vitamin B3), biotin, tetrahydrofolate and vitamin K. Most of these vitamins are involved in the immune system (Kau *et al.*, 2011).

Vitamins and minerals also play a crucial role in the maintenance of immune-competence. These include vitamin A, beta-carotene, folic acid, vitamin B6, vitamin

B12, vitamin C, vitamin E, riboflavin, iron, zinc and selenium (Grimble, 1997). Dietary deficiencies of vitamin A have been shown to induce impairment of mitogen-induced proliferation, reduce antibody responses to pneumococcal polysaccharide, and decrease NK cell and IFN- γ activity (Kubena *et al.*, 1996). Vitamin C is an antioxidant that plays a pivotal role in maintaining the antioxidant/oxidant balance in immune cells and in protecting them from oxidative stress (Victor *et al.*, 2004). Deficiencies of vitamin C induces a number of subtle changes in immune system and lymphocyte functions, but the major effect of vitamin C deficiency occurs in phagocytic cells (Anderson *et al.*, 1990). In both clinical and experimental scurvy, the locomotion of phagocytic cells is severely impaired, possibly because these cells cannot produce tubulin (Beisel, 1996). This vital intracellular protein allows cells to change shape, and to move about. Without an adequate tubulin infrastructure, phagocytic cells cannot migrate to sites where an inflammatory process is needed in order to prevent the dissemination of a localized infection. Although the microbicidal activity of phagocytes generally remains effective in the presence of ascorbic acid deficiency, the cells cannot move toward the microorganisms to engulf and destroy them (Beisel, 1996). Vitamin E is the major lipid-soluble antioxidant in the body and is required for protection of membrane lipids from peroxidation. Since free radicals and lipid peroxidation are immunosuppressive, it is believed that vitamin E may act to optimise and even enhance the immune response. In fact, vitamin E deficiency decreased spleen lymphocyte proliferation, natural killer cell activity, antibody production following vaccination, and phagocytosis by neutrophils (Meydani and Beharka, 1998). On the other hand, vitamin E supplementation of the diet of laboratory animals enhances antibody production, lymphocyte proliferation,

natural killer cell activity, and macrophage phagocytosis. In another report, vitamin E prevented the retrovirus-induced decrease in production of IL-2 and interferon- γ (IFN- γ) by spleen lymphocytes and in natural killer cell activity (Wang *et al.* 1994). The high level of vitamin E caused increased IL-2 and IFN- γ production of spleen lymphocytes (Han and Meydani.1999). Vitamin D is an important immune system regulator. The active form of vitamin D, 1, 25-dihydroxyvitamin D₃ [1, 25(OH)₂D₃], has been shown to inhibit the development of autoimmune diseases, including inflammatory bowel disease (IBD). An additional factor that determines the effect of vitamin D status on immune function is dietary calcium. This mineral has independent effects on the severity of IBD severity, and addition of 1, 25 (OH)₂D₃ to low-calcium diets was shown to improve IBD symptoms (Cantorna *et al.*, 2004).

Selenium is essential for the efficient operation of many aspects of the immune system in both animals and humans. Low dietary intake of selenium and consequent deficiency in farm animals can result in a wide range of diseases that are often associated with a concurrent vitamin E deficiency (Turner and Finch, 1991). Selenium can influence the cell function through antioxidant activities, thyroid hormone metabolism and regulation of the activity of redox-active proteins (McKenzie *et al.*, 2002). All of these general effects on metabolism can be associated with more specific processes that will affect the immune system. Thus, selenium influences both the innate and the acquired immune systems. Selenium-deficient lymphocytes are less able to proliferate in response to mitogen, and in macrophages, leukotriene B4 synthesis, which is essential for neutrophil chemotaxis, is impaired by this deficiency. The humoral system is also affected by selenium deficiency where by a decrease in

production of IgM, IgG and IgA is observed. One of the most widely investigated associations between selenium and the immune system is the effect of the micronutrient on neutrophil function. Neutrophils produce superoxide-derived radicals that contribute to killing of microbes. Although selenium deficiency does not affect neutrophil numbers in many species, certain aspects of their function because subnormal (Turner and Finch, 1991). For example, neutrophils from selenium-deficient mice, rats and cattle are able to ingest pathogens in vitro, but are less able to kill them than are neutrophils from selenium-sufficient animals (Boyne and Arthur, 1986). Selenium deficiency also impairs thyroid hormone metabolism and attenuates the ability of neutrophils to respond to foreign organisms. Selenium deficiency can favor the formation of pro-inflammatory mediators that would predispose to diseases. Magnesium functions in the development, distribution, and function of immune cells and soluble factors that are critical for humoral and cell-mediated immunity. Magnesium not only affects more than 300 enzymes, but it also influences the metabolism of many other nutrients. Single nutrient deficiencies of copper and iron are known to adversely affect several aspects of the immune response, because they serve as important cofactors for enzymes that are vital for lymphocyte and macrophage function (Boyne and Arthur, 1986). Zinc deficiency in animals is associated with a wide range of immune impairments (Fraker *et al.*, 1993). In effect, a deficiency of zinc in the diet leads to a marked decrease in the number of nucleated cells and a reduction in the number and proportion of lymphoid cell precursors (Osati-Ashtinani, 1998). Reduced intestinal zinc absorption can cause thymic atrophy, impair lymphocyte development, decrease the numbers of CD4 cells and reduce lymphocyte responsiveness (Fraker *et al.*, 1986). Moderate or mild zinc

deficiency or experimental zinc deficiency results in decreased thymulin activity, decreased natural killer cell activity, lowered ratio of CD4:CD8, and decreased lymphocyte proliferation (Shankar and Prasad, 1998). Experimental Zinc deficiency decreased IL-2, IFN- γ and TNF- α production by mitogen-stimulated lymphocytes (Beck *et al.*, 1997). Low plasma zinc level causes the development of lower respiratory tract infections and diarrhea among infants (Bahl *et al.*, 1998). There are now a number of studies showing that zinc supplementation decreases the incidence of childhood diarrhea and respiratory illness (Jackson, 2000). Zinc administration to preterm low-birth-weight infants increased the number of circulating T lymphocytes, stimulated lymphocyte proliferation, increased cell-mediated immune function and decreased the incidence of gastrointestinal and upper respiratory tract infections (Lira *et al.*, 1998).

Omega-3 Polyunsaturated Fatty Acids (*n*-3 PUFA) in Swine Nutrition

Definition and Biosynthesis of *n*-3 PUFA

Omega-3 fatty acids (*n*-3 fatty acids) refer to a group of polyunsaturated fatty acids (PUFA) that includes α -linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The term *n*-3 is a structural descriptor, which refers to the position of the double bond that is closest to the methyl terminus of the acyl chain of the fatty acid. All *n*-3 fatty acids have this double bond on carbon 3, counting the methyl carbon as carbon one. Like other fatty acids, *n*-3 fatty acids have systematic and common names, but they are frequently referred to by a shorthand nomenclature that denotes the number of carbon atoms in the chain, the number of double bonds, and the position of the first double bond relative to the methyl carbon. The simplest *n*-3 fatty acid is α -Linolenic acid (ALA; C18:3*n*-3). Alpha-Linolenic acid is synthesized from linoleic acid (C18:2*n*-6) by delta-15 desaturase. Animals do not possess delta 12 and

delta 15 desaturase enzymes and, therefore, cannot synthesize ALA. This fatty acid must be supplied in the diet. Although animals cannot synthesize ALA, they can metabolize it by further desaturation and elongation (Figure 1). Desaturations occur at carbon atoms below carbon number 9 (counting from the carboxyl carbon) and occurs mainly in the liver. Alpha-Linolenic acid can be converted to stearidonic acid (C18:4n-3) by delta-6 desaturase and then stearidonic acid can be elongated to eicosatetraenoic acid (C20:4n-3), which is subsequently desaturated by delta-5 desaturase to yield EPA(C20:5n-3). It is important to note that the conversion of ALA to EPA is in competition with the conversion of linoleic acid to arachidonic acid (AA; C20:4n-6), because the same enzymes are used. The preferred substrate for delta-6 desaturase is ALA. Linoleic acid is much more prevalent in most human diets than ALA, and thus metabolism of *n*-6 fatty acids is quantitatively more important. The activities of delta-6 and delta-5 desaturases are regulated by nutritional status, hormones and by feedback inhibition by the end products. The conversion of EPA to docosahexaenoic acid (DHA; C22:6n-3) involves addition of 2 carbons to EPA to form docosapentaenoic acid (DPA; C22:5n-3). This molecule is then desaturated at the delta-6 position to form tetracosahexaenoic acid (C24:6n-3), which is subsequently translocated from the endoplasmic reticulum to peroxisomes, where 2 carbons are removed by limited β -oxidation to yield DHA (Calder, 2012). Short term studies with isotopically-labeled ALA and long term studies using greater amounts of ALA have demonstrated that the conversion of ALA to EPA, DPA and DHA is generally poor in humans (Arterburn *et al.*, 2006). Eicosapentaenoic acid, DPA and DHA are found at high concentration in fish that consume algae.

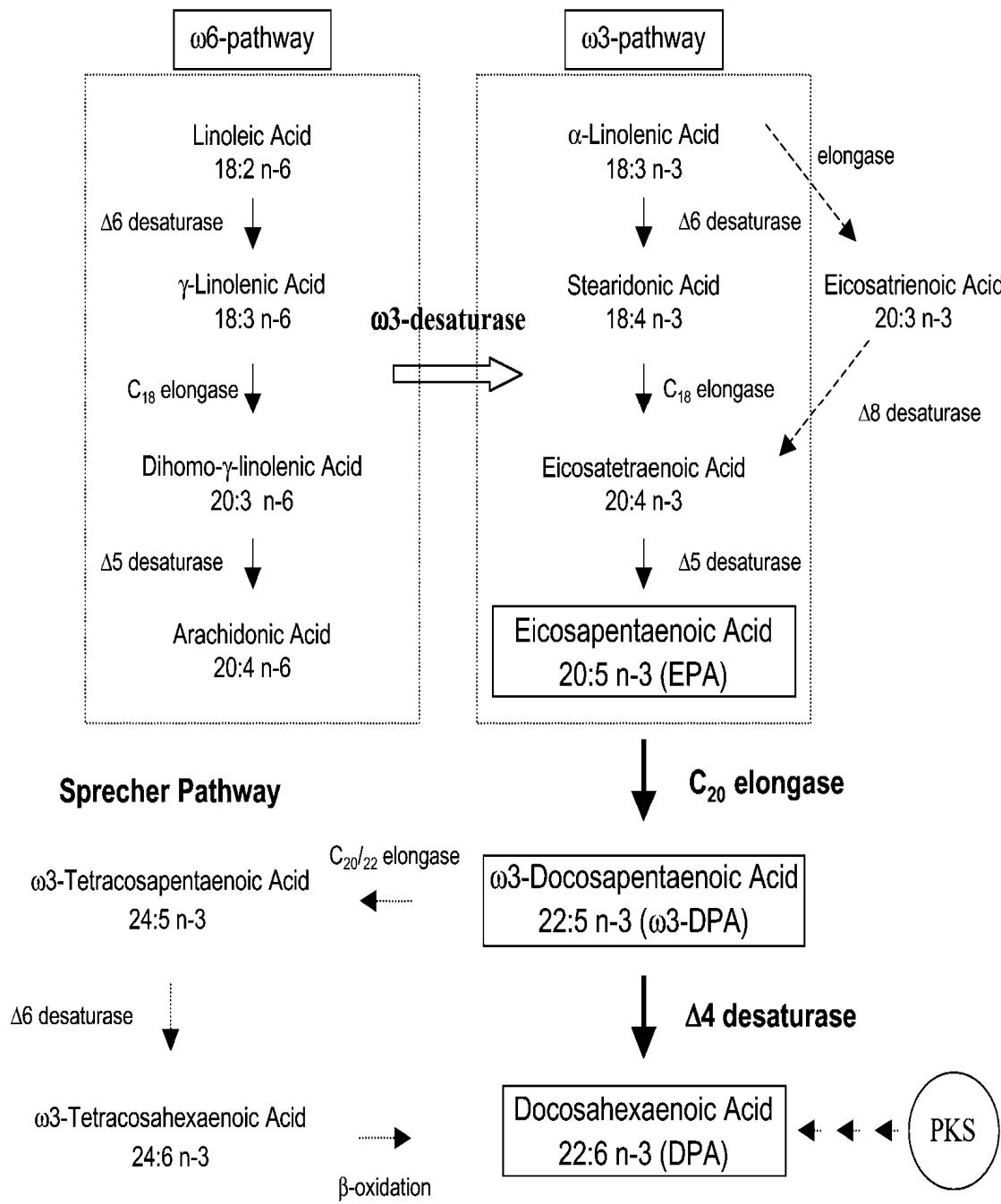


Figure 1-1. Omega-3 PUFA biosynthesis (adapted from Pereira et al., 2004).

Because ALA is produced in plants, green leaves and some plant oils, nuts and seeds contain moderate to high amounts of ALA. Alpha-linolenic acid is the major *n*-3 fatty acid consumed in most human diets. However, the main PUFA in most Western

diets is linoleic acid which is typically consumed in 5 to 20-fold greater amounts than ALA. Sea foods are a good source of long chain *n*-3 PUFA. These fatty acids are found in the flesh of both lean and oily fish, with much greater amounts in the latter, and in the livers of some lean fish. Humans who consume small amount of fish in their diet deposit less than 0.2 to 0.3 g of long chain *n*-3 PUFA per day (British Nutrition Foundation, 1999). A single lean fish meal could provide about 0.2 to 0.3 g of long chain *n*-3 PUFA, while a single oily fish meal can provide 1.5 to 3.0 g of these fatty acids. Fish oil is prepared from the flesh of oily fish or from the livers of lean fish. In a typical fish oil supplement, EPA and DHA make about 30% of the total fatty acids. Consequently, a one gram fish oil capsule will provide about 0.3 g of EPA and DHA. However, the amount of *n*-3 PUFA can vary between fish oils, as can the relative proportions of the individual long chain *n*-3 PUFA. In fish oil capsules, fatty acids are usually present in the form of ethyl esters (Calder, 2012).

Effects of *n*-3 PUFA on Growth

Polyunsaturated fatty acids (PUFA) comprise a group of fatty acids with more than one double bond in their carbon chains. These fatty acids have the ability to affect a diverse number of physiological processes, including the regulation of plasma lipid concentration, immune function and retinal development. There are two major classes of PUFA found in the diet, *n*-3 and *n*-6. Both *n*-3 and *n*-6 PUFA are essential, but their metabolic products elicit different cellular functions (Meers *et al.*, 2004). The long-chain *n*-3 PUFA, eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), are found in fish oil and have a wide range of biological effects that are believed to confer benefits to human health (British Nutrition Foundation, 1999). As a

result, it is generally recommended that long-chain *n*-3 PUFA consumption be increased in the human diet.

Feeding pigs with a diet containing a high α -linolenic acid level leads to significant enrichment of animal tissues with *n*-3 fatty acids. Because unsaturated fatty acids inhibit lipogenesis than saturated fatty acids do, dietary fish oil may reduce the adipocyte size and fat pad weights in domestic animals (Fickova *et al.*, 2002). Supplemental fish oil does not appear to affect growth responses (feed intake, daily gains, feed conversion efficiency) or carcass quality (slaughter weight, dressing percentage, lean percentage, nutrient composition of the loin) in pigs when the ratio of *n*-6 : *n*-3 PUFA is higher than 2 (Jaturasitha *et al.*, 2002). In one study (Duan *et al.*, 2014), growth performance of pigs fed with diets with an *n*-6:*n*-3 PUFA ratio of 5:1 was the best, but the group fed diets with an *n*-6:*n*-3 PUFA ratio of 1:1 had the highest muscle mass and the lowest adipose tissue mass. Excessive dietary *n*-3 fatty acids can cause soft pork, but also increase the susceptibility of the meat to oxidation. Therefore, concomitant supplementation of vitamin E is necessary to prevent fishy flavor due to lipid oxidation (Wood and Enser, 1997).

It is well documented that lipids play an important role in skeletal biology and bone health. Dietary fat may influence bone metabolism by altering the biosynthesis of prostaglandins. Prostaglandins are locally produced in osteogenic cells and regulate both bone formation and bone resorption. Eicosapentaenoic acid treated-cells tended to have increased levels of alkaline phosphatase activity and osteocalcin, suggesting that PGE₂ may decrease bone mass by inhibiting osteoblast activity. Additionally, PGE₂ may modulate IGF-1 synthesis and affect its action to support anabolic responses in the

bone (McCarthy *et al.*, 1994). Thus, dietary supplementation of *n*-3 PUFA can affect bone remodeling by directly inhibiting the biosynthesis of PGE₂ and indirectly influencing IGF-1 secretion. On the other hand, cells exhibit greater collagen synthesis when enriched with *n*-3 PUFA (Watkins *et al.*, 2001). The fibroblast also displays a high collagen formation when exposed to moderate levels of EPA (Hankenson *et al.*, 2000). Because DHA is involved in the fluidity, physical flexibility and compressibility of cell membranes, *n*-3 fatty acids are needed to maintain the development of brain and the normal function of retina (Neuringer *et al.*, 1988). Furthermore, *n*-3 PUFA are known to increase the litter size, and reduce the preweaning mortality of piglets. They also affect the conception rate and the number of pigs born alive (Reese, 2003).

Effects of *n*-3 PUFA on Inflammatory Responses

Long chain *n*-3 PUFA decrease the expression of adhesion molecules on the surface of monocytes (Hughes *et al.*, 1996), macrophages (Miles *et al.*, 2000), lymphocytes (Sanderson *et al.*, 1998) and endothelial cells (Yamada *et al.*, 2008). Eicosapentaenoic acid reduces the expression of VCAM-1, ELAM-1 and ICAM-1 on the surface of LPS-stimulated human umbilical vein endothelial cells (Calder, 1997). Murine peritoneal macrophages cultured in the presence of EPA or DHA were less adherent to artificial surfaces than those cultured with other fatty acids (Calder, 1997). Consumption of 1.8 g EPA and DHA per day by patients with peripheral vascular disease decreased the adhesive interaction of their monocytes to endothelial monolayers in culture (Luu *et al.*, 2007)

Chemotaxis is the process by which leukocytes move towards the infection sites in response to the release of specific chemicals. These chemicals, also known as chemokines, include IL-8 and the arachidonic acid-derived eicosanoid Leukotriene B4

(LTB₄). Studies with fish oil supplementation of healthy humans have demonstrated a decrease in chemotaxis of neutrophils and monocytes towards various chemoattractants including LTB₄, bacterial peptides and human serum (Luostarinen *et al.*, 1992). The near-maximum inhibition of chemotaxis occurs at an intake of 1.3 g EPA and DHA per day (Schmidt *et al.*, 1991).

Long chain *n*-3 PUFA also suppress the production of arachidonic acid-derived eicosanoids including PGE₂ and LT (Peterson *et al.*, 1998). Arachidonic acid is released from the phospholipids through the action of phospholipase A₂ enzyme, which is activated by inflammatory stimuli. The free arachidonic acid then acts as a substrate for cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 enzymes. Cyclooxygenases lead to PG and thromboxane formation, whereas lipoxygenases lead to LT formation. Cytochrome P450 enzymes catalyze the formation of hydroxyeicosatetraenoic and epoxyeicosatrienoic acids. All these eicosanoids have long been recognized as key mediators and regulators of inflammation, acting via G protein-coupled receptors (Tilley *et al.*, 2001). An EPA intake of 2.7 g per day significantly decreases PGE₂ production (Rees *et al.*, 2006). Eicosapentaenoic acid is also a substrate for the COX, LOX and cytochrome P450 enzymes that produce eicosanoids, but the mediators produced have different structures and functions from those made from arachidonic acid. Instead of serving as substrate for PGE₂ and LTB₄ biosynthesis, EPA is converted to PGE₃ and LTB₅ which are much less biologically active than those produced from arachidonic acid (Bagga *et al.*, 2003). Omega-3 PUFA compete with *n*-6 fatty acids not only for enzymes, but also for the receptors on the leukocytes. They decrease the production of inflammatory eicosanoids from

arachidonic acid and increase the production of anti-inflammatory eicosanoids (Wada *et al.*, 2007). Moreover, long chain *n*-3 PUFA lead to the formation of endocannabinoids, which have marked anti-inflammatory properties in cell culture systems (Meijerink *et al.*, 2011).

Resolvins and protectins were recently discovered as lipid mediators produced from long chain *n*-3 PUFA. The synthesis of resolvins and protectins also involves the COX and LOX pathways and leads to the resolution of inflammation. Resolvin E1, D1 and protectin D1 all inhibit transendothelial migration of neutrophils, ultimately preventing the infiltration of neutrophils into the sites of inflammation. Resolvin D1 inhibits IL-1 β production, whereas protectin D1 inhibits TNF- α and IL-1 β production (Serhan *et al.*, 2002).

Through their effects on eicosanoid production, *n*-3 PUFA affects the concentrations of cytokines in biological fluids. Eicosapentaenoic acid and DHA (2 g/day) suppress the secretion of TNF- α , IL-1 β and IL-6 by endotoxin-stimulated macrophages, and decrease circulating TNF- α , IL-1 β and IL-6 concentrations in blood (Sadeghi *et al.*, 1999). Lymphocyte-derived cytokine production (IL-2, IL-4 and IL-10) is also reduced by long chain *n*-3 PUFA (Calder, 1997).

Besides the humoral immunity, *n*-3 PUFA influence the cell mediated immunity as well. A number of studies have shown that ALA, EPA and DHA inhibit the proliferation of lymphocytes in lymphoid tissues and peripheral blood. Eicosapentaenoic acid appears to be the most inhibitory. Eicosapentaenoic acid and DHA have been reported to suppress phorbol ester-stimulating superoxide generation by neutrophils. Docosahexaenoic acid allegedly inhibits IFN- γ -stimulated tumoricidal

action of macrophages by suppressing IFN- γ dependent signals. Furthermore, *n*-3 PUFA affect the killing of microbial or tumor cells in macrophages by modulating the production of reactive oxygen species and nitric oxide (Calder, 1997). Not only do *n*-3 PUFA affect the response of lymphocytes to antigen, but they may also affect the ability of antigen presenting cells to present antigen (Calder, 1997). The activities of cytotoxic cells (CLT) and natural killer cells are diminished by *n*-3 PUFA as well, because *n*-3 PUFA inhibit NK cell activity directly and suppress the degranulation of CLT. In addition, there is evidence that circulating levels of immunoglobulin G and immunoglobulin M may be decreased by dietary supplementation of *n*-3 PUFA (Calder, 1997).

The health benefits of *n*-3 PUFA may come at a price. The ability of *n*-3 PUFA to improve the host's survival from gram-positive and gram-negative bacterial infections is due to their inhibitory effects on excessive immune responses caused by exotoxin and endotoxin produced by these extracellular pathogens. However, *n*-3 PUFA can diminish the host's resistance to intracellular bacteria, because they inhibit the production of certain key cytokines (IL-12 and IFN- γ) that play essential roles in the host's defense against intracellular pathogens. *Omega-3* PUFA can have a direct cytotoxic action on certain parasites, but they may affect the host's antiviral defense in both positive and negative ways, and the magnitude of their effects appears to be much smaller for antiviral than antibacterial responses. Therefore, more studies are needed to investigate the mechanism of *n*-3 PUFAs benefit the host immunity against a large variety of pathogens (Anderson and Fritzsche, 2002).

Mechanisms of Action of *n*-3 PUFA

*Omega-3 PUFA affect inflammation by regulating prostaglandin biosynthesis and their effects on nuclear factor kappa-β (NF-κB) and peroxisome proliferator-activated receptor-γ (PPAR-γ) activation which may be mediated via the G-protein coupled receptor GPR 120 (Calder, 2012). Because the primary PUFA in cell membranes is arachidonic acid (AA), most eicosanoids produced in cells are prostanoids of the 2 and 4 series (PGE₂, PGF_{2α}, and leukotriene B₄). Eicosapentaenoic acid is a substrate for prostanoids of the 3 and 5-series. In general, AA-derived eicosanoids have pro-inflammatory effects (Calder and Kew, 2002), whereas EPA-derived eicosanoids have anti-inflammatory effects (Calder and Kew, 2002). High intakes of *n*-3 PUFA result in their incorporation into membrane phospholipids, where they partially replace AA (Crawford, 2000). By decreasing the availability of AA, EPA suppresses the biosynthesis of AA-derived eicosanoids and favors the formation of EPA-derived 3-series prostanoids and 5-series leukotrienes. Additionally, *n*-3 PUFA compete with *n*-6 PUFA for desaturases and elongases, and have greater affinities for these enzymes than *n*-6 PUFA. Therefore, dietary intake of *n*-3 PUFA reduces the desaturation and elongation of linoleic acid (LA) to AA (Rose and Connolly, 1999) and decreases the production of AA-derived eicosanoids. Furthermore, *n*-3 PUFA suppress COX enzymes or compete with *n*-6 PUFA for eicosanoid biosynthesis (Ringbom *et al.*, 2001). Compared with AA, EPA is the preferential substrate for lipoxygenase; hence an increased EPA intake leads to greater formation of EPA-derived lipoxygenase products at the expense of AA-derived lipoxygenase products (Grimm *et al.*, 2002). Finally, *n*-3 PUFA enhance eicosanoid catabolism, which is postulated to be mediated through*

induction of peroxisomal enzymes (von Schacky *et al.*, 1993). Changes in fatty acid composition of inflammatory cell membrane phospholipids alters the availability of substrates for synthesis of eicosanoids, endocannabinoids, resolvins and protectins (Calder, 2012).

A second aspect of the alteration of cell membrane phospholipid fatty acids with marine *n*-3 PUFA involves the so called 'lipid rafts' which are now fairly well studied in T cells (Yaqoob, 2009). Rafts are structures that are formed by the movement of receptors, accessory proteins and enzymes within the plane of the cell membrane. This movement occurs in response to cell activation and is essential for the intracellular signals to be properly transduced into the cytosol and nucleus. Lipid rafts are intimately involved in T-lymphocyte responses to activation (Harder, 2004). Cell culture and animal feeding studies have shown that exposure to marine *n*-3 PUFA modifies the raft formation in T-cells in a way that impairs the intracellular signaling mechanisms (Zeyda and Stulnig, 2006).

Dietary PUFA and their metabolites may exert some of their anti-tumor effects by affecting gene expression or activity of signal transduction molecules involved in the control of cell growth, differentiation and apoptosis (Larsson *et al.*, 2004). Nuclear factor kappa B (NF- kBs) is the main transcription factor involved in up-regulation of the genes encoding inflammatory cytokines, adhesion molecules and COX-2 (Siga, 2006). Nuclear factor kappa B are normally confined in the cytoplasm through their association with IkBs. When cells are activated by inflammatory stimuli, the IkBs are rapidly phosphorylated and degraded to free the NF-kBs. The free NF-kBs then migrate to the nucleus where they bind to cognate DNA binding sites and activate inflammatory gene

transcription (Calder, 2012). Eicosapentaenoic acid or fish oil decreased endotoxin-induced activation of NF- κ Bs in human monocytes and this was associated with decreased I κ B phosphorylation (Zhao *et al.*, 2004). Likewise, DHA reduced NF- κ Bs activation in response to endotoxin in cultured macrophages (Lee *et al.*, 2001) and dendritic cells (Kong *et al.*, 2010), an effect that involved decreased I κ B phosphorylation (Lee *et al.*, 2001). These observations suggest that marine *n*-3 PUFA may inhibit inflammatory gene expression via inhibition of activation of the transcription factor NF- κ Bs in response to exogenous inflammatory stimuli (Calder, 2012).

Another transcription factor that has been identified as being regulated by fatty acids is PPAR- γ (Three isoforms: PPAR- γ 1, PPAR- γ 2, and PPAR- γ 3. Jump, 2002). This is a member of the PPAR superfamily, which also includes PPAR- α and PPAR- β . These ligand-activated transcription factors were first found to be implicated in the regulation of lipid metabolism and homeostasis but have recently been shown to be involved in cell proliferation, cell differentiation, and inflammatory responses (Grimaldi, 2001). The preferred natural ligands of NF- κ B are PUFA, including LA, α -LNA, AA, and EPA (Houseknecht *et al.*, 2002). Proliferator-activated receptor- γ knockout mice show enhanced susceptibility to chemically-induced colitis (Desreumaux *et al.*, 2001) and PPAR- γ agonists reduce colitis in other mice models (Su *et al.*, 1999). Thus, up-regulation of PPAR- γ is a likely target for controlling inflammation (Desreumaux *et al.*, 2001). While PPAR- γ directly regulates inflammatory gene expression, it also interferes with the translocation of NF- κ B to the nucleus (Vanden Berghe *et al.*, 2003). Eicosapentaenoic acid has been shown to significantly increase PPAR- γ 1 messenger RNA concentrations in isolated adipocytes (Chambrier *et al.*, 2002). These effects were

linked to decreased production of the inflammatory cytokines TNF- α and IL-6 upon endotoxin stimulation (Kong *et al.*, 2010). Thus, activation of PPAR- γ may be one of the anti-inflammatory mechanisms of action of marine *n*-3 PUFA and this may be linked to the inhibition of NF- κ B activation described above (Calder, 2012).

Several G-protein coupled cell membrane receptors can bind fatty acids. These include GPR40 and GPR120. Inflammatory macrophages express GPR120 abundantly but do not express GPR40 (Oh *et al.*, 2010). A synthetic agonist of GPR120 inhibited the macrophage response to endotoxin, an effect that involved maintenance of cytosolic I κ B and a decrease in production of TNF- α and IL-6 (Oh *et al.*, 2010). These effects are similar to those of EPA and DHA and indicate that GPR120 may be involved in anti-inflammatory signaling. Oh et al. (2010) studied the effect of EPA and DHA on GPR120-mediated gene activation and found that both EPA and DHA enhanced the anti-inflammatory signaling. The ability of DHA to inhibit the responsiveness of macrophages to endotoxin was abolished in GPR120 knockout cells. These findings indicate that the inhibitory effect of DHA (and probably also of EPA) on NF- κ B occurs in part via GPR120. Thus, there appear to be at least two mechanisms by which marine *n*-3 PUFA inhibit NF- κ B activation—one involving GPR120 and the other involving PPAR- γ , although the two may be linked (Calder, 2012).

Besides the mechanisms discussed above, incorporation of *n*-3 PUFA in cell membrane phospholipids increases its fluidity, flexibility, compressibility and permeability (Neuringer *et al.*, 1988). Additionally, the activity of a number of enzymes are affected by changes in membrane fatty acid composition, including ornithine decarboxylase, 3-hydroxy-3-methylglutaryl coenzyme-A reductase, COX-2 and

lipoxygenases (Larsson *et al.*, 2004). Further studies are needed to identify new mechanisms and verify these mechanisms in animal models to better understand the effects of *n*-3 PUFA intake on animal health *in vivo*.

CHAPTER 2

MATERIALS AND METHODS

Experimental Animals and Diets

Forty crossbred pigs (averaging 27 ± 2 d of age and 7.8 ± 0.6 kg of BW) were balanced for initial BW and gender across two treatment groups in a complete randomized block design. The animal protocol for this research was approved by the institutional Animal Research Committee (ARC) of the University of Florida. To avoid potential differences due to farrowing season, the study was conducted using piglets born within one week at the Swine Research Unit of the University of Florida (Gainesville, FL) during the month of March 2013. Piglets were housed in 8 pens (5 animals per pen; size = 2.4 m x 1.8 m).

Table 2-1. Ingredient and calculated compositions of experimental diets (as-fed basis)

Composition	<u>Experimental diets^a</u>	
	Control	Omega
Corn	61.90	61.90
Soybean meal	25.00	25.00
Vegetable oil	3.00	-
Gromega	-	3.00
Min.- Vit. mix	10.00	10.00
L-Lysine.HCL	0.10	0.10
Calculated composition		
ME, kcal/kg	3282.38	3282.38
CP, %	19.53	19.53
CF, %	3.39	3.39
Lysine, %	1.40	1.40
Calcium, %	0.78	0.97
Phosphorus, %	0.63	0.63

^aDiets were: Control (3% vegetable oil) and omega (3% Gromega Ultra 345, provided by JBS United, Inc., Sheridan, IN).

Four pens were assigned to receive a control diet (3% vegetable oil, n = 20), while the other 4 pens were assigned to receive the n-3 PUFA -supplemented (3% Gromega, JBS United, Sheridan IN, n = 20) diet for four weeks. Piglets were provided ad libitum access to feed and water and the nursery temperature was maintained within the thermoneutral zone for the piglets.

Table 2-2. Fatty acid profile (g/100 g of total fat) of experimental diets (as-fed)^a

Experimental Diets^b

Fatty acid	Control	Omega
C14:0	0.21	2.61
C15:0	0.00	2.61
C16:0	14.68	19.76
C16:1,9c	0.32	2.98
C17:0	0.13	0.42
C17:1	0.00	0.39
C18:0	4.26	4.71
C18:1,9c	24.72	23.89
C18:2n-6	49.79	37.04
C18:3n-3	4.53	2.15
C18:4n-3	0.00	0.47
C20:0	0.38	0.40
C20:1n-9	0.00	0.71
C20:5n-3	0.00	1.30
C22:0	0.41	0.26
C22:5n-3	0.00	0.26
C22:6n-3	0.00	0.96
C24:0	0.26	0.34
$\Sigma n - 6$	49.79	37.04
$\Sigma n - 3$	4.53	5.54
$\Sigma n - 6/\Sigma n - 3$	10.99	6.69
ΣSFA	20.33	28.79
ΣUFA	79.36	70.15

^aFatty acid analysis was performed by the University of Missouri Analytical Laboratory.

^bDiets were: Control (3% vegetable oil) and omega (3% Gromega Ultra 345, provided by JBS United, Inc., Sheridan, IN).

The n-3 PUFA- and vegetable oil-enriched diets were formulated to meet the NRC (2012) nutrient requirements, and were based primarily on corn, soybean meal, vitamin and mineral premix and L-lysine supplements which were calculated to contain 3,282.38 Kcal/g ME, 19.53% CP, 3.39% CF and 1.40% Lys. Complete ingredient compositions and FA profiles of experimental diets are summarized in Tables 2-1 and 2-2, respectively.

Measurement of Growth and Feed Intake

Individual piglet body weights and pen feed consumption were recorded weekly throughout the 4-week experiment. These observations were used to calculate average daily gain and average daily feed intake. The G:F ratios were calculated to estimate feed efficiency.

Blood Collection and Analysis

On d 0, 14 and 28 of the experiment, jugular venous blood samples (~8 mL from each experimental pig) were collected into evacuated heparinized tubes (BD Franklin Lakes, NJ) and centrifuged (3,000 x g for 15 min) to separate plasma. The plasma samples were stored at -80°C until analysis. Concentrations of IGF-I and TNF- α in plasma were analyzed using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN). Hormone and cytokine analyses were performed in single assays and intra-assay CV were 4.0 and 4.7% for IGF-I and TNF- α , respectively. The least detectable concentrations were 0.06 ng/mL for IGF-1 and 5.50 pg/ML for TNF- α , respectively. On d 27 of the experiment, additional blood samples were collected for complete blood cell counts (CBC), and hematological traits were determined as described by Quiroz-Rocha *et al.* (2009).

Fecal Evaluation

Fecal scores were measured at each pen on weeks 1, 2, 3, and 4 post-weaning. The scale used to assess fecal consistency was based on a numerical scale of 1 to 3, where 1 represented a normal (hard) feces, 2 represented a soft (moist) feces, and 3 represented diarrhea (watery liquid). The final score for each pen was calculated by averaging the two fecal consistency scores.

Statistical Analysis

Effects of diets on growth, IGF-I, TNF- α and fecal characteristics were analyzed using the MIXED procedure of SAS with repeated measures (Littell *et al.*, 1998). For individual measurements (body weight), fixed effects included diet, sex, diet \times sex interaction, week after weaning, diet \times week interaction, sex \times week interaction and diet \times sex \times week interaction. The pig, nested within sex and diet, was considered a random variable, and therefore the pig variance was used to test the effects of diet, sex, and diet \times sex interaction. A similar model was used to test the effect of diet on plasma IGF-I and TNF- α concentrations, except that week after weaning was replaced by day of blood sample collection. For collective measurements (feed intake, average daily gain, feed efficiency, and fecal consistency scores), the statistical model included the independent effects diets, pen (diet), week relative to weaning, diet \times week interaction. In these models, the pen was used as experimental unit to test the main effect of diet. Single blood samples were collected for CBC counts, and, therefore, the statistical models for blood counts contained only the main effect of diet. Significant differences were declared at $P < 0.05$ and tendencies were discussed at P greater than 0.05 but less than 0.10.

CHAPTER 3 RESULTS

Body Weight

Body weight increased ($P < 0.01$) from 8.0 ± 0.6 to 16.5 ± 0.6 kg between weeks 0 (week 0 = week of weaning) and 4 in male pigs fed the vegetable oil-enriched diet and from 8.0 ± 0.6 to 17.6 ± 0.6 kg in the males consuming the *n*-3 PUFA-supplemented diet (Figure 3-1A).

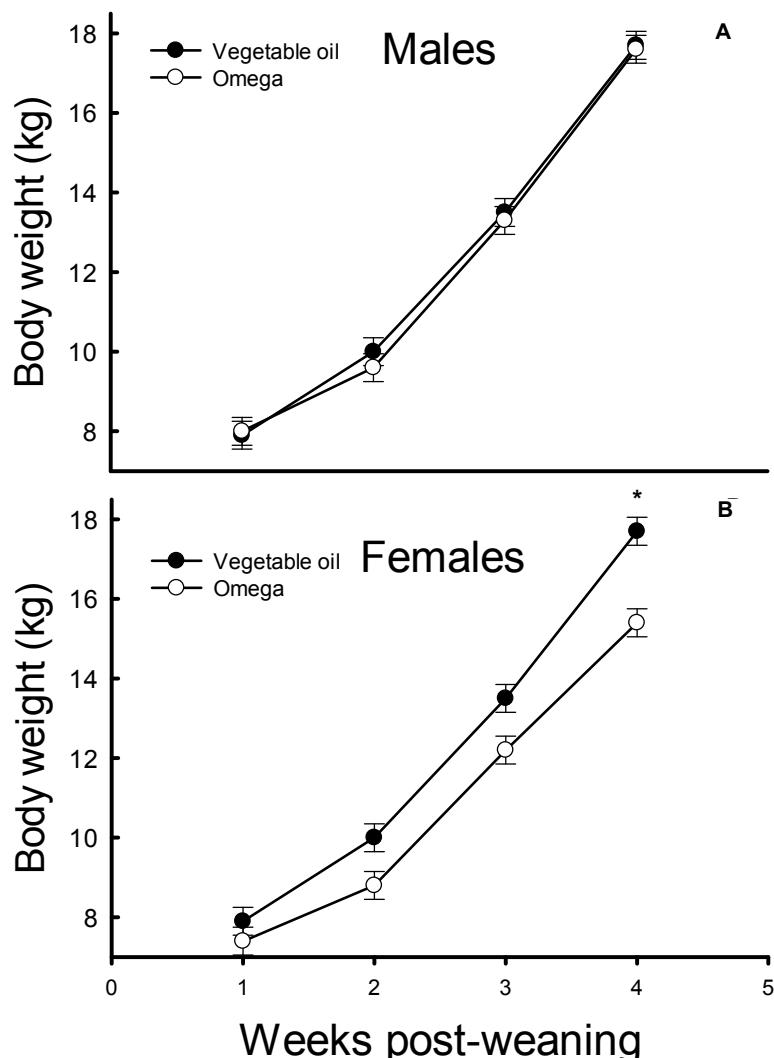


Figure 3-1. Body weights of male (A) and female (B) pigs fed diets enriched with vegetable oil ($n = 20$) or *n*-3 PUFA ($n = 20$) for four weeks after weaning. Asterisk indicates significant difference between the least squares means at the specified collection week.

Corresponding values for female piglets were 7.9 ± 0.6 and 17.7 ± 0.6 kg for the vegetable oil-enriched diet, and 7.4 ± 0.6 to 15.4 ± 0.6 kg for the *n*-3 PUFA-supplemented diet (Figure 3-1B). Female piglets fed the *n*-3 PUFA-enriched diet were lighter ($P < 0.04$) than those consuming the vegetable oil-enriched diet at week 4 post-weaning (Figure 3-1B).

Average Daily Gain, Feed Intake and Feed Efficiency

Newly weaned pigs gained ($P < 0.01$) 529.6 ± 9.8 g between weeks 2 and 4 post-weaning (Figure 3-2). Corresponding values for average daily feed intake and gain feed ratio were 800.6 ± 12.6 and 0.66 ± 0.02 , respectively (Figures 4 and 5). Under conditions of this study, inclusion of 3% *n*-3 PUFA in the diet did not affect daily gain, feed intake or feed efficiency.

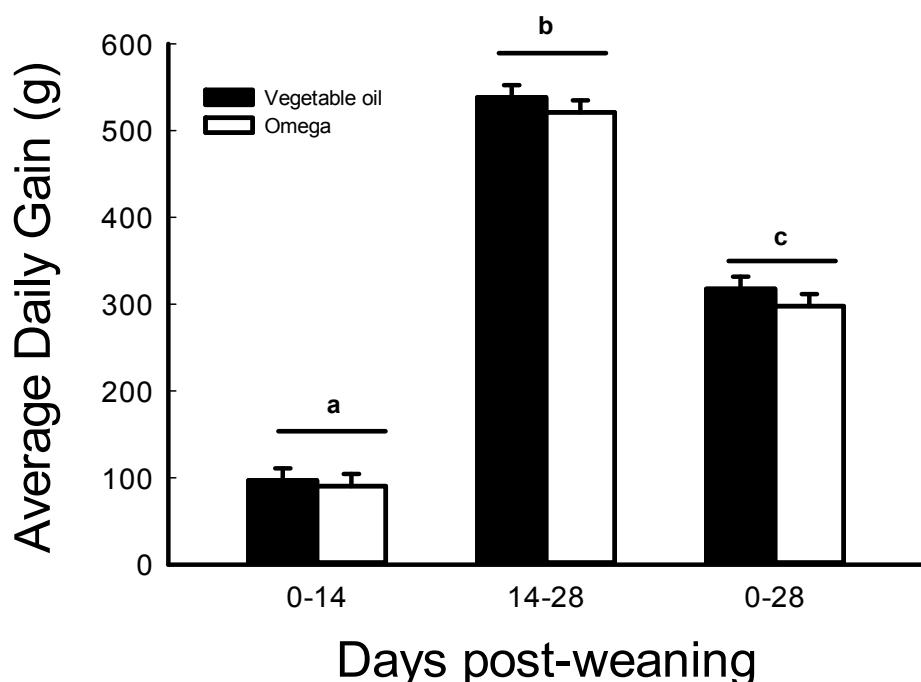


Figure 3-2. Average daily gains of weanling piglets fed diets enriched with vegetable oil ($n = 20$) or *n*-3 PUFA ($n = 20$). Pairs of histograms with different superscripts differ at $P < 0.01$. There were no differences in daily weight gain due to the diet.

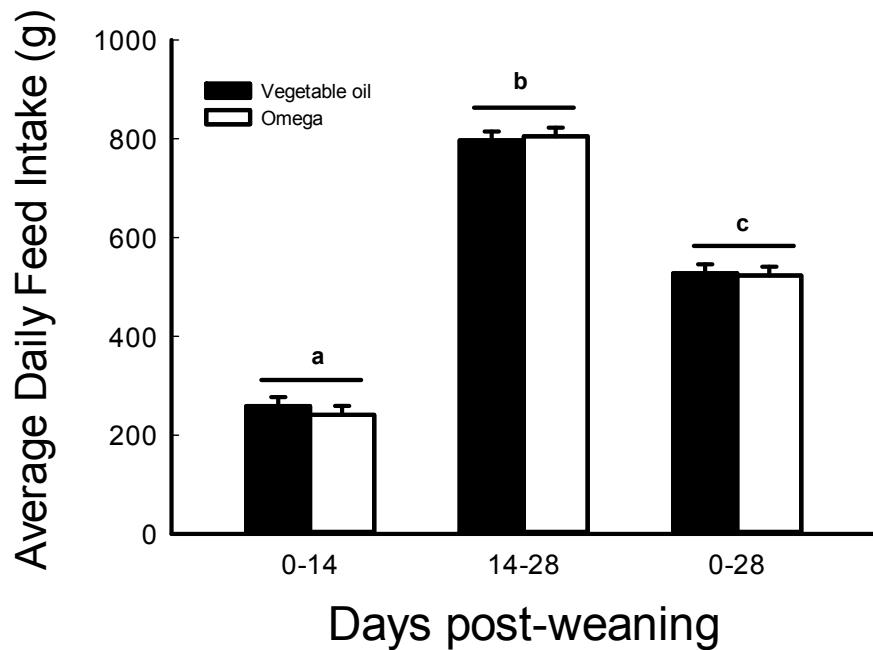


Figure 3-3. Average daily feed intakes of weanling piglets fed diets enriched with vegetable oil ($n = 20$) or n -3 PUFA ($n = 20$). Pairs of histograms with different superscripts differ at $P < 0.01$. There were no differences in daily feed intake due to the diet.

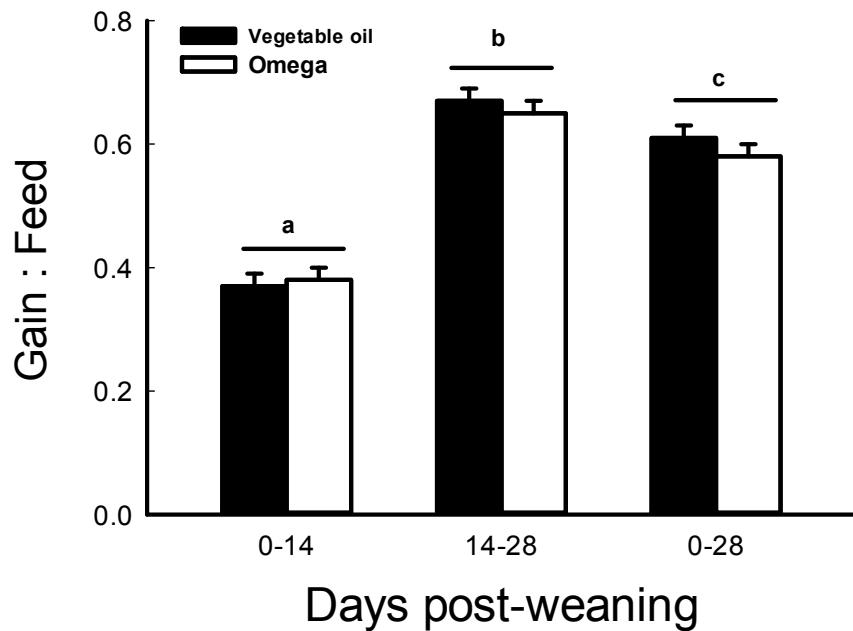


Figure 3-4. Average gain : feed of weanling piglets fed diets enriched with vegetable oil ($n = 20$) or n -3 PUFA ($n = 20$). Pairs of histograms with different superscripts differ at $P < 0.01$. There were no differences in gain : feed ratio due to the diet.

Plasma IGF-I and TNF- α

Concentrations of IGF-I and TNF- α in plasma are presented in Figures 3-5A and 3-5B. Peripheral IGF-I concentration decreased ($P < 0.01$) from 87.2 ± 17.0 ng/mL to 68.3 ± 21.1 ng/mL between days 0 and 14 post-weaning, and then increased to 155.2 ± 20.9 ng/mL at d 28. There were no differences in peripheral IGF-I concentration due to diet or gender of the piglet (Figure 3-5A).

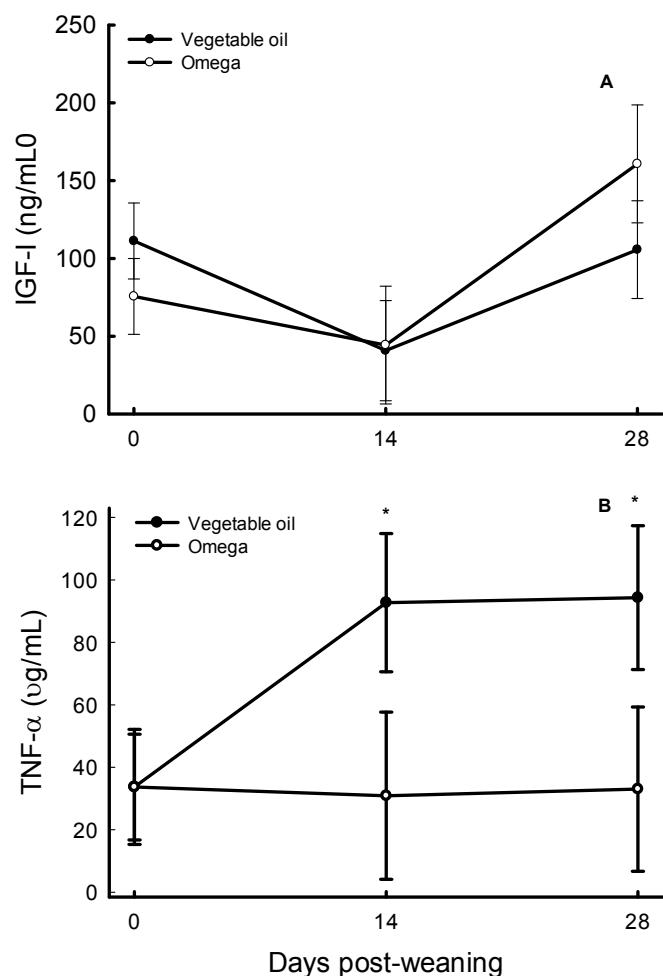


Figure 3-5. Concentrations of IGF-I (A) and TNF- α (B) in plasma of weanling piglets fed diets enriched with vegetable oil ($n = 20$) or n -3 PUFA ($n = 20$). Plasma IGF-I concentration was affected by day of blood collection ($P < 0.01$), but not dietary treatment ($P > 0.44$). Plasma TNF- α concentration was affected by dietary treatment ($P < 0.01$), but not day of blood collection ($P > 0.28$). Asterisks indicate significant difference ($P < 0.01$) between the least squares means at the specified collection week.

There was a positive correlation between plasma IGF-I concentration and body weight (Pearson's correlation coefficient = +0.56, $P < 0.01$). This positive relationship persisted after adjustment for the dietary effect (partial correlation coefficient = +0.56; $P < 0.01$). In piglets fed the vegetable oil-enriched diet, became elevated ($P < 0.01$) plasma TNF- α concentration between days 0 and 14 post-weaning (37.6 ± 14.5 pg/mL $< 102.9 \pm 16.6$ pg/mL; Figure 3-5B). Contrastly, pigs fed the *n*-3 PUFA diet showed no increase in TNF- α concentration between day 0 and 14 and thus, a diet effect ($P < 0.05$) was noted for TNF- α (Figure 3-5B).

Hematological and Fecal Characteristics

Blood platelet and eosinophils concentration was higher ($P < 0.05$) in weanling piglets fed the *n*-3 PUFA-enriched diet as compared to those consuming the vegetable oil-enriched diet ($674.0 \pm 80.4 \times 10^3 / \text{mm}^3 > 378.5 \pm 80.4 \times 10^3 / \text{mm}^3$, $P < 0.05$; $0.438 \pm 0.05 \% > 0.208 \pm 0.05 \%$, $P < 0.05$). Dietary *n*-3 PUFA had no detectable effects on the other hematological traits (Table 3-1).

Table 3-1. Hematological traits of weanling pigs fed diets with vegetable oil or *n*-3 PUFA^a

Trait	Experiment Diets ^b			<i>P</i> ^c
	Control	Omega	SEM	
WBC ^d 03 / mm ³	9.7	15.7	2.3	0.13
Lymphocytes, %	5.9	6.5	0.5	0.44
Neutrophils, %	7.6	8.0	1.0	0.81
Eosinophils, %	0.2	0.4	0.0	0.02
Monocytes, %	0.4	0.7	0.1	0.20
RBC ^e x 10 ³ / mm ³	9.7	6.6	2.0	0.32
Hemoglobin	10.4	9.2	0.7	0.28
Hematocrit, %	34.1	31.4	2.1	0.37
Platelets x 10 ³ / mm ³	378.5	674.0	80.4	0.04

^aMeans represent 4 pigs per dietary treatment.

^bDiets were: Control (3% vegetable oil) and omega (3% Gromega Ultra 345, provided by JBS United, Inc., Sheridan, IN).

^cP-values for control compared to Omega diet.

^dWhite blood cells.

^eRed blood cells.

Table 3-2. Fecal consistency scores^a of weanling pigs fed diets with vegetable oil or n-3 PUFA^b

Week Post-weaning	<u>Experimental diets^c</u>		SEM	P ^d
	Control	Omega		
1	2.6	2.5	0.2	0.67
2	1.9	1.9	0.2	1.00
3	2.0	1.9	0.2	0.68
4	1.3	1.1	0.2	0.68

^aThe scale used for assessing fecal consistency was based on a numerical scale of 1 to 3, where 1 represented a normal (hard) feces, 2 represented a soft moist feces, and 3 represented diarrhea (watery liquid).

^bMeans represent average fecal scores for 4 pens per dietary treatment.

^cDiets were: Control (3% vegetable oil) and omega (3% Gromega Ultra 345, provided by JBS United, Inc., Sheridan, IN).

^dP-values for control compared to Omega diet.

Fecal consistency score decreased ($P < 0.01$) from 2.6 ± 0.1 at week 1 to 1.2 ± 0.1 at week 4, but did not differ between the two dietary treatments (Table 3-2). There were no differences in fecal consistency scores due to the diets.

CHAPTER 4 DISCUSSION

Weaning imposes tremendous stress on piglets and is accompanied by marked changes in gastrointestinal physiology, microbiology and immunology (Pluske *et al.*, 1997; Brooks *et al.*, 2001). The biochemical and histological changes that occur in the small intestine cause excessive secretion of pro-inflammatory cytokines and induce severe intestinal inflammation. *Omega-3 PUFA* are known to possess anti-inflammatory properties in humans (Calder, 2010, 2012), swine (Carroll *et al.*, 2003; Liu *et al.*, 2003; Mateo *et al.*, 2009) and chickens (Korver and Klansing, 1997). To test the hypothesis that nutritional management strategies that attenuate intestinal inflammation may repartition nutrients to tissue accretion, we examined the effects of dietary *n-3 PUFA* on growth and immune responses of weanling pigs raised without an added bacterial or environmental challenge.

Inclusion of 3 % *n-3 PUFA* in the weanling piglet's diet did not significantly improve average daily gain, average daily feed intake or gain:feed ratio. Our results are consistent with an earlier study (Eastwood *et al.*, 2009) which detected no linear or quadratic effects of dietary flax seed meal (rich in alpha linolenic acid, an *n-3 PUFA*) on basal body weight gain, feed intake or feed efficiency in weanling pigs. These findings indicate that, in the absence of significant immunological challenges, dietary *n-3 PUFA* do not affect feed intake and growth response in nursery pigs (Liu *et al.*, 2003). Additionally, the fact that the control diet contained a relatively high amount of *n-3 PUFA* (Table 3) may have prevented us from detecting true *n-3 PUFA* effects on growth parameters. The improvement of body weight gain detected between days 14 and 28 post-weaning (Figure 3) likely resulted from an increase in feed intake and a decrease

in basal inflammatory challenges during the second phase of growth. The observation that female piglets consuming the *n*-3 PUFA-supplemented diet were lighter at week 4 than those consuming the vegetable oil-enriched diet, the mechanism warrants further investigation.

Peripheral concentration of IGF-I decreased immediately following weaning and increased again by day 28 post-weaning. These findings indicate that weaning causes a significant metabolic stress in weanling pigs and that this stress decreases with increasing weeks after weaning. There is little information on the effect of dietary *n*-3 PUFA on peripheral concentration of growth factors in the pig. In the present study, inclusion of 3 % *n*-3 PUFA into the piglet's diet had no detectable effects on plasma IGF-I concentration during the first four weeks after weaning. These observations are consistent with previous studies (Carroll *et al.*, 2003; Liu *et al.*, 2003) which showed no beneficial effects of dietary fish oil on basal IGF-I concentration in weaned pigs. Thissen and Verniers (1997) reported that interleukin-6 (IL-6) and TNF- α decreased both growth hormone (GH) and IGF-I mRNA in rat hepatocyte primary cultures. We did not examine GH or IGF-I transcript modulation by inflammatory cytokines, and therefore, whether or not the lack of *n*-3 PUFA effects on plasma IGF-I concentration detected in the present study was indicative of cytokine-mediated uncoupling of GH and IGF-I gene expression in weanling pigs warrants further investigation.

Tumor necrosis factor- α , a cytokine produced primarily by monocytes and macrophages, is thought to be one of the principal mediators of inflammation (Bemelmans *et al.*, 1994). In the present study, plasma TNF- α concentrations were lower in weanling piglets supplemented with *n*-3 PUFA than those fed the vegetable oil

supplement. These findings are consistent with previous *in vitro* (Lo *et al.*, 1999; Novak *et al.*, 2003; Zhao *et al.*, 2004) and *in vivo* (Carroll *et al.*, 2003; Gaines *et al.*, 2003; Malekshahi Moghadam *et al.*, 2012) studies and suggest that *n*-3 PUFA inclusion in the diet could significantly improve the piglets' immune status. Whereas exact mechanisms of *n*-3 PUFA suppression of TNF- α are yet to be fully elucidated, we speculate that suppression of TNF- α production by *n*-3 PUFA may be attributed, in part, to their inhibitory effects on the nuclear factor kappa B (NF- κ B) activation and or translocation to the nucleus (Chong-Jeh *et al.*, 1999; Novak *et al.*, 2003; Zhao *et al.*, 2004). Nuclear factor- κ Bs are normally confined in the cytoplasm through their association with I κ Bs. When cells are activated by inflammatory stimuli, the I κ Bs are rapidly phosphorylated and degraded to free the NF- κ Bs. The free NF- κ Bs then migrate to the nucleus where they bind to cognate DNA binding sites and activate inflammatory gene transcription (Calder, 2012). On the other hand, eicosapentaenoic acid is a substrate for prostanoids of the 3 and 5-series, which are less inflammatory than arachidonic acid (AA)-derived prostaglandins. Additionally, eicosapentaenoic acid was shown to significantly increase PPAR- γ 1 messenger RNA levels in isolated adipocytes (Chambrier *et al.*, 2002). These effects were linked to decreased production of the inflammatory cytokines TNF- α and IL-6 upon endotoxin stimulation (Kong *et al.*, 2010). Thus, activation of PPAR- γ may be another intracellular mechanism by which marine *n*-3 PUFA regulate NF- κ B activation and TNF- α production in cell models (Calder, 2012).

Hematological traits of swine are influenced by a variety of environmental and physiological factors including diet, age, sex and housing (Wilson *et al.*, 1972; Friendship *et al.*, 1984). In the present study, most of the blood characteristics

examined did not differ between pigs fed the two dietary treatments (Table 4). Blood samples for complete blood cell count (CBC) were collected at 4 weeks after weaning, and it is possible that by this sampling time, the weanling piglets had already recovered from most physiological and dietary challenges normally associated with weaning in pigs. Alternatively, the piglets used in this study were raised in a thoroughly cleaned environment (pressured washed followed by steam cleaning and then sprayed with a disinfectant) and may not have acquired adequate intestinal that would cause clinical disease. This hypothesis was further supported by our inability to detect salmonella and enterotoxigenic *E. coli* in fecal samples collected at week 4 post-weaning (data not shown).

CHAPTER 5 CONCLUSION

In the pig, the period following weaning is generally characterized by sub-optimal growth, deteriorated feed efficiency, and a high incidence of diarrhea (Heo *et al.*, 2012; Pluske *et al.*, 2013). Results of this study provided no evidence for *n*-3 PUFA modulation of growth or hematological parameters of nursery piglets raised in the absence of significant immunological and environmental challenges, but might decrease the female piglets' growth rate. However, dietary *n*-3 PUFA may improve the immune status of weanling pigs, as reflected by considerably lower plasma TNF- α in pigs consuming *n*-3 PUFA than those fed vegetable oil. Furthermore, the gradual increase of body weight, feed intake and feed efficiency following weaning may reflect a slow adaptation to post-weaning diets and a gradual improvement of the gastrointestinal microbiota.

APPENDIX
LEAST SQUARES MEANS SIGNIFICANT DATA

Table A-1. Least squares means (+SEM) for body weights (kg) of male and female pigs fed diets enriched with vegetable oil ($n = 20$) or n-3 PUFA ($n = 20$)

Week post-weaning	Male pigs		Female pigs	
	Vegetable oil	Omega	Vegetable oil	Omega
1	8.0 + 0.6	8.0 + 0.6	7.9 + 0.6	7.3 + 0.6
2	9.2 + 0.6	9.6 + 0.6	9.9 + 0.6	8.8 + 0.6
3	12.2 + 0.6	13.3 + 0.6	13.5 + 0.6	12.2 + 0.6
4	16.5 + 0.6	17.6 + 0.6	17.7 + 0.6	15.4 + 0.6

Table A-2. Least squares means (+SEM) for daily body weight gains (g) of weanling pigs fed diets enriched with vegetable oil ($n = 20$) or n-3 PUFA ($n = 20$)

Days post-weaning	Vegetable oil	Omega
0-14	97.0 + 13.9	90.5 + 13.9
14-28	538.4 + 13.9	520.9 + 13.9
0-28	317.7 + 13.9	298.7 + 13.9

Table A-3. Least squares means (+SEM) for daily feed intakes (g) of weanling pigs fed diets enriched with vegetable oil ($n = 20$) or n-3 PUFA ($n = 20$)

Days post-weaning	Vegetable oil	Omega
0-14	258.8 + 17.8	241.1 + 17.8
14-28	796.6 + 17.8	804.7 + 17.8
0-28	527.7 + 17.8	522.9 + 17.8

Table A-4. Least squares means (+SEM) for gain : feed ratios of weanling pigs fed diets enriched with vegetable oil ($n = 20$) or n -3 PUFA ($n = 20$)

Days post-weaning	Vegetable oil	Omega
0-14	0.37 + 0.02	0.38 + 0.02
14-28	0.68 + 0.02	0.65 + 0.02
0-28	0.61 + 0.02	0.58 + 0.02

Table A-5. Least squares means (\pm SEM) for plasma insulin-like growth factor-I (IGF-I) and tumor necrosis factor alpha (TNF- α) concentrations in weanling pigs fed diets enriched with vegetable oil ($n = 20$) or n -3 PUFA ($n = 20$)

Days post-weaning	IGF-I		TNF- α	
	<u>Vegetable oil</u>	<u>Omega</u>	<u>Vegetable oil</u>	<u>Omega</u>
0	93.8 \pm 22.7	80.6 \pm 25.4	37.6 \pm 14.5	33.0 \pm 17.2
14	84.9 \pm 26.0	51.7 \pm 33.2	102.9 \pm 16.6	29.1 \pm 21.7
28	142.3 \pm 25.4	168.1 \pm 33.2	99.0 \pm 17.2	36.7 \pm 21.4

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BIOGRAPHICAL SKETCH

Qizhang Li was born in Nanjing, China, in 1987. She is daughter of Xiaosu Li and Minglang Xia. She graduated from Nanjing NO.13 high school in 2006, and started her Bachelor of Agriculture that same year at Yangzhou University in Animal Sciences Department. She graduated in 2010 and after that, she spent one year working as a laboratory technician in the same department. She joined the University of Florida In fall 2012, and began working on her Master of Science degree under the guidance of Dr. Lokenga Badinga. She graduated with a M.S. in animal sciences in 2014. Her research at the University of Florida has focused on the effects of *omega-3* polyunsaturated fatty acid on growth and immunity of weanling piglets.