Impact of Dietary Proteins on Growth Performance, Intestinal Morphology, and mRNA Abundance in Weanling Pigs

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(ABSTRACT)

The objectives of these studies were to investigate the effects of two special proteins, spray-dried plasma protein (SDPP), a high quality protein source, and Peptiva®, a mixture of peptides manufactured from marine products, on growth performance, nitrogen balance and enzyme and nutrient transporter mRNA expression in the brushborder membrane in weanling pigs. The results indicated that 6 % SDPP increased ADG and ADFI in the first 10 d after weaning ($P < 0.05$) without carry-over benefits in subsequent phases. There were potential additive effects of SDPP and Cu on growth promotion. Trends for interaction of diet and pen sanitation were observed for G:F with more pronounced response to SDPP ($P = 0.07$) and Cu ($P = 0.11$) supplementation in the sub-sanitary pens. In the duodenum, reduced crypt depth with Cu supplementation ($P < 0.01$) and a trend for greater villous length with SDPP supplementation ($P = 0.09$) were observed. Pigs reared in the sub-sanitary pens had lower ADG ($P < 0.05$) as well as shorter villous length and less crypt depth ($P < 0.05$) than those from sanitary pens.

To investigate the potential impact of dietary proteins on gene expression in the intestine, 54 weanling pigs were fed either 6 % SDPP, 0.5 % Peptiva®, or soy control diets, and were killed 3 or 10 d after weaning. Northern blot results revealed significant diet by intestinal segment interactions ($P < 0.05$) for aminopeptidase A and aminopeptidase N. Aminopeptidase A was evenly distributed along the small intestine in the Peptiva® group, but decreased dramatically in the ileum in other groups. Aminopeptidase N increased from the proximal to the distal intestine in the soy protein and SDPP groups, whereas in the Peptiva® group, relative abundance was highest in the jejunum and lowest in the duodenum. Most of the enzyme and nutrient transporter mRNA abundance was observed in the distal segements of the small intestine and changed as the animals matured. Due to the low abundance of cytokine mRNA expression in the intestine, mRNA levels of cytokine were quantified by Real-Time PCR. The results indicated that the pigs fed the SDPP diet tended to have lower pro-inflammatory cytokine IL-1-β and TNF-α compared to other treatments. Tumor necrosis factor-α and IL-10 mRNA abundance increased from the proximal to the distal intestine, and was higher ($P < 0.05$) in the ileum than in the duodenum and jejunum. The mRNA abundance of IL-1-β, IL-10, and TNF-α also increased as the animals matured ($P < 0.01$). In summary, SDPP increased growth performance of weanling pigs, which were associated with changes in intestinal morphology and function. Peptiva® influenced aminopeptidases distribution along the small intestine. The mRNA abundance for digestive enzymes, nutrient transporters, and cytokines were differentially regulated along the small intestine as pigs matured.

Key Words: Cytokine, Enzyme, Peptide, Pigs, Spray-dried plasma protein, Transporter.

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Chapter I

Introduction

Protein is a critical nutrient for animal growth and health. The quality of protein depends on AA balance as well as digestion and absorption in the small intestine. Soy protein is the most commonly used protein source in the swine industry. However, young pigs are not well adapted to soy protein because of the presence of anti-nutritional factors, such as glycinin and β-conglycinin (Sissons and Smit, 1976) and the immaturity of the digestive system. Spray-dried plasma protein (SDPP) has proven to be a high-quality protein source for weanling pigs. Most prior studies indicated that SDPP consistently improved growth performance when included in phase I pig diets at the expense of dried skim milk (Kat et al., 1994; de Rodas et al., 1995), soybean meal (Fakler et al., 1993; Coffey and Cromwell, 1994) or whey (Hansen et al., 1993). Reported effects of SDPP on G:F were less consistent than those reports for weight gain and feed intake, because generally, increased feed intake can partly explain the improved weight gain. Animal nutritionists are very interested to know the mode of action of SDPP and to find appropriate substitutes for SDPP, due to the high cost of SDPP and public concerns about feeding animal products back to animals. Since the identification of peptide transporters and increased efforts directed at peptide research, utilization of small peptides as a protein source in the animal industry becomes a reality; however, limited information regarding their effects on weanling pig performance is available.

The primary function of the small intestine is to extract nutrients from food and pass them into the body in a usable form. Another important function is to defend against potentially harmful microorganisms, while at the same time allowing unimpeded access to equally foreign materials ingested as food. In addition, the intestinal mucosal immune system must distinguish

between pathogenic microorganisms that enter from the external environment and the resident microflora that colonize the intestine.

The small intestine is the major site of digestion and absorption, and is also one of the first organs directly exposed to the luminal environment. Luminal nutrients, in addition to being substrates for digestion by their corresponding enzymes, can function as extracellular regulatory signals for digestive enzymes and gene expression in epithelial cells. Both micro (minerals and vitamins) and macronutrients (AA and fatty acids) have been proven to be regulation signals in manipulating gene expression at the transcriptional and translational level (Fafournoux et al., 2000; Paoloni-Giacobino et al., 2003). Manipulating mucosal immune function through dietary changes is also of interest to scientists. It has been proposed that SDPP may benefit the intestinal immune system because of a high concentration of immunoglobulin. The objectives of this study were to investigate the effects of the special protein sources (soy protein, SDPP, and a commercial peptide source) on growth performance, nitrogen balance, and intestinal morphology in weanling pigs, the potential dietary protein and ontogenetic regulation of membrane-bound enzyme and nutrient transporter mRNA expression, and the effects of dietary protein sources on cytokine gene expression in the small intestine.

Chapter II

Literature Review

Special Protein Sources for Weanling Pigs

Benefits of Spray-Dried Plasma Protein in Weanling Pigs

In modern swine production systems, weaning pigs from the sow occurs at a very early age. In most production settings, pigs are weaned at less than three weeks of age and in many systems weaning age ranges from 12 to 18 days (Maxwell and Carter, 2001). This transition from a diet of sow's milk to a diet of dry feed in a new housing environment is associated with a period of reduced feed consumption and slow growth.

Spray-dried plasma protein (SDPP) has been identified to be a high quality protein source for young weanling pigs. Most previous studies indicated that SDPP supplementation in diets improved growth performance of animals, especially when they are reared in sub-optimal or under stressful conditions. Many researchers have attempted to understand the mode of action of dietary SDPP in early weanling pigs. The high cost of SDPP and public concern of feeding animal products back to animals create an important need to understand the physiological pathways that may respond to SDPP feeding in weanling pigs, and to find potential substitutes for SDPP supplementation in weanling pig diets. Several potential physiological pathways have been proposed, including high palatability, good protein quality and high digestibility, benefits to the immune system, and protection of the small intestine from bacterial attachment.

Feed Intake. It is well known that inclusion of SDPP in the diet of weanling pigs improves growth performance (Kats et al., 1994; Angulo and Cubilo, 1998; Grinstead et al., 2000), even though the range of improvement is variable depending on the age of piglets, the

level of SDPP added in the diet, and the environment in which the pigs are reared. The results on G:F are inconsistent; a few studies noted improvement in G:F (Gatnau and Zimmerman, 1991), while others did not (Hansen et al., 1993; de Rodas et al., 1995). Usually, the high level of ADFI can explain most of the SDPP-induced growth improvement. Studies by Gatnau and Zimmerman (1991), Sohn et al. (1991), Hansen et al. (1993) and de Rodas et al. (1995) suggested that the improvements in ADG associated with SDPP resulted from increased ADFI. Part of the increased ADFI is due to greater palatability, because weanling pigs prefer diets containing SDPP to those containing dried skim milk (Ermer et al., 1994). A multiple regression analysis of 68 separate experiments indicated that overall SDPP induced changes in ADG, ADFI, and G:F in the first two wk after weaning of + 26.8 %, + 24.5 %, and + 3.2 %, respectively (review by van Dijk et al., 2001a). The response of both ADG and ADFI is more or less consistent up to 6 % SDPP in the diet. As for G:F, the response to SDPP seems beneficial at levels below 6 % (van Dijk et al., 2001a). In order to separate the effects of improved weight gain from increased feed consumption, Jiang et al. (2000a) conducted a pair-fed study. Piglets were pair-fed a control diet containing extruded soy protein or 10 % SDPP for 24 d. The feed intake levels were controlled to be the same for both treatments. The results indicated that ADG and protein conversion efficiencies were greater in the SDPP group. This study provided direct evidence that increased feed intake or greater palatability is not the sole reason for improved growth performance with SDPP supplementation, since feed intake for both treatments was held constant.

Digestibility. SDPP is a high quality protein source for piglets, and may have high digestibility and nitrogen retention. Thomson et al. (1995) reported that nitrogen intake, digestibility and retention were greater for mice fed SDPP compared with a control group. In

contrast, studies by Hansen et al. (1993) reported lower DM and nitrogen digestibility for piglets consuming diets containing SDPP instead of dairy proteins. Knabe (1994) found low apparent ideal AA digestibility for SDPP. The results were in agreement with Chae et al. (1999), who reported lower digestibility of soybean meal and SDPP-based diets than a dried skim milk-based diet in 10 d old pigs. Several other studies have shown that increased growth performance by addition of SDPP was due to increased feed consumption rather than digestibility or nitrogen retention (Sohn et al., 1991; Hansen et al., 1993; Ermer et al., 1994). In a pair-feeding experiment, Jiang et al. (2000a) found that protein conversion efficiency was greater in pigs fed SDPP diets compared with those fed a soy protein control diet. The circulating urea concentration was 40 % lower in the SDPP-fed pigs compared to the control group. The authors suggested that SDPP increased the efficiency of dietary protein use for lean tissue growth and this response was mediated in part by decreased AA catabolism. Based on previous studies, it seems that enhanced growth performance is not always positively related to high digestibility or nitrogen retention, depending on what control diets were used. Generally, digestibility and nitrogen retention of SDPP diets are higher than soy-protein but lower than dairy protein (dried skim milk, dried whey). Therefore the improved growth performance observed with SDPP supplementation does not necessarily result in or relate to high digestibility or nitrogen retention.

Immunity and Insulin-like Growth Factor-1 (IGF-1). Spray-dried plasma protein contains 15 to 20 % immunoglobulins and 0.8 ng/mg immuno-active IGF-1 (Thomson et al., 1994). Although it is unlikely that the immunoglobulins presented in SDPP are absorbed across the intestinal wall in 2 to 4 wk old piglets, the high concentration of immunogluobulins may prevent bacterial attachment to epithelial cells and help maintain intestinal health and integrity. A reduction in damage to the intestinal wall could explain why Gatnau and Zimmerman (1991)

found a lower incidence of diarrhea in pigs fed a diet with 10 % SDPP than in those fed traditional protein sources. A more functional gut wall might also explain why the pigs housed in the conventional nursery responded more to SDPP than pigs housed in the cleaner, more environmentally controlled experimental nursery. Similarly, van Dijk et al. (2002) reported that pigs fed SDPP diets generally had a more favorable fecal score and a healthier appearance, even though the excretion of *E. coli* in feces was not reduced. The mortality of pigs was not reduced in SDPP fed piglets after a pathogenic *E. coli* challenge. Numerous studies (Stahly et al., 1994; van Dijk et al., 2001b; van Dijk et al., 2002) suggested that the improved ADG and ADFI by SDPP supplementation was more profound when piglets were in less favorable circumstances compared to non-stressed conditions and non-infected piglets. Gatnau (1990) and Thomson et al. (1994) divided the SDPP into three fractions (low molecular weight fraction, albumin, and immunoglobulin) to evaluate the mode of action of SDPP in weanling pigs. Both studies suggested that the beneficial effects of SDPP appear to be associated with the immunoglobulin fraction, because this fraction mimicked the benefits of SDPP in piglets. Some studies have indicated that the increased performance with SDPP supplementation is related to immunity, while others indicated that improved growth resulting from dietary SDPP supplementation was independent of immune challenge (Dritz et al., 1996) and antimicrobial agents (Cu and antibiotics) in the diets (Rojas et al., 1994; Coffey and Cromwell, 1995).

More recently, using molecular technology, Touchette et al. (2002) reported that feeding SDPP resulted in reduced tumor necrosis factor alpha (TNF-α) and IL-1-β mRNA expression in the adrenal gland, spleen, hypothalamus, pituitary gland, and liver. Expression of IL-6 mRNA was also reduced in the spleen and pituitary gland. These results demonstrate that the immune system appears to be less active with SDPP supplementation compared to pigs fed a diet without

SDPP. It is well known that immune activation always competes for nutrients, and animal growth performance is retarded as a result of immune activation. Decreased immune activation in the pigs fed SDPP may mean more energy and nutrients are available for growth. However, in the same study, pigs fed the SDPP diet exhibited an over-response of the immune system following lipopolysaccharide administration, resulting in major damage to the mucosa of the gastrointestinal tract compared to the control diet. No explanation was provided to interpret the results of this experiment. We suspect that different immune reactions to SDPP supplementation may be due to the level of pathogen challenge or pig health conditions. Spray-dried plasma protein may help inhibit immune activation under minor or moderate stress conditions, but can not be used as treatment to cure pathogenic diseases.

Spray-dried plasma protein contains high levels of IGF-1, but addition of 4 % SDPP to the diet did not increase circulating IGF-1 levels (de Rodas et al., 1995). However, the growth performance of piglets was improved. Plasma GH tended to be higher in pigs fed SDPP than in pigs fed soybean meal based diets. Whether the higher growth rate shown by pigs fed SDPP in this study is due to higher GH concentration remains to be determined.

Intestinal Morphology. Epithelial cells are the first line of dietary exposure, and they display dramatic adaptations to dietary changes. There are several ways that SDPP may affect intestinal morphology. First, the immunoglobulins contained in SDPP may prevent bacterial attachment and thus protect intestinal integrity. Secondly, pigs fed SDPP usually have higher feed intake. Failure to consume enough feed is one of the most significant problems of weaning stress, and it usually induces intestinal atrophy (Pluske et al., 1996a and 1996b). The local intestinal immunity also seems to have a direct impact on epithelial morphology. However, our knowledge of the effects of SDPP on intestinal morphology is very limited, and the results of

previous studies are inconsistent. An experiment conducted by Touchette et al. (2002) indicated that feeding SDPP did not affect villous height but reduced crypt depth in pigs. Spencer et al. (1997) reported that pigs fed a SDPP diet had longer villi and a greater villous length to crypt depth ratio (VCR) than pigs fed a no-SDPP diet. On the other hand, Jiang et al. (2000b) reported that SDPP did not affect crypt cell proliferation, crypt depth or villous height, but instead reduced intravillous lamina propria cell concentration. The total intestinal mass, jejunum and ileum protein and DNA mass were reduced with SDPP supplementation, compared to the soybean meal control group (Spencer et al., 1997). Further research is necessary for understanding the effects of SDPP on intestinal morphology and the underlying mechanisms.

High Dietary Copper in Weanling Pigs

Copper is an essential trace element for all classes of swine. Weanling piglets have a dietary requirement of 5 to 7 ppm to prevent deficiency symptoms and support normal growth. However, it is well established that elevated dietary Cu supplementation within the range of 100 to 250 ppm generally results in growth promoting effects in weanling pigs (Cromwell et al., 1989; Davis et al., 2002; Armstrong et al., 2004), much like that seen with sub-therapeutic levels of antibiotic feed additives (Stahly et al., 1980; Roof and Mahan, 1982; Edmonds et al., 1985). However, feeding dietary Cu levels in excess of 250 ppm for extended periods of time may result in Cu toxicity. Furthermore, elevated dietary Cu levels result in elevated tissue levels and elevated fecal excretion (NRC, 1998). The results of Roof and Mahan (1982) indicated that addition of 250 ppm Cu as $CuSO_4$ resulted in an approximately 14-fold increase in fecal Cu excretion.

Organic complexes of Cu have become commercially available and may be more bioavailable and more effective in stimulating growth in pigs. For example, Coffey et al. (1994)

demonstrated that 100 ppm supplemental Cu from a commercial Cu-lysine complex provided the same magnitude of growth promotion in weanling pigs as 200 ppm Cu from $CuSO_4$. Recently Harper et al. (2001) reported that 200 ppm supplemental Cu from an organic Cu complex (Bioplex® Copper, Alltech Inc.) appeared to be more efficacious in growth stimulation for weanling pigs than 200 ppm $CuSO_4$. The study report also suggested that some level of Cu supplementation from Bioplex® intermediate between 50 and 200 ppm may elicit growth promotion responses while reducing fecal Cu excretion below that observed with higher supplementation rates.

It was originally proposed that elevated dietary Cu stimulated growth through an antibacterial-like action in the gastrointestinal tract (Fuller et al., 1960; Cromwell et al., 1989). The results of Shurson et al. (1990) suggested a strong physiological relationship between microbiological status and the impact of dietary Cu in pigs. High dietary Cu improved the growth performance in conventionally reared pigs, but reduced the ADG and ADFI in germ-free pigs. The villous height and crypt depth were reduced by high dietary Cu in germ-free pigs, and the effect was reversed in conventionally reared pigs. Based on this data, elevated dietary Cu appears have a direct impact on intestinal microflora. More recent evidence suggests that Cu may act systemically as well (Zhou et al., 1994) because intravenous injection of Cu, which by-passes the gastrointestinal tract, had similar impacts as high dietary Cu. Indeed, a correlation between growth stimulation and Cu source bioavailability has been observed (Cromwell et al., 1989). Other potential growth promoting actions of Cu include stimulation of small intestinal lipase and phospholipase A activities (Luo and Dove, 1996), and increases in apparent DM digestibility, fat digestibility, and nitrogen retention (Dove, 1995; Luo and Dove, 1996). Radechi et al. (1992) reported that the turnover rate of jejunal mucosa was reduced and the

intestinal cell generation interval was longer in pigs fed 250 ppm Cu. The reduced turnover rate of intestinal mucosa may result in lower energy requirements for the maintenance of the small intestine (Radechi et al., 1992). Consequently, more nutrients and energy are available for growth.

Potential Additive Effects of SDPP and Cu under Different Environments

The growth stimulatory effects of both SDPP and Cu appear to be related to the microbial flora in the intestinal lumen. The high concentration of immunoglobulins in SDPP may prevent bacterial attachment in the intestine and protect intestinal integrity (Gatnau and Zimmerman, 1991). It was originally proposed that elevated Cu stimulated growth through an antibacterial like action in the gastrointestinal tract (Fuller et al., 1960). From those points of view, both the efficacy of SDPP and dietary Cu would interact with the level of sanitation management or environmental conditions. In fact, Coffey and Cromwell (1995) reported that the response to SDPP in weanling pigs was more pronounced under a commercial farm environment than in an experimental nursery. This observation was supported by Stahly et al. (1994) and Bergstrom et al. (1997), and their results suggested that healthy segregated early-weanling pigs reared in an off-site nursery respond less to SDPP in the transition diet than pigs with a poor health status reared in an on-site nursery. The poor response of germ-free pigs and the positive response of conventionally reared pigs to high dietary Cu (283 ppm) provides support that Cu exhibits some relationship with microbacteria in the lumen (Shurson et al., 1990). A similar situation has been reported for the response to antibacterial growth promoters (Cromwell, 2001). Moreover the response to SDPP and antibacterial growth promoters are considered additive (Coffey and Cromwell, 1995). Torrallardona et al. (2003) suggested that SDPP can be an alternative to antibiotics since it provided a level of protection against an experimental challenge with *E. coli*

K99 in pigs. Since both SDPP and Cu impact intestinal microbacteria flora and both are potential alternatives to antibiotic growth promotion, the potential exists for organic complex Cu and SDPP supplementation to produce additive responses.

Peptides as a Dietary Protein Source

Traditionally, it was thought that all dietary proteins needed to be hydrolyzed to free AA in order to be absorbed. This concept changed when the first intestinal oligopeptide transporter, PepT1, was simultaneously cloned and identified by two separate groups in rats and rabbits (Boll et al., 1994; Fei et al., 1994). The peptide transporter was then identified and characterized in domestic animals such as pigs (Klang et al., 2005), chickens (Chen et al., 1999), turkey (Van et al., 2005), and ruminant animals (Chen et al., 1999). The PepT1 protein is located at the brushborder membrane of intestinal epithelial cells (Leibach and Ganapathy, 1996), and has been shown to have rather broad substrate specificity, compared to the relatively narrow substrate specificity of most free AA transporters. A wide range of di- or tripeptides, regardless of their molecular weight, electrical charge and hydrophobicity could be transported by ovine and chicken PepT1, demonstrating the importance of peptide transport in domestic animals (Pan et al., 2001; Chen et al., 2002). A recent study indicated that PepT1 can potentially transport all the 400 dipeptides and 8,000 tripeptides in vitro (Adibi, 2003). Moreover, the intestinal peptide transport system mediates the absorption of a broad range of peptidomimetic drugs, including β-lactam antibiotics and the anti-cancer agent bestatin, thereby playing an important pharmacological role for oral drug delivery (Daniel, 1996; Inui et al., 2000; Brodin et al., 2002).

Absorption of intact peptides from the gastrointestinal tract appears to constitute part of the end products of dietary protein entering the blood (Pan et al., 1998). Peptide-bound AA account for 52 to 78 % of the total plasma AA pool in ruminants (DiRienzo, 1990; Koeln et al.,

1993), 10 % in humans (Christensen et al., 1947), 9 to15 % in rats (Seal and Parker, 1991), and 11 to14 % in guinea pigs (Gardner et al., 1983). The small peptides in the blood can then serve as sources of AA to meet tissue needs. Direct evidence of tissue utilization of peptide-bound AA has been reported both in vitro and in vivo. For example, intravenous injection of radiolabeled or hydrolysis-resistant peptides were cleared from the blood, accompanied by the appearance of radioactivity or intact hydrolysis-resistant peptides in tissues including liver, muscle, kidney, gut, lung, brain, and pancreas (Adibi, 1987; Stehle et al., 1991). Moreover, an in vitro study conducted 20 yr ago by Grimble et al. (1987) indicated that nitrogen absorption from a mixture of 71 to 85 % small peptides and free AA perfusion was significantly higher than 100 % free AA mixture in human jejunum. A series of studies in our lab suggested that peptide-bound methionine can be a source of methionine for protein synthesis in mammary tissue (Wang et al., 1996), in cultured C_2C_{12} myogenic and MAC-T bovine mammary epithelial cells (Pan et al., 1996), and for protein accretion and cell proliferation in ovine skeletal muscle (Pan and Webb, Jr., 1998). Most of the di- and tripeptides (total 17 kinds) promoted 15 to 76 % greater [^{3}H]-leucine incorporation into secreted protein than did free methionine. There was a negative [^{3}H]-leucine correlation between the rate of incorporation promoted by peptides and the number of AA residues in the peptides (Wang et al., 1996).

There are a number of physiological situations in which measured transorgan fluxes of free AA do not totally account for the protein accreted or secreted by the tissue. For example, in lactating dairy cows, the uptake of certain essential AA across the mammary gland appears to be insufficient for their output as milk protein (Metcalf et al., 1994); and in growing sheep, the extensive hepatic removal of absorbed essential AA such as histidine and phenylalanine results in an apparent insufficiency of these AA to support observed net protein gain in the animals

(Wolff et al., 1976; Bricherstaffe et al., 1974). There is a potential utilization of protein or peptides in the supply of AA for tissue and secreting protein synthesis. By using a dual-labeled tracer technique involving close-arterial (external pudic artery) infusion of [^{13}C]-labeled dipeptides, Backwell et al. (1994) found that [^{13}C]-phenylalanine and [^{13}C]-leucine from intravenously infused glycyl-L-[1-^{13}C]-phenylalanine and glycyl-L-[1-^{13}C]-leucine were incorporated into milk casein of goats. Peptides contribute 10 to 20 % of the phenylalanine requirement for casein biosynthesis in the lactating dairy goat (Blackwell et al., 1996). Several mechanisms were proposed to explain how enterocytes and renal cortex tubular cells absorb peptides. Those mechanisms included, 1) uptake of intact peptides via carrier-mediated transport systems followed by intracellular hydrolysis, 2) uptake of intact peptides by simple diffusion followed by intracellular hydrolysis, 3) hydrolysis of peptides during their translocation across cell membranes, and 4) extracellular hydrolysis of peptides via plasma membrane-bound peptidases followed by the absorption of the constituent AA by AA carriers. However, the mechanism by which tissues assimilate peptide-bound AA from the circulation and how those peptides are used is still poorly understood.

Since the identification of PepT1 in intestinal epithelial cells, numerous studies have shown that absorption of digested protein products in the small intestine occurs primarily in the form of small peptides rather than AA (Ganapathy et al., 1994) based on the fact that the end products of protein digestion in the lumen of the intestine are mainly small peptides (Leibach and Ganapathy, 1996; Ganapathy and Leibach, 1999). Moreover, peptide absorption from the lumen is faster and more efficient than AA transportation (Johnson, 1997a). Theoretically, it is therefore possible that incorporation of small peptides or hydrolyzed protein into the diet would be beneficial for animal growth. However, very limited information is available about the

utilization of peptides as protein sources in animal diets because of the lack of manufacturing and the difficulty of peptide detection methods. Most of the previous studies were conducted in fish, possibly because fish have a shorter small intestine than most domestic animals, and it makes sense that small peptides are more easily absorbed without further digestion compared to intact protein, especially for young animals. Zambonino Infante et al. (1997) fed sea bass larvae with 20 or 40 % enzymatic hydrolysate (75 % di- and tripeptides) in replacement of native fish meal protein for 21 d. The results indicated that growth and survival were significantly greater ($P < 0.05$) in larvae fed peptide diets compared to the control group, with the best performance exhibited by those fed the 20 % peptide diet. The larval weight in the 20 % peptide group was 1.55 times higher than the control intact protein group; survival was also enhanced by 30 %. The authors suggested that the improved growth was related to the enhanced proteolytic capacity of the pancreas and the earlier development of intestinal digestion, because the larvae fed 20 % peptides had increased chymotrypsin and reduced trypsin activity than the control group. The brushborder enzymes, aminopeptidases, maltase and glutamyl transpeptidases were also altered by small peptide incorporation in the diets, indicating an earlier maturation process of the intestine in the fish group fed peptide (Cahu and Zambonino Infante, 1995a; 1995b; Cahu et al., 1999). Those short peptides, especially di- and tripeptides are absorbed quickly and efficiently by the intestine without any prior pancreatic digestion (Zambonino Infante et al., 1997). Dabrowski et al. (2003) conducted a study in which teleost fish were fed a casein-gelatin protein source, a mixture of dipeptides or a mixture of synthetic L-AA for several wk. This study showed that the synthetic dipeptide-based diet could support growth in the early stage of ontogenesis of teleost fish, whereas a free AA based diet failed. The fish fed the free AA based diet exhibited an increased rate of ammonia excretion, suggesting that deamination is involved in

the metabolism of dietary AA. In mammals, Sasaki et al. (1989) and Scheppach et al. (1994) reported that hydrolysate containing short peptides was effective in stimulating enzyme activity in brushborder membranes and in facilitating nutritional rehabilitation. Because of the limitations (solubility, heat stability, and storage) of utilizing free glutamine in a clinical setting in humans, synthetic glutamine dipeptides have been considered as a perfect substitute for the replacement of a deficiency in glutamine (Furst, 2001).

There are few commercial peptide products available for use in the diet of domestic animals. Peptiva® is produced by Vitech BioChem Corporation (San Fernando, CA), by blending appropriate amount of porcine mucosa peptides, fish peptides, and microbial peptides. It is claimed to contain feed stimulating peptides, small intestine activity peptides, exorphine peptides, immune modulating peptides and anti-microbial peptides (www.vitechusa.com). No scientific report is yet available on utilization of this product in the animal industry. The other commercially available peptide product DPS (dried porcine soluble), is manufactured by Nutra-Flo® Company (Sioux City, IA) and consists of the residues remaining after extraction of heparin from porcine intestines. Previous studies indicated that DPS supplementation improved the growth performance of weanling pigs (Zimmerman et al., 1997; Lindemann et al., 1998) and feed intake of lactating sows (Johnston et al., 2003). The ADFI was increased (125g, 136g, and 129g) with increasing dietary concentrations of DPS (0 %, 1.5 %, and 3.0 %, respectively) during the entire lactation period ($P < 0.10$; Johnson et al., 2003). Lindemann et al. (1998) reported a similar improvement in voluntary feed intake of nursery pigs fed up to 6 % DPS. However, Bregendahl et al. (1998) and DeRouchey et al. (2000) found no effect of dietary DPS on feed intake in nursery pigs. Peptide research is very active in China, and many commercial products are available. One study conducted by Wang et al. (2003) indicated that addition of 3

% Mini-Peptides (Tianxi Technology Inc., Shandong, China) in a weanling pig diet increased weight gain and feed efficiency by 34 % and 8.4 %, respectively, compared to the soy control group. Dietary supplementation of Mini-Peptides also increased villous height and activities of duodenal luminal amylase, lipase, and trypsin in 21-d old pigs (Wang et al., 2003). Even though results of a few previous studies and most company field trials found some benefit of the inculsion of peptide products in animal feeds, more research is needed before routine application is a reality.

In summary, SDPP is an expensive high quality protein source for young weanling pigs. The model of action of SDPP is still elusive, but is more or less related to the impact on small intestine and immune function. Peptide protein could be a potential substitute for SDPP, but more research is necessary to verify this assertion.

Digestive and Absorptive Function of Small Intestine at the Molecular Level

The intestinal epithelial cells have two major functions: the first is to secrete digestive enzymes to facilitate processing of ingested food stuffs and to extract macromolecular nutrients and fluid; the second is to act as a physical barrier, separating the contents of a harsh luminal environment from the layers of tissue comprising the internal milieu. The combinations of these two functions facilitate a controlled and selective movement of components between the lumen and the underlying mucosa, restricting the passage of potentially harmful microorganisms from the intestinal tract into the surrounding tissue (Pitman and Blumberg, 2000). The epithelial cells are initiated from the crypts and removed by apoptosis upon reaching the villous tip a few days later (Radtke and Clevers, 2005). The whole renewal process takes only 3 to 5 d in most mammals; this rapid turnover causes the small intestine to be the most active organ of metabolism; it accounts for approximately 40 % of total AA utilization, 90 % of glutamate usage

(Wu et al., 1995; Stoll et al., 1998), and 17 to 25 % of the whole-body oxygen consumption (Yen et al., 1988). The small intestine is also the first organ system that is directly in contact with the ingested diet, and displays the most rapid and dramatic changes in response to luminal factors. Luminal nutrients, in addition to being substrates for digestion and absorption, can function as extracellular signals for regulating gene expression in the small intestine. For example, Ferraris (2001) suggested that AA and peptide transport rates in the intestine usually vary with dietary protein level (decreasing rates with lower dietary protein) until minimum required dietary protein concentrations are reached. Further decreases in the concentration of dietary protein below minimum requirements are accompanied by increases in essential but not nonessential AA transport.

As mentioned before, digestion and absorption capability in the small intestine is critical for protein quality. On the other hand, luminal nutrients can be signals for regulating intestinal morphology and function. This section briefly reviews the digestion and absorption function of the small intestine at the molecular biological level, more specifically the genes that are involved in the brushborder membrane digestion and assimilation process, followed by dietary and developmental regulation of gene expression at the brushborder membrane. The interactions of luminal nutrients, microbial environment, and intestinal immune function are also covered.

Genes Involved in Brushborder Membrane Digestion

Protein digestion begins in the stomach with the action of pepsin, an endopeptidase with specificity for peptide bonds involving aromatic L-AA. Pepsin activity terminates when the gastric contents mix with alkaline pancreatic juice in the small bowel (Johnson, 1997a). Pancreatic proteases take over after the chyme enters the small intestine. There are two general classes of pancreatic proteases (endopeptidases and exopeptidases). Endopeptidases recognize

specific AA in the middle of the peptide, whereas exopeptidases recognize one or two terminal AA. Exopeptidases that attack peptides from the N-terminus (removing either single AA or a dipeptide) are termed (dipeptidyl) aminopeptidases, whereas peptidases attacking the C-terminus are termed carboxypeptidases (van der Velden and Hulsmann, 1999). The end products of pancreatic digestion (disaccharides, small peptides) are further digested at the brushborder membrane of the small intestine by membrane-bound enzymes (Sherwood et al., 2005), which mainly include two categories, disaccharidases and aminopeptidases.

Aminopeptidases. Aminopeptidases are defined by two motifs in the catalytic domain, a zinc-binding motif and an exopeptidase motif. Aminopeptidases participate in a wide range of biological processes. Some of them act as ectoenzymes that may participate in the degradation of extracellular matrices, and some act as intracellular enzymes that may regulate the metabolism of bio-active molecules including hormones and neurotransmitters. Aminopeptidase N (APN, EC 3.4.11.2) is a widely studied membrane-bound ectoenzyme belonging to the zinc metallopeptidase family (Olsen et al., 1988; 1989). Aminopeptidase N hydrolyzes preferentially natural or synthetic substrates with a N-terminal alanine residue. Other AA, especially neutral ones, may also be removed hydrolytically, with the exception of proline (van der Veldon and Hulsmann, 1999). Structural studies indicated that the N-terminus of APN is located inside while the C-terminus is located outside of the cell (a type II integral membrane protein) (Look et al., 1989). The extracellular domain contains a pentapeptide consensus sequence characteristic of members of the zinc-binding metalloprotease family.

Aminopeptidase N is widely distributed in most mammalian tissues and has been identified in renal proximal tubular epithelial cells, small intestinal epithelium, biliary cannaliculae, synaptic membranes of the central nervous system, bone marrow stroma cells,

fibroblasts, osteoclasts, placenta, and granulocytes (Shipp and Look, 1993; Jardinaud et al., 2004). Several functions of APN have been described based on distribution in different tissues (Shipp and Look, 1993). First, APN expressed on the brushborder of the intestine may be involved in the final stages of digestion of small peptides. Second, APN may function to reduce cellular responses to peptide hormones, such as enkephalins, tachykinins, and bradykinin. Third, a recent report implicates APN in the processing of peptides presented by the major histocompatibility complex class II molecule (Larsen et al., 1996). Fourth, APN may be involved in tumor invasion and metastasis by degradation of collagen type IV (Jardinaud et al., 2004).

Aminopeptidase A (APA, EC 3.4.11.7) is another zinc-dependent membrane-bound metallopeptidase, which specifically cleaves the N-terminal glutamyl or aspartyl residue from peptide substrates including angiotensin II (Ang II; Nagatsu et al., 1970). Human APA has been determined to be a 140 to 160 kDa type II integral membrane protein, which is identical to mouse B cell differentiation antigen BP-1 (Wu et al., 1990) or the human kidney differentiation antigen gp160 (Nanus et al., 1993). The N-terminal extracellular domain of APA from residues 43 to 602 is the active site containing a zinc metallopeptidase domain; whereas the C-terminal end is essential for activation and correct folding of APA (Rozenfeld et al., 2004). Aminopeptidase A is mainly localized to the brushborder of renal proximal tubules and enterocytes, and has also been identified in other tissues (Troyanovskaya, 2000). In humans, APA is involved in the regulation of blood pressure by converting angiotensin II to angiotensin III by cleavage of the N-terminal aspartyl acid residue.

Dipeptidyl (amino) peptidase IV (DPP IV; EC 3.4.14.5) is a typical serine protease consisting of 766 AA with type II membrane topology (Tanaka et al., 1992). In contrast to APA and APN, DPP IV does not contain zinc in its catalytic center. Based upon its structural

homology with other non-classic serine proteases, DPP IV is assigned to the prolyl oligopeptidase family. Members of this family exhibit a catalytic site in which the essential residues are arranged in the unique sequence of Ser-Asp-His. Recognizing peptides with proline or alanine in the second position, DPP IV selectively removes two AA from the N-terminus. Since N-termini-containing X-Proline (X represents any AA) are not easily cleaved by other peptidases, the action of DPP IV is a rate-limiting step in the degradation of such peptides. Several biologically active peptides (bradykinin, cytokines, and chemokines, such as IL-1β, IL-2, and TNF-β) have the X-Proline sequence at their N-terminus and therefore DPP IV may play an important role in modulating their action (Karl et al., 2003). Dipeptidyl peptidase IV is expressed on renal proximal tubular epithelial cells, epithelial cells in the small intestine, and biliary cannaliculae (Shipp and Look, 1993). In the small intestine, DPP IV exhibits a characteristic pattern of distribution along the proximal to distal axis, with somewhat higher expression in the distal region (Sterchi, 1981; Darmoul et al., 1994), and high expression in the differentiated enterocytes of the villous tip, in comparison with the crypt cells. Low levels of DPP IV are present in the normal colonic epithelium, but are aberrantly highly expressed in colonic tumor (Zweibaum et al., 1984). Suzuki et al. (1993) showed that small intestinal DPP IV activity, mRNA levels and gene transcription can be increased by diets rich in proline. Much attention has been given to elucidating the effects of DPP IV on T lymphocyte levels and the immune system, due to its unique substrate specificity. More complete and comprehensive information about the properties and function of DPP IV were reviewed by Lambeir et al. (2003).

Glycohydrolases. Four brushborder glycohydrolases have been identified in the mammalian small intestine, namely sucrase isomaltase complex (SI), maltase glucoamylase

complex (MGA), lactase phlorizin hydrolase (LPH), and trehalase (Oosthuizen et al., 1998). Lactase phlorizin hydrolase is expressed maximally in the proximal and middle small intestine, and declines significantly in the distal segments of the intestine (Lee et al., 2002). Most studies of LPH are more or less clinically related to lactose intolerance. Sucrase isomaltase is located on the intestinal brushborder membrane, and is responsible for all sucrase activity, almost all isomaltase activity and approximately 80 % of maltase activity. The MGA complex contributes minimal isomaltase activity, about 20 % maltase activity and all glucoamylase activity (Semenza and Auricchio, 1989; Nichols et al., 1998). Much research has been conducted to understand the SI, LPH and trehalase, and the studies have revealed a considerable structural homogeneity among the corresponding enzymes in most mammalian species in which they have been analyzed (Semenza and Auricchio, 1989). For the purpose of this dissertation, only MGA is discussed.

Maltase-glucoamylase is an exoamylase that hydrolyzes maltose and maltooligosaccharides from the non-reducing end, and is only expressed in the brushborder membrane of enterocytes in the small intestine. Maltase-glucoamylase plays a key role in the final surface digestion of oligosaccharide nutrients in preparation for the assimilation of released monosaccharides (Nichols et al., 1998), especially when luminal α-amylase activity is deficient, as is the case when animals are immature or malnourished. In contrast to SI and LPH, structural features of MGA appear to vary significantly among species. Results from studies of maltase-glucoamylase in humans, pigs, and chickens have shown that the enzyme consists of a single polypeptide chain with a molecular weight of 335, 240, and 240 kDa, respectively (Noren et al., 1986; Hu et al., 1987; Nichols et al., 1998), while in mice MGA displays an apparent molecular weight of more than 500 kDa (Quezada-Calvillo et al., 2002). Noren et al. (1986) demonstrated

that the enzyme complex from pig intestinal mucosa contains two identical catalytic sites which differ only in their heat stability.

Genes Involved in Protein Assimilation

The products of dietary protein digestion are absorbed in the small intestine in the form of small peptides and free AA (Johnson, 1997a). Absorption of peptides is mediated predominantly by a single peptide transporter (PepT1), while absorption of AA involves participation of several transport systems. The AA transport systems in the intestine differ in their substrate specificity and driving force. Unlike glucose, AA do not have a large storage pool in the body. The extra AA are metabolized quickly, and it is critical that the requirement of AA is well balanced. A number of AA transporters have been isolated in enterocyte cells. Those transporter proteins typically possess 10 to 12 membrane-spanning domains and exhibit broad substrate specificity (several AA may be transported by one type of transporter) as well as sterospecificity (L-isomers are preferentially transported) allowing them to recognize, bind and transfer AA into or out of the cells (Palacin et al., 1998). Knowledge of the intestinal AA transporters has accumulated quickly in the last decade, and new transporters are still in the process of been identified. The nomenclature system is somewhat confusing because different research groups give different names to the same transporter. A new nomenclature system for the human SLC (solute carrier family) system has been widely adopted (Verry et al., 2004; Seow et al., 2004). The simplest nomenclature system based on the subtrate specificity (anionic, cationic and zwitterionic) is used in this review.

Current Knowledge of the Molecular Aspects of AA Transporters

CAT family of cationic AA transporters. Cationic AA transporter is a Na^+-independent transporter of the cationic AA arginine, lysine, ornithine and histidine when positively charged

(known as system y^+). Four homologous genes defining the family of cationic AA transporters (CAT-1, -2, -3, and -4) have been identified (Verrey et al., 2004). All of them contain 14 putative transmembrane (TM) segments and are glycosylated. The first CAT1 member was cloned from host cells infected by murine ecotropic leukemia virus (Albritton et al., 1989). Three additional related proteins, CAT-2A, -2B, and -3 have since been identified in different mammalian species, with CAT-2A and -2B being splice variants that differ only in a stretch of 42 AA (Deves and Boyd, 1998; Closs, 2002). The function of CAT4 has remained elusive, while it is known that all other CAT proteins mediate Na^+ -independent transport of cationic L-AA. The distribution of CAT1 is ubiquitous. The only tissue known to lack expression of high-affinity CAT is mammalian adult liver (Malandro and Kilberg, 1996). The CAT-1 co-localizes with caveolin in endothelial cells and is restricted to the basolateral membrane in epithelial cells. Expression of CAT1 mRNA is enhanced when cells are shifted from a quiescent or differentiated state to an undifferentiated, rapidly growing state. The regulation of CAT1 was proposed to be mediated by protein kinase C (Graf et al., 2001). The CAT-1 seems to be the major system y^+ transporter in most cells. Homozygous CAT-1 knockout mice died 1 d after birth, were 25 % smaller than their wild-type littermates and suffered from severe anemia, while the heterozygous mice exhibited no phenotypic abnormalities (Perkins et al., 1997).

The CAT-2 splice variants exhibited quite distinct expression patterns. The low affinity CAT-2A is most abundant in liver, but is also expressed in skeletal muscle, pancreas, cardiomyocytes, cardiac microvascular endothelial cells and vascular smooth muscle (Verrey et al., 2004). Significant expression of CAT-2B is found only after cytokine or lipopolysaccharide treatment in most cell types. It is often induced together with the inducible isoform of NO-synthase (iNOS) and is generally co-expressed with CAT-1. Cationic AA transporter 3 is

thought to play a major role during embryogenesis. Cationic AA transporter 4 is expressed in brain, testis and placenta, and the function is not known (Sperandeo et al., 1998).

Glutamate/Neutral AA Transporters (SLC1). The solute carrier family 1 (SLC1) includes five high-affinity glutamate transporters, EAAC-1, EAAT-1, -2, -4, and -5, as well as three zwitterionic AA transporters, ASCT1, ASCT2 and ATB^0 (Kanai and Hediger, 2003). The high affinity glutamate transporters mediate the transport of L-Glu, L-Asp and D-Asp, accompanied by the co-transport of three Na^+ and one H^+, and counter-transport of one K^+ (Zerangue and Kavanaugh, 1996a), whereas ASC transporters mediate Na^+-dependent exchange of small neutral AA such as alanine, serine, cystine, and threonine. The SLC1 family members have a unique, highly conserved long hydrophobic stretch near the C-terminus. The model features eight predicted alpha-helical transmembrane domains, a large cellular glycosylated loop between transmembrane domains three and four, a "re-entrant loop" between transmembrane domains seven and eight similar to the ion-permeating pore of ion channels, and a "loop" which is predicted to extend partially into the "translocation pore" between transmembrane domain seven and eight (reviewed by Kanai and Hediger, 2003). Northern blot analyses have localized human and rat EAAT mRNA to both brain and peripheral tissues. The EAAT1, -2, and -4 mRNAs are predominantly expressed in the brain, EAAT5 is prominent in the retina, and EAAC1 is widely distributed in both brain and peripheral tissues (Erickson et al., 1995; Fairman et al., 1995; Kanai et al., 1995). The first EAAC1 (EAAT3) was isolated from rabbit small intestine (Kanai and Hediger, 1992), and is thought to be the primary EAAT isoform responsible for supplying anionic AA for general cellular metabolic processes. It is predominantly expressed in neurons, apical membrane of proximal tubules of kidney, duodenum, jejunum, and ileum, but not in the colon. Electrophysiological analysis of transporters revealed high affinity uptake for L-glutamate

and L-aspartate, and inhibition of this uptake by D-aspartate and DL-threo-3-hydroxy-aspartate. The EAAC1 knockout mice developed dicarboxylic aminoaciduria, confirming the role of EAAC1 in the reabsorption of glutamate from the renal proximal tubules (Peghini et al., 1997).

The ASC neutral AA transporters exhibit the properties of the classical Na^+ -dependent AA transport system ASC. The ASC transporters have a high-affinity for alanine, serine, threonine and cysteine (Arriza et al., 1993; Shafqat et al., 1993; Utsunomiya-Tate et al., 1996). The two ASC transporters ASCT1 and ASCT2 exhibit distinct substrate selectivity. In addition to common substrates of ASC transporters, ASCT2 also accepts glutamine and asparagine as high-affinity substrates, and methionine, leucine and glycine as low-affinity substrates, whereas ASCT1 does not transport these substrates. In contrast to glutamate transporters which require K^+ to fulfill their relocation step, ASC transporters are independent of K^+ or H^+ , but are Na^+ -dependent AA exchangers (Zerangue and Kavanaugh, 1996b). One extracellular AA is exchanged for one intracellular AA, resulting in no net accumulation of AA, while glutamate transporters can mediate both uptake and exchange. The ASCT1 is preferentially expressed in the brain glial cells, while ASCT2 was shown to be present in the brushborder membranes of proximal tubules of kidney, and enterocytes of intestine (review by Kanai and Hediger, 2003).

Heterodimeric AA Ttransporters (HATs). Knowledge about HATs has accumulated dramatically in the past few years. More and more attention has been paid to this transporter because several clinical diseases, such as primary inherited aminoacidurias and cystinuria, are related to HAT gene malfunction. Heterodimeric AA transporters are composed of a heavy subunit (NBAT or 4F2hc) and the corresponding light subunit, linked by a disulfide bridge (Deves and Boyd, 2000; Chillaron et al., 2001). The two heavy subunits (NBAT and 4Fh2c) from the SLC3 family are type II membrane glycoproteins with a single transmembrane domain,

with an intracellular NH_2 terminus and an extracellular COOH-terminus (Chillaron et al., 2001). The NBAT is mainly expressed in the epithelial cells of the kidney proximal tubule and the small intestine brushborder membrane. In contrast, 4F2hc is ubiquitous, with a basolateral location in epithelial cells. Six of the known light chains (y^+LAT-1, y^+LAT-2, LAT-1, LAT-2, asc-1, and SCT) associate with the widely distributed 4F2hc that are expressed strongly in growing cells and are found basolaterally in polarized transporting cells of the kidney and small intestine. One light chain, $b^{0,+}$AT, associates with the related NBAT, localizing to the apical pole of epithelial cells in kidney proximal tubule and small intestine. There are two other light chains of HATs (asc2 and AGT-1) that were recently identified, and they seem to interact with an as yet unknown heavy chain (Verrey et al., 2004). A common functional feature of the heterodimeric transporters is that they apparently function as obligatory exchangers. Research indicated that the specificity and the characteristics of the HAT are mainly determined by the light chains. For the purpose of this review, only the NBAT/ $b^{0,+}$AT heterodimer will be covered. Current knowledge of other HAT transporters are available from many excellent reviews (Chillaron et al., 2001; Wagner et al., 2001; Verrey et al., 2004; Palacin et al., 2005).

The NBAT cDNA contains an open reading frame of 2049 nucleotides encoding a protein of 683 AA (Malandro and Kilberg, 1996). The $b^{0,+}$AT protein has been shown to be covalently linked to NBAT and expressed in jejunal microvilli in rats (Furriols et al., 1993; Pickel et al., 1993). Studies demonstrated that NBAT/ $b^{0,+}$AT acts as a tertiary active transporter, mediating the electrogenic exchange of dibasic AA (influx) for neutral AA (efflux) with a stoichiometry of 1:1 (Chillaron et al., 1996). Under physiological conditions, luminal L-cystine and the cationic AA are taken up preferentially because of their high apparent extracellular affinity, the intracellular reduction of cystine to cysteine and the membrane

potential, whereas the neutral AA are transported outwards because of their intracellular accumulation via an as yet unidentified, apical, B^0 –type Na^+ co-transporter. The $b^{0,+}AT$ is the catalytic subunit and has been shown recently to function alone as a transporter after reconstitution in liposomes. The heavy chain NBAT is required for trafficking to the cell membrane (Bauch and Verry, 2002).

Peptide transporters. There is little doubt that the absorption of AA by the gut mucosa is physiologically important; however, AA appear in portal blood faster and reach a higher level when peptide forms of protein hydrolysate contact the gut mucosa as compared to an equal-molar solution of free AA (Johnson, 1997a). Absorption of intact small peptides from mammalian intestine was recognized for years, and there is substantial progress in understanding peptide transporter molecular structure, physiological function and their regulation as well as mechanisms of regulation. The unique feature of peptide transporters is that they depend on a proton gradient as the driving force rather than Na^+ gradient (Fei et al., 1994). Two peptide transporters have been identified, PepT1 and PepT2, which have a broad range of substrates, including 400 dipeptides and 8,000 tripeptides that could be produced from the digestion of dietary and body proteins and a wide range of drugs, which have dipeptide and tripeptide like structures (Adibi, 2003). Preference for zwitterionic or charged substrates may vary (Amasheh et al., 1997; Steel et al., 1997). Adibi (2003) offered an explanation of the process of peptide absorption: 1) a Na^+/K^+ exchanger located in the brushborder membrane that maintains an intracellular alkaline pH, 2) Na^+/K^+ adenosine triphosphatase (ATPase) located in the basolateral membrane that maintains a negative membrane potential, and 3) several cytoplasmic peptidases that prevent intracellular accumulation of absorbed peptides. These enzymes convert most of the absorbed oligopeptides to AA that are either used by the absorbing cells or are released into the

portal circulation via the AA transporters located on the basolateral membrane of these cells. The oligopeptides that escape hydrolysis by the cytoplasmic peptidases are transported across the basolateral membrane into the portal circulation by a peptide transporter that appears to be different from PepT1.

These two peptide transporters, PepT1 and PepT2, differ in their tissue distribution, substrate kinetics, and possible specific roles in different tissues (Chen, 2001). The PepT1, which is expressed mainly in the small intestine, is important for nutrient absorption and may have clinical and pharmaceutical applications (Daniel, 1996; Adibi, 1997). PepT2, expressed in the kidney, the central nervous system, and several other peripheral tissues, is believed to have different functions in different tissues and is also very important for pharmaceutical applications (Daniel and Adibi, 1994; Wang et al., 1998; Lin et al., 1999). By sequencing and putative analysis, peptide transporters are predicted to contain 12 transmembrane domains and a very large extracellular loop between transmembrane domain nine and ten (Fei et al., 1994). The promoter of the PepT1 gene does not have a typical TATA box, but contains regulatory elements such as GC-rich boxes and also binding sites for activators and regulators of transcription. The potentially important regulatory elements for the expression of the PepT1 gene in the small intestine are the AA-responsive element (AARE) at – 431 to – 437 bp upstream and the transcription factor cdx-2 binding site at –795 to – 812 bp upstream (Fei et al., 2000). Many studies have been conducted to understand the regulation of PepT1 in the brushborder membrane because of the importance in protein assimilation and transport of peptidomimetic drugs. PepT1 expression could be altered by substrates (small peptides), hormones (epidermal growth factor, insulin, thyroid hormone), as well as certain physiological conditions (fasting, diabetes) (Adibi, 2003).

Regulation of Enzyme and Nutrient Transporter Expression in the Brushborder Membrane

Many factors such as neuron, hormone, and luminal nutrients, could affect intestinal function and morphology. From a molecular biological view point, regulation of enzyme and transporter proteins involves transcriptional factors, and translational and protein processing controls. Regulation at the transcription level results in changes expressed as variations in mRNA abundance. Regulation of protein translation and processing changes the number of functional proteins on the membrane. These regulations result in changes in overall transporter protein function (Chen, 2001). This part of the review addresses the effects of development and dietary factors on expression of intestinal membrane-bound digestive enzymes and nutrient transporters.

Dietary Regulation. It is well known that nutrient substrates are among the most significant factors regulating intestinal digestive enzyme and transport activity. Results from earlier research showed that the activity of most digestive enzymes and transporters, such as hydrolases, monosaccharide and AA transporters, are all closely related to dietary intake, although little is known about the mechanisms of dietary regulation (Ferraris et al., 1992; Tolza and Diamond, 1992; Biviano et al., 1993). Changing the diet from milk-based to a wheat and barley-based diet induced an increase in alkaline phosphatase and sucrase activities in pigs. The activity of DPP IV, APA, and APN in the small intestine also increased in wheat-fed pigs (Boundry et al., 2002). Generally, specific substrates induce enzyme activity and transporter expression that are involved in the digestion and absorption of those substrates (Ferraris, 2001). However, some luminal compounds could act as modifiers of enzymes that are involved in different metabolic pathways. For example, Kushak and Winter (1999) reported that tributyrin significantly (20 to 50 %) inhibited both fetal and postnatal APN activity in the small intestine of fetuses and children.

Amino acid digestion and absorption are more complicated and more difficult to study compared to glucose. In addition, many peptides and AA are involved in physiological regulation. According to the theory of Ferraris and Diamond (1989), the substrate regulation of transporters is different for essential and nonessential AA. The regulation of transporters for the nonessential AA follows the regulatory pattern for sugar. The activity of transporter increases monotonically with dietary nitrogen level. For the essential AA, nonmonotonic regulation patterns were observed for leucine, lysine, methionine, histidine, and alanine. Below the maintenance levels, uptake decreases or remains constant with increasing dietary nitrogen. Above the maintenance level, uptake increases with increasing dietary nitrogen (Ferraris and Diamond, 1989). However, there is evidence that other nutrients and AA could impact digestive enzymes and transporter activity involved in another AA metabolic pathway. For example, Stein et al. (1987) reported that the acidic AA aspartate is a good inducer of basic AA transport in mouse jejunum. The basic AA arginine but not lysine is a good inducer of the acidic AA transporter. Prolonged incubation of cells in an AA-free medium resulted in a time- and protein synthesis-dependent stimulation of system A transport activity, which was decreased upon restoration of extracellular AA availability. Gazzola et al (2001) reported that AA starvation increased AA transporter A2 mRNA levels nearly tenfold and AA resupplementation down regulated both AA transporter A2 mRNA and transport activity. Cationic AA transporter 1 mRNA levels in Fao cells increased for 24 h after AA starvation and decreased after resupplementation of AA (Hyatt et al., 1997). Using Caco-2 cells, human intestinal system y^+ arginine transport activity was rapidly and reversibly activated by AA, increasing twofold after one h of AA incubation, and returning to baseline three h after cells were reincubated in AA-free media. A high protein diet (50 % casein) increased EAAC1 mRNA two to threefold in the

middle small intestine of rats, compared to a 4 % casein diet (Erickson et al., 1995). Little change was observed in the proximal and distal regions of the intestine. In growing lambs (fed at two times NE for maintenance), ileal epithelium contained 313 % more EAAC1 protein, and liver contained 240 % more SGLT1 protein than lambs fed at maintenance (Howell et al., 2003). mRNA for SGLT1 did not differ between the two groups.

Since the identification of the first peptide transporter in rats (Fei et al., 1994), the nutritional importance of peptides has become more and more acknowledged, and understanding of the regulation of peptide transporters has accumulated in the last decade. Shiraga et al. (1999) studied the effects of dietary protein on rat PepT1 by using brushborder membrane vesicles (BBMV). After rats were fed protein-free, 5 %, 20 % or 50 % casein diets, BBMV was prepared from the ileal section of the small intestine and Gly-Sar uptake was measured. Four days after the start of dietary treatment, the Gly-Sar transport activity in the ileum was decreased in the protein-free diet group. In contrast, Gly-Sar uptake increased in BBMV from rats fed 50 % casein. The Gly-Sar transport activity was about twofold greater in rats fed 50 % casein compared with those fed the protein-free diet. Northern blot and immunoblot analysis revealed that both PepT1 mRNA and protein levels increased about twofold in rat fed 50 % casein compared with rats fed the protein-free diet. A more recent study also showed upregulation of PepT1 under special dietary conditions (Ihara et al., 2000). Rats were given starving or semi-starving diets (50 % of control), or total parenteral nutrition (TPN) for 10 days. Total RNA from small intestinal sections was isolated and northern blot analysis was performed to examine PepT1 and SGLT1 mRNA abundance. Results showed that PepT1 mRNA in the starved group increased to 179 % of the control value in the proximal portion of the small intestine. The PepT1 mRNA level in the TPN and semi-starved group also increased to 161 % of control. In contrast,

SGLT1 mRNA levels did not change in any of the treatments. Returning to a normal diet and feeding pattern by the starved group resulted in a drastic decrease in PepT1 mRNA level. Western blot analysis of PepT1 protein expression confirmed the same pattern as mRNA abundance in all treatment groups. The study demonstrated that, under various malnourished conditions, PepT1 expression was greatly enhanced. Chen et al. (2005) reported that chicken PepT1 mRNA level was lowest in chickens fed a low protein diet (12 %), highest in chickens fed a 24 % CP diet, and intermediate for chicks fed the 18 % CP diet ($P < 0.05$). Northern blot analysis of mRNA from the intestine of rats on a normal diet (17 % casein) showed that mRNA for PepT1 was relatively evenly distributed along the longitudinal axis of the small intestine. Changing the diet from low (4 % casein) to high (50 % gelatin) protein resulted in a 1.5 to two fold increase in PepT1 mRNA level in the middle and distal small intestine. The results also indicated that the distal region of the small intestine was the primary site for adaptive response (Erickson et al., 1995). Another finding was that PepT1 protein expression in the jejunum of diabetic rats significantly increased, due to increased stability at the translational level (Gangopadhyay et al., 2002).

In most previous studies, researchers fed animals high and low protein diets, and then measured the transporter activity or gene expression levels. These kinds of results could be difficult to interpret because AA contents were reduced, and it is difficult to interpret which AA induced the response. Secondly, animal health condition could be affected by AA deficiency, and third, hormonal and neuronal factors could also be involved.

Fasting and Restricted Feeding. Caloric restriction has become an active area of research in medical science because it has been proven that caloric restriction is effective in treating diabetes, and also extends the life of animals. In domestic animals, fasting or restricted feeding

generally induced weight loss and significantly affected intestinal function and morphology. Total intestinal surface area was decreased with fasting (Mayhew, 1990), and the fasting-related reduction in surface area was mainly at the villous level (Ferraris and Carey, 2000). Villous height decreased with fasting because of significant reductions in the number of cells along the crypt-villous axis. The decrease in cell number likely resulted from decreases in cell proliferation and migration rates, as well as from an increase in rates of cell loss and apoptosis (Boza et al., 1999). Expression of PepT1 was significantly enhanced in rat small intestine under various conditions of dietary restriction (Ihara et al., 2000). Aminopeptidase N and DPP IV activities were also significantly elevated to 177 % and 166 %, respectively in rats. Sucrase and maltase activities were significantly decreased with dietary restriction (Ihara et al., 2000). As stated before, nutrient deficiency always involves other issues, and careful consideration should be applied when addressing the effects of fasting and malnutrition on intestinal gene expression.

Developmental Regulation. The intestine undergoes dramatic structural and functional changes after birth, such as increasing dry mass and absorptive surface area, and changing of membrane permeability and fluidity (Buddington et al., 2001). In addition to these nonspecific changes, the absorptive capability per cell (per mg) and the expression of transporters also alters with aging. Because of the large number of hydrolases and nutrient transporters in the small intestine, and the number of animal species, it is difficult to make a general statement for the developmental regulation of enzymes and transporters in the small intestine. The fetal small intestine of many mammals is known to actively transport glucose or to express SGLT1 and GLUT2 mRNA at significant levels (Wang et al., 1994), while GLUT5 is expressed in significant quantities only after weaning is completed, fructose being a necessary factor to initiate GLUT5 expression in the small intestine (Ferraris , 2001). Diet seems to have some

impact on the developmental regulation pattern; however Jiang and Ferraris (2001) reported that the hard-wired age regulation is genetically programmed and less influenced by external factors in rats. It has been shown that AA is much more effectively absorbed from the intestine in peptide form in newborns than in adults (Himukai et al., 1980; Guandalini and Rubino, 1982). Rate of AA absorption in pigs was highest at birth, and decreased by an average of 30 % during the first 24 h of suckling (Buddington et al., 2001). van Winkle and Campione (1996) have described the regulation of both CAT1 and CAT2 in the developing mouse embryo, detecting expression as early as the one-cell stage. High levels of CAT1 mRNA were observed in rat fetal intestine and decreased upon birth, and then returned to a high level during adulthood. There was an increase in glutamate transport activity throughout development from fetus to adult (Blakely et al., 1991). More information about AA transporter developmental regulation can be found in the review by van Winkle (2001).

Shen et al. (2001) reported that rat PepT1 mRNA level and protein expression follow the same pattern throughout the developmental stages examined. At d 20 of fetal life, both PepT1 mRNA and protein were evident, but at low levels in all sections. Both PepT1 mRNA and protein reached peak levels by d 3 to 5 of age. PepT1 mRNA then declined rapidly to 11 to 13 % of the maximum by d 14. Both PepT1 mRNA and protein levels then rose so that by d 24, about the time of weaning, PepT1 mRNA and protein levels were 23 to 58 % and 59 to 88 % of the maximum, respectively. At d 75 postpartum (adult), PepT1 mRNA was approximately 25 % and PepT1 protein level was 70 % of levels observed at d 3 to 5. Significant PepT1 expression was observed in the colon at d 1 to 5 and then rapidly decreased to an undetectable level after d 14.

Distribution along the Intestinal Axis. Because of the unique morphological characteristics of the intestine, the distribution of transporters is different along the intestinal axis

from proximal to distal segments, and from the crypt to villous. The NBAT in rats exhibited greater expression in the proximal part of the intestine (Toloza and Diamond, 1992; Rome, et al., 2002). Glutamate transporter (EAAC1) showed a more pronounced gradient of mRNA along the proximal to distal intestinal axis, with the highest levels observed distally, while PepT1 mRNA was evenly distributed. The transporter expression patterns were similar in all parts of the small intestine (proximal, median and distal) at all ages (Rome et al., 2002). In contrast, Erickson et al. (1995) reported that D2/NBAT mRNA displayed an even intestinal distribution in rat, and a different diet had no effect on the steady state levels of intestinal NBAT. Generally, only mature enterocytes have transport capability. The specific distribution pattern may relate to physiological function, even though further research is needed to confirm this.

Mechanisms of Regulation. Regulation of nutrient transporters is still a puzzle, because some of the transporters are upregulated by dietary substrates (glucose and nonessential AA), while others (vitamins and minerals) are proven to be downregulated, and still others (essential AA) are not affected. Several considerations may explain this difference in dietary regulation: 1) biosynthetic cost, and the cost of synthesizing and maintaining transport function, 2) caloric benefits of a nutrient tends to upregulate its transporters, 3) a fixed daily requirement of a certain essential AA tends to cause low level of dietary substrates to upregulate transporter expression, and 4) potential toxic nutrients at high concentrations tend to downregulate its transporters (Ferraris and Diamond, 1997). Animals also have certain reserve capacities because of unpredictable factors (feed limitations, dietary composition and different nutrient requirements). The safety factor (capability/intake) of intestinal transport is approximately two while factors as high as seven have also been observed (Lam et al., 2002).

Two types of regulation of nutrient transporters are proposed by Ferraris (2001), nonspecific and specific mechanisms. Changes in cell size or total number of cells are the most common nonspecific mechanism of regulation. Both result in altered total intestinal length and total mass as well as the surface area of absorption. Increases in intestinal surface area, mediated by increases in height of microvilli of rat enterocytes, were produced by a high-calorie galactose-rich diet. Sodium ion, Cl^-, and H^+ concentration gradients are the energy sources for some AA transporters. Changes in the electrochemical gradient may be responsible for changes in the capability of nutrient transporters because of alterations in the membrane potential. As mentioned before, the unique characteristic of the small intestine is that the enterocytes mature while migrating from the crypt to villous tip. The balance of cell division, maturation, migration and cell exfoliation is usually in a constant state. Changes in the ratio of mature (absorbing) to immature (nonabsorbing) enterocytes also alter the transport ability. Finally, Syme and Smith (1982) reported that in protein malnourished rats, the top 60 % of a short villous column absorbed valine, whereas in well-fed rats, only the top 40 % of a tall villous absorbed valine. In both diet groups, only enterocytes 30 h or older absorbed valine, and the author suggested that the shortened villi of malnourished rats were occupied by cells older than those found at the same height in the longer villi of well-nourished rats. Similar phenomena were observed in diabetic mice; the enterocytes in basal and lower villi were recruited for absorption. Nutrient uptake in the newborn pig occurs along the entire crypt/villous axis, but only in the upper villous regions during adulthood (Buddington et al., 2001). The specific mechanisms include, changing the turnover number of transporters from inactive to active form and changing the density of transporters by changes in the rate of synthesis or rate of degradation (Ferraris, 2001). Previous

studies indicated that induction of transporters only occurs in developing crypt cells and not in mature villous cells.

Intestinal Mucosal Immunity (Cytokines)

In addition to the assimilation function, the small intestine also plays a pivotal role in pathogen prevention and initiates immune activation. The small intestine has all the cellular components required for induction and implementation of every type of immune response. The B and T lymphocytes, macrophages, mast cells, plasma cells, and various types of antigen-presenting cells exist thoughout the length of the gut (Joneja, 2004). The gastrointestinal immune system is quite different from systemic immunity in that the intestinal mucosal immune system must balance two opposing functions: to mount an immune response to pathogens, while maintaining tolerance to antigens derived from commensal bacteria and food (Pitman and Blumberg, 2000). The imbalance of those two opposite functions causes malfunction, such as food intolerance, inflammation, and diseases.

The gut of monogastric animals is inhabited by microbial populations throughout the life of the host. The acid secreted in the stomach and the swift flow of contents in the duodenum and jejunum ensure that the proximal regions contain only transient bacterial cells in the healthy host (Savage, 1977). The terminal ileum and the large bowel are hospitable places for bacterial proliferation, and a numerous and complex bacterial community (Microflora) reside in this site. The commensal enteric flora makes significant metabolic contributions to the health of the host (Nataro, 2005), producing vitamin K, vitamin B and short-chain fatty acids for epithelial cell growth and additional energy. In addition, the normal commensal microflora stimulates maturation of the mucosal immune system and helps resist colonization by enteric pathogens (Nataro, 2005). However, it is also well known that germ-free animals generally grow better

than conventionally raised animals. In addition, overgrowth of the microflora in the lumen, or over-activation of the mucosal immune system induces infection and diseases.

Cytokines are a group of compounds which exert profound influence on the functional state of immune cell populations by inducing an altered pattern of gene expression upon binding to specific cellular receptors (Turnbull and Rivier, 1999). Intestinal epithelial cells are capable of expressing a number of cytokines, thereby possessing the ability to affect local immune responses (Rogler and Andus, 1998; Wittig and Zeitz, 2003). Diseases such as ulcerative colitis and Crohn's disease are characterized by alterations in the balance of proinflammatory and regulatory cytokines (Rogler and Andus, 1998). In addition to involvement in development of inflammation and immunity, cytokines are also involved in a variety of biological processes, including cell activation, growth, and differentiation (Murtaugh and Foss, 2002). Cells of the innate immune system, such as macrophages and monocytes, are able to mount a rapid response to a danger signal by secreting several proinflammatory cytokines such as IL-1, IL-6, IL-8, IL-12, and TNF-α. The cytokine milieu subsequently directs the development of adaptive immunity mediated by T and B lymphocytes. For the purpose of this dissertation, a brief review about the cytokines in the brushborder membrane, and the impact of cytokines on metabolism and growth of animals is addressed.

Based on function on immune activation, cytokines are divided into proinflammatory and anti-inflammatory factors. The proinflammatory factors include IL-1, IL-2, TNF-α, and IFN-γ. The anti-inflammatory factors include IL-4, IL-10, IL-1α and TGF-β (transforming growth factor) (Rogler and Andus, 1998). Interleukin 6 is of particular interest, because generally it is refered to as a proinflammatory cytokine (Williams, 2001; Andreakos et al., 2002), while it was also claimed to be an anti-inflammatory cytokine, because it stimulates synthesis of acute-phase

proteins, and downregulates proinflammatory cytokine production (Xing et al., 1998; Wang et al., 2000).

Proinflammatory Cytokines. Interleukin 1 is a potent mediator activated by many immune and inflammatory cells. It has two agonists, IL-1α and IL-1β, and they share about 25 % sequence homology, and exhibit the same activities in numerous biological test systems (Dinarello, 1991). Two distinct membrane-bound mammalian IL-1 receptors have been described and designated IL-1R1 and IL-1R2 (Sims and Dower, 1994). Each receptor binds IL-1α and IL-1β, but with differing affinities. It has been proposed that the biological actions of IL-1 are mediated exclusively through IL-1R1 (Sims et al., 1993), with IL-1R2 functioning solely as a decoy receptor that limits the availability of IL-1 for interaction with IL-1R1 (Colotta et al., 1993; Sims and Dower, 1994). Effects of IL-1 are controlled by an IL-1 receptor antagonist, which is produced by the same cells as IL-1 itself and competitively counteracts the actions of IL-1 (Dinarello and Thompson, 1991). Functions of IL-1 include promoting inflammation, activating the coagulation pathway, stimulating the liver to produce acute phase proteins, catabolism of fat for energy conversion, inducing sleep, stimulating the synthesis of collagen and collagenase for scar tissue formation, stimulating the synthesis of adhesion factors on endothelial cells and leukocytes for diapedesis, and activating macrophages.

Tumor necrosis factor is an important mediator of inflammation. It shares many pro-inflammatory activities with IL-1. Tumor necrosis factor also occurs in α- and β-forms, which share about 50 % sequence homology (Turnbull and Rivier, 1999). Tumor necrosis factor-β (lymphotoxin-α) is produced predominantly by activated lymphocytes. In contrast, TNF-α (also known as cachectin) is expressed on a wide variety of hemopoietic and nonhemopoietic cells as a 26-kDa membrane-associated molecule. Actions of TNF- α are exerted through interactions with

two distinct receptors: the 55-kDa (TNF-R1) and 75-kDa (TNF-R2) receptors (Bazzoni and Beutler, 1996). Receptors for IL-1 and TNF- α occur not only in membrane-bound forms, but also as truncated soluble products, which are capable of binding their ligands. The ability of IL-1 and TNF soluble receptors to bind their ligands limits the availability of either IL-1 or TNF- α for interaction with their membrane-bound receptors, and therefore confers antagonistic properties to these truncated receptors. Tumor necrosis factor α is the principle cytokine that mediates acute inflammation**.** Functions include acting on endothelial cells to stimulate inflammation and the coagulation pathway; stimulating endothelial cells and macrophages to produce chemokines that contribute to diapedesis, chemotaxis and the recruitment of leukocytes, stimulating macrophages to secrete IL-1 for redundancy, activating neutrophils and promoting extracellular killing by neutrophils, stimulating the liver to produce acute phase proteins, and acting on muscles and fat to stimulate catabolism for energy conversion (Turnbull and Rivier, 1999).

Anti-inflammatory. The IL-10 is an 18.5 kDa cytokine with broad immunoregulatory activity, impacting on both the innate and cell-mediated branches of the immune system (Lindsay and Hodgson, 2001). IL-10 is released in a late stage (24 to 48 h after stimulation) in comparison to other T-cell cytokines. Research indicated that IL-10 plays a pivotal role in maintaining intestinal mucosal immune function, and preventing pathological inflammation, such as Crohn's disease. The immunoregulatory activity of IL-10 is based upon its ability to inhibit both cytokine synthesis and antigen presentation (Lindsay and Hodgson, 2001; Murtaugh and Foss, 2002). In addition, IL-10 inhibits the synthesis of a vast array of macrophage-derived cytokines that play a crucial role in inflammation, such as IL-1, IL-6, IL-12, TNF-α, IFN-α and, chemokine IL-8. Finally, IL-10 induces the production of cytokine inhibitors, such as IL-1 receptor antagonist, and the release of soluble TNF-α receptors. Result from a previous study

indicated that the IL-10 present in the submucosa of inflamed gastrointestinal tissue was significantly increased compared to the non-inflamed gut (Papadakis and Targan, 2000; Lindsay and Hodgson, 2001). The IL-10 knock-out mice developed a spontaneous ileocolitis by two to three mo of age with similarities to Crohn's disease.

Interleukin-6 is a single 21 to 28 kDa glycoprotein produced by both lymphoid and nonlymphoid cells that regulates immune responses, acute-phase protein synthesis, and hematopoiesis (Turnbull and Rivier, 1999). One IL-6 receptor has thus far been identified. This IL-6 specific receptor is responsible only for binding of its ligand. Interleukin 6 functions to stimulate the liver to produce acute phase proteins; stimulates the proliferation of B lymphocytes; and increases neutrophil production**.** Interleukin 6 is produced by many cells including T-lymphocytes, macrophages, monocytes, endothelial cells, and fibroblasts (Pitman and Blumberg, 2000).

It is well known that the function of cytokines, especially proinflammatory cytokines goes way beyond that of the immune system. They are also directly involved in the regulation of nutrient metabolism and growth (Johnson, 1997b). The detrimental effect of immunological stress on the nutritional status of the animal is of considerable economic importance, because nutrients are diverted away from growth to support immune-related processes (Spurlock, 1997). Sophisticated whole-animal studies involving euglycemic hyperinsulinemic clamps in combination with primed constant tracer infusion established clearly that IL-1 (Ling et al., 1994) and TNF (Lang et al., 1992; Ling et al., 1994) reduced the apparent rate of glucose uptake by skeletal muscle, heart, and liver. Tumor necrosis factor also decreased the expression of GLUT4 and cellular membrane GLUT4 content. With regards to AA assimilation, it seems that TNF treatment has been associated with increased AA transport (increased V_{max} of system A, N, and

ASC) by hepatocyte plasma membrane vesicles (Pacitti et al., 1993). The IL-6 also stimulates uptake of AA by hepatocytes in vitro (Andus et al., 1991). Accelerated muscle protein degradation and increased hepatic acute phase protein synthesis are hallmarks of an inflammatory response. The cytokines IL-1, IL-6, and TNF are able to stimulate hepatic synthesis of acute phase proteins in vivo and in vitro (Richards et al., 1991). On the other hand, both TNF and IL-1 have been linked to depressed protein synthesis and increased degradation rates in rodent as a model of an immune challenge (Zamir et al., 1992).

Manipulation of the luminal constituents through dietary means could be a potential therapy for intestinal related diseases. Few studies have been conducted to elucidate the mechanisms underlying the pathway of how luminal nutrients alter cytokine production, and how cytokines target the immune system (Sanderson, 2001; Gil, 2002). Dietary nucleotides have been shown to enhance the gene expression of IL-6 and IL-8 in fetal small intestinal explants (Gil, 2002). Dietary n-3 polyunsaturated fatty acid decreased IL-1-β, IL-2, IL-6 and TNF-α in the human peripheral blood mononuclear cells compared with a control diet (Blok et al., 1996). The blood flow of small intestine was even increased by immune enhancing diets (L-arginine, ω-3 fatty acid and RNA fragments) in rats (Matheson et al., 2003).

In summary, there is a very close relationship between dietary factors and small intestine function. The small intestine is a major site of nutrient digestion and assimilation. On the other hand, dietary factors alter intestinal structure and assimilation as well as mucosal immune function. Moreover, immune activation could change nutrient absorption and metabolism. From this point of view, the small intestine is the perfect model to study the actions of SDPP in pigs. Impacts of SDPP and Peptiva® on intestinal morphology and function may provide a new aspect of nutrigenetic knowledge.

Chapter III

Assessment of a marine based hydrolyzed protein and spray-dried plasma protein as supplements in the diet of early weanling pigs

ABSTRACT

Inclusion of spray-dried plasma protein (SDPP) in the diet of weanling pigs improves growth but increases diet cost. The objective of this research was to investigate replacement of SDPP with a hydrolyzed peptide protein on growth performance and nitrogen balance in weanling pigs. A total of 140 early weaned pigs (17 ± 2 d of age) was divided across five dietary treatments. There were seven replicate pens of pigs with four pigs per pen for each treatment. The experimental diets were: 1) a control diet with no SDPP or Peptiva® , 2) a diet formulated to be similar to diet 1 in nutrient and energy content but containing 6 % SDPP, 3) a diet identical to diet 2 except half (3 %) of plasma protein was replaced with Peptiva®, 4) a diet identical to diet 2 except all (6 %) of the plasma protein was replaced with Peptiva®, and 5) a diet containing 6 % Peptiva® with adjustments using soy protein concentrate to have nutrient and energy concentrations similar to diets 1 and 2. The results indicated that pigs fed diets with SDPP had greater ($P < 0.05$) ADG and ADFI compared to pigs fed the control diets in phase I without carry-over benefits in subsequent diet phases. Based on the results of the first experiment, the purpose of experiment two was to investigate whether the benefits of SDPP in pigs were related to digestibility and nitrogen balance. There were three dietary treatments: a corn-soy-whey control diet or similar diets containing 6 % SDPP or 6 % Peptiva®. Weanling pigs were placed in metabolic cages (8 cages with 2 pigs each / treatment) and fed their respected diets at the level of approximately 3 % of BW. Total feces, urine, and orts were collected for two 5-d collection periods after a 7-d adjustment period. Dry matter content of feed and feces, and nitrogen

concentration of feces, feed and urine were determined. Analytical data were used to calculate DM digestibility, protein digestibility and nitrogen balance. The results indicated that supplementation of 6 % SDPP or 6 % Peptiva® had no impact on DM digestibility, nitrogen retention, or protein biological value (P = 0.22 to 0.39). In summary, inclusion of SDPP in the phase I diet improved growth performance. Situations in which supplementation of these products improves performance appears to be unrelated to the effects they may have on digestibility or nitrogen balance.

Key Words: Digestibility, Nitrogen balance, Peptide, Pigs, Spray-dried plasma protein.

Introduction

Numerous studies have been conducted to investigate the benefits of adding spray-dried plasma protein (SDPP) to diets of weanling pigs. A multiple regression analysis of published studies indicated that the overall, mean SDPP-induced changes in ADG, ADFI, and G:F in the first 2 wk after weaning were + 26.8 %, + 24.5 %, and + 3.2 %, respectively (see review by van Dijk et al., 2001a). The high cost of SDPP and public concern about feeding animal products back to animals create an important need to understand the physiological pathways that may respond to the benefits of SDPP in weanling pigs, and to find potential substitutes for SDPP supplementation in weanling pig diets.

Peptide absorption from the intestinal lumen has been recognized for several years, and peptide transport is thought to be faster and more efficient than AA transport for intestinal absorption. This is based partly on the fact that peptides are more abundant than AA in the lumen (Leibach and Ganapathy, 1996; Ganapathy and Leibach, 1999). A report by Zhao et al. (1997) further supports the importance of peptide absorption. Their research indicated that

protein absorption by the proximal small intestine was greater and more efficient for hydrolyzed peptides than intact soy protein in dogs. Partial substitution of di- and tripeptides for native protein in the diet of sea bass also improved larval development, and growth performance and survival were significantly improved by peptide supplemented diets compared to intact protein (Zambonino infante et al., 1997). However, limited information is available on the effect of supplemental peptides in livestock. The objectives of this study were to determine the effects of substitution of SDPP (AP 920®, American Proteins Corp., Ames, IA) with a commercial marine based hydrolyzed protein rich in peptides (Peptiva®, Vitech Biochem, San Fernando, CA) on growth performance and nitrogen balance in weanling pigs.

Materials and Methods

Experiment One

A five wk growth performance trial involving 140 crossbred weanling pigs (Yorkshire x Landrace x NPD Hamline®) was conducted at the Virginia Tech Tidewater Agricultural Research and Extension Center Swine Unit in Suffolk, VA. The pigs were weaned at 17 $\pm$ 2 d of age (6.4 ± 0.11 kg BW) and randomly allotted to five dietary treatments from outcome groups based on litter of origin, BW and sex. For each treatment, there were seven replicate pens of pigs with four pigs per pen. Care and welfare of the pigs were in accordance with published guidelines (FASS, 1999), and were approved by the university animal care committee. Pigs were housed in a clean, disinfected pig nursery with supplemental heat and a negative pressure ventilation system. Pens were equipped with plastic-coated woven wire flooring, a nipple drinker and a standard nursery feeder. Feed and water were available *ad libitum.* The five dietary treatments (Table 3.1) included: 1) a control diet with no SDPP or Peptiva® , 2) a diet formulated to be similar to diet 1 in nutrient and energy content but containing 6 % SDPP, 3) a

diet identical to diet 2 except half (3 %) of plasma protein was replaced with Peptiva®, 4) a diet identical to diet 2 except all (6 %) of the plasma protein was replaced with Peptiva®, and 5) a diet containing 6 % Peptiva® with adjustments using soy protein concentrate to have nutrient and energy concentrations similar to diets 1 and 2. Aside from these alterations, the diets were similar to commercial early-weaned pig diets and contained identical levels of corn, soybean meal, dried whey, lactose, and fishmeal. A common basal diet containing these ingredients was prepared initially to ensure that common ingredient levels were identical for each experimental diet. The experimental diets were fed for the initial 10 d after weaning. All pigs were then fed a common phase II nursery diet (corn-soy-dried whey based) formulated to meet or exceed NRC (1998) nutritional requirements for the subsequent 10 d, followed by a common phase III nursery diet (corn-soy based) for the final 15 d (Table 3.2). Pigs were weighed and feed consumption and feed efficiency were determined at diet changes on d 10, d 20 and at the conclusion of the nursery study on d 35.

Fecal Firmness Scores. On d 5, 10, 20, and 35 of the experiment, a visual appraisal of fecal firmness within each pen was conducted. The purpose was to determine possible treatment effects on fecal consistency or occurrence of diarrhea. Pen fecal firmness data was recorded based on a scale of 1 to 5. A score of 1 indicates exceptionally firm feces, 2 = normal consistency of formed feces, 3 = soft, partially formed feces, 4 = loose, semi-liquid feces, 5 = very loose, watery feces.

Experiment Two

A total of 48 early-weaned crossbred pigs were used in two identical trials (24 pigs in each trial). For each trial, pigs were randomly assigned at weaning to dietary treatments from outcome groups based on sex and BW. Littermates were balanced across treatments. Upon

assignment to dietary treatments, pigs were placed in stainless steel metabolism cages with two pigs, one barrow and one gilt in each cage. The two pigs in each cage represented a single experimental unit with a total of four cages per treatment in each trial. The two trials combined provided a total of eight replications for each treatment. Each metabolism cage was equipped with a removable high-density plastic feeder and a nipple drinker. Perforated flooring, a stainless steel screen and funnel in each cage allowed for total separation and collection of feces and urine. The metabolism cages were located in an environmentally controlled metabolism room at the main Virginia Tech campus (Blacksburg, VA). The room was maintained with continuous lighting and an ambient temperature of 27 º C. The three dietary treatments were the same as the first, second and fifth diets in Exp.1 (Table 3.1), which included: 1) a soy protein based control diet, 2) the control diet supplemented with 6 % SDPP, and 3) the control diet supplemented with 6 % Peptiva®. All diets were formulated to have similar nutrient and energy concentrations by adjusting with soy protein concentrate. To minimize non-treatment variation in the experimental diets, a common basal diet containing corn, soybean meal, dried whey, lactose and fishmeal was initially prepared using a vertical screw mixer. This common basal diet was then used to prepare each experimental diet using a Hobart paddle mixer. All diets were prepared and fed in meal form.

Sampling and Analysis. There was a 7-d adjustment period followed by two 5-d collection periods in each trial. Total feces and urine were collected during the 5-d collection periods. Pigs were fed their respective diets in two equal portions at 12-h intervals (0800 and 2000 hr) to provide a daily intake equal to approximately 3 % of BW (160 g per pig per day). Water was provided *ad libitum*. During each collection period, feces were collected and weighed daily and dried in a forced air oven (60° C, 24 h). Dried samples from each collection period for

each cage were composited, weighed, subsampled, and then ground to pass through a 1-mm screen using a cyclone type sample mill (U-D Corp., Boulder, CO). Total urine for each collection period was collected in plastic containers at room temperature. The pH of the urine was maintained below 4.0 by appropriate addition of 6 N HCl. Total urine for each collection period was weighed, subsampled and then stored at 4° C until analysis. Dry matter content of feed and fecal samples were determined by drying at 105° C overnight. Nitrogen concentration of feces, feed and urine was determined by the Kjeldahl method. Data from these analyses were used to calculate digestibilities for DM, crude protein and nitrogen balance.

Statistical Analysis

All data were subjected to analysis of variance using the MIXED procedure of SAS (1995). Adjusted Tukey's multiple comparison tests were used if a significant F-statistic difference was detected. Dependent performance variables in Exp. 1 included BW, ADG, ADFI, G:F, and fecal firmness score. In all cases the pen served as the experimental unit. For the second experiment, dependent performance variables included DM digestibility, crude protein digestibility, and nitrogen balance. The cage served as the experimental unit.

Results

Experiment 1

In general, all the pigs were in a good state of health and performed well throughout the entire experimental period. Dietary supplementation of 6 % SDPP significantly improved ($P < 0.05$) ADG and ADFI of weaned pigs during the initial 10 d (Table 3.3), as compared to the control diet and the diets containing 3 % SDPP plus 3 % Peptiva®, or the diets supplemented with 6 % Peptiva® (diets 4 and 5). In addition, pig BW was significantly elevated ($P < 0.05$) at the end of 10 d for pigs fed the diet with 6 % supplemental SDPP when compared to all other

dietary treatments. There was a numerical advantage (15 % increase) in G:F ratio for pigs fed the 6 % SDPP diet but, due to variation in the data, there was no statistical difference ($P = 0.14$) among treatments. During phase II, pigs fed the diet with 6 % Peptiva® adjusted for energy and AA content during phase I (diet 5) had an improved G:F ($P = 0.02$) relative to the control treatment group (Table 3.3). For all other performance parameters during phase II and phase III, there were no significant differences ($P > 0.05$) for pigs fed the various dietary treatments during phase I. Furthermore, for the overall nursery period, there were no statistically significant differences among pigs fed different dietary treatments during the initial post-weaning period.

Throughout the experiment there were no signs of diarrhea or enteric health problems. At the end of phase I on d 10, visual observations indicated that pigs fed the diet containing 6 % SDPP had slightly firmer feces ($P < 0.05$) than those fed diets with 6 % Peptiva® (Table 3.4). No difference in fecal firmness was observed among dietary treatments at any other observation times.

Experiment Two

Because the dietary treatment responses were similar for each sub-trial, and within each sub-trial, there was no sampling period by dietary treatment interaction. The data from both trials were pooled for analysis and are presented in Table 3.5. It was observed that pigs readily consumed the experimental diets during each feeding. However, as the pigs were eating, greater spillage occurred with the SDPP supplemented diet than the other two dietary treatments. All diets were fed in meal form and this observation may have been related to the finer texture associated with the SDPP. As a consequence total daily DM intake and daily nitrogen intake was lower ($P < 0.05$) for the SDPP supplementatal diet. Related to reduced nitrogen consumption was a reduction in daily nitrogen absorption ($P < 0.05$) and daily nitrogen retention

($P < 0.05$) in the SDPP diet as compared to the control (Table 3.5). Earlier studies have demonstrated that moderate differences in consumption level have no impact on crude protein and AA utilization in pigs (Sauer et al., 1982; Hayden et al., 1984). Therefore, in this study percent absorption, retention and biological value provide reliable measures of diet effects on nitrogen balance. Percent absorbed nitrogen ranged from 88.51 to 89.34 % and was not different among treatments ($P > 0.05$). Similarly, percentage nitrogen retention ranged from 69.08 to 72.01 % and was not different among the treatments ($P > 0.05$). The calculation of biological value represents the proportion of absorbed nitrogen from the diet that is retained by the pig. In this study biological value ranged from 77.62 to 80.92 % and was not different among the three treatment diets ($P > 0.05$). The digestibility coefficient for DM was also similar among the three treatments ($P > 0.05$).

Discussion

Generally, our data is in agreement with most previous research that inclusion of SDPP in the diet of weanling pigs improves growth performance in phase I (Kats et al., 1994; Angulo and Cubilo, 1998), and most of the improvement in ADG is a result of increased ADFI. The increased consumption of diets containing SDPP may be due to greater palatability, because weanling pigs prefer diets containing SDPP to dried skim milk (Ermer et al., 1994). However, Exp.1 also indicated that there was a numerical advantage (15 % increase) in G:F for pigs fed the 6 % SDPP diet compared to the soy protein based diet, even though the difference was not statistically significant ($P = 0.14$). Jiang et al. (2000a) indicated that increased feed intake is not the sole reason for improved growth performance by SDPP addition, because ADG and protein conversion efficiencies were greater in a 10 % SDPP pair-fed group than in a soy control group. Our data also indicates that pigs fed SDPP diet have firmer stools than groups fed diets without

SDPP. Similarity, van Dijk et al. (2001b) observed that pigs fed the SDPP diet generally had a more favorable fecal score and a healthier small intestine than those fed soybean meal plus whey powder. However, the same authors (van Dijk et al., 2002) indicated that the immunoglobulins present in the SDPP diet did not affect intestinal proliferation and fecal excretion of *E. coli* strain that pigs were challenged with. Further research is needed to understand the potential protective effects of SDPP on intestinal health in weanling pigs.

One of our assumptions for this experiment was that the benefits of SDPP in weanling pigs diets is related to high quality and high digestibility of protein. However, under the conditions of this nitrogen balance experiment, the digestibility of DM, protein and nitrogen retention were not affected by diets. This is in agreement with Burnham et al. (1995), who reported that apparent digestibility of DM and nitrogen were not improved by SDPP compared with wheat gluten, although growth performance was improved. Chae et al. (1999) also reported that SDPP did not improve the apparent digestibility of protein and energy compared to a soybean meal based diet. Several reasons could be responsible for the lack of effects on digestibility and nitrogen balance in this study, but growth performance was improved by SDPP supplementation. First, the benefits of SDPP during phase I in the performance experiments were perhaps not related to digestibility or nitrogen retention, but instead other factors such as palatability, or passive immunity and intestinal morphology (van Dijk et al., 2001a). Second, the supplemental level of SDPP in the nitrogen balance experiment was only 6 %, which provided only about 20 % of total dietary protein. The chance of having digestibility difference among treatments is very low, due to the experimental variation and high baseline digestibility of the control diet. We chose a dietary addition rate for SDPP of 6 % because this is a similar level as used in commercial practice due to the high cost of this ingredient. Lastly, the environment may

account for most of the contradictory results between growth performance, nitrogen digestibility and balance. The growth experiment was conducted in a conventional nursery situation, while the nitrogen balance trial was conducted under a very clean and well-controlled experimental metabolism room. Some previous studies (Coffey and Cromwell, 1995; Bergstrom et al., 1997; Campbell et al., 2003) indicated that pigs housed in a conventional nursery responded more to SDPP than pigs housed in a cleaner, more controlled experimental nursery. The authors suggested that these responses might have been related to immunological effects of SDPP.

It is well known that animals cannot thrive under the conditions of a pure AA supplemented diet without providing intact protein, but can survive with synthetic dipeptide-based diets (Dabrowski et al., 2003). Absorption of intact peptides from mammalian intestine has been recognized for several years, and the importance of peptide transporters is well documented based on the fact that peptides are more abundant than AA in the intestinal lumen. Previous in vitro studies using Caco cell lines and Chinese hamster ovary cells suggested that peptide transport is more important and efficient than amino acid transport for intestinal absorption (Chen, 2001). Direct evidence was provided by Zhao et al. (1997), whose results suggested that intestinal transit was inhibited by intact protein more potently than hydrolyzed peptides, and hydrolyzed peptide absorption by the proximal half of the small intestine was greater and more efficient than intact soy protein in the dog. Limited applied research has been conducted to investigate the possibility or the potential benefits of utilizing peptides in livestock. Our experiments indicated that the commercial source of peptides (Peptiva®) did not provide any additional benefits on growth performance, digestibility or nitrogen balance. The Peptiva® product used in these experiments contains a lower level of protein and ME (52% CP and 2692 kcal/kg ME) compared with soy protein concentrate and SDPP (64 and 78 % CP, respectively;

4100 and 3895 kcal/kg ME, respectively). In addition, Peptiva® contains a higher level of crude fiber (4.16 %) compared to the other two protein sources. Higher dietary fiber may affect the digestibility and growth performance of early weanling pigs. Even though the theory and biochemistry of absorption of peptides, and the potential benefits of hydrolyzed or synthesized peptides are well developed, limited information is available about utilization of peptides in livestock diets. Under our experimental condition, no performance benefits of supplemental Peptiva® were observed.

Implications

Even though SDPP has proved to be a high quality protein source for weanling pigs, SDPP is quite expensive compared to other protein sources. Also, consumer concerns on feeding animal products back to animals might inhibit the usage of SDPP in pig diets in the future. Better understanding of the mechanisms of SDPP in piglets may help nutritionists find cost effective and safe alternative supplements for the animal industry. Several biological explanations (palatability, digestibility, immunity, and intestinal morphology) have been proposed, but none of them can be solely attributed to the observed benefits of SDPP. Our studies did not show any benefits of utilizing the dietary peptide source, Peptiva®, in weanling piglets. Further research is needed to apply the peptide transport theory to the livestock industry.

Table 3.1. Phase I dietary treatments for evaluation of Peptiva® as a replacement for SDPP in early weanling pig diets (Exp.1) [a]

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Ground corn	37.50	37.50	37.50	37.50	37.50
Soybean meal, dehulled	15.00	15.00	15.00	15.00	15.00
Dried whey	20.00	20.00	20.00	20.00	20.00
Lactose	5.00	5.00	5.00	5.00	5.00
Fish meal, menhaden	2.50	2.50	2.50	2.50	2.50
SDPP (AP920) [b]	---	6.00	3.00	---	---
Peptiva® [c]	---	---	3.00	6.00	6.00
Soy protein concentrate	12.18	4.87	4.87	4.87	7.29
Soybean oil	2.53	1.60	1.60	1.60	3.70
Dicalcium phosphate	1.47	1.24	1.35	1.45	1.35
Calcium carbonate	0.50	0.67	0.61	0.54	0.58
Salt	0.25	0.25	0.25	0.25	0.25
Synthetic lysine	0.177	0.044	0.044	0.044	0.23
D-L-Methionine	0.14	0.076	0.076	0.076	0.14
Vitamin premix [d]	0.25	0.25	0.25	0.25	0.25
Trace mineral premix [e]	0.15	0.15	0.15	0.15	0.15
Corn starch	2.35	4.85	4.80	4.77	0.06
Totals	100.00	100.00	100.00	100.00	100.00
Calculated analysis [f]					
Crude protein, %	22.00	22.00	22.00	22.00	22.00
Total lysine, %	1.50	1.50	1.38	1.23	1.50
Methionine + cystine, %	0.86	0.86	0.73	0.67	0.86
Calcium, %	0.90	0.90	0.90	0.90	0.90
Total phosphorus, %	0.80	0.80	0.80	0.80	0.80
ME, Mcal/kg	3.415	3.415	3.377	3.340	3.415

[a] Phase I diets of experiment were fed for the initial 10 d post-weaning.

[b] SDPP: spray-dried plasma protein, provided by American Protein Corp, Ames, IA

[c] Peptiva®: provided by Vitech Biochem, San Fernando, CA

[d] Vitamin premix provided per kg of diet: 12,400 IU of vitamin A as acetate, 2,067 IU of stabilized vitamin D3, 82 IU of vitamin E as DL-tocopheryl acetate, 5.5 mg of vitamin K as menadione sodium bisulfate, 11 mg of riboflavin, 36 mg of D-Pantothenic acid as calcium pantothenate, 63 mg of niacin as nicotinamide, 0.28 mg of D-biotin, 1.65 mg folic acid, 2.07 mg pyridine, 1.25 mg thiamine, and 0.05 mg of vitamin B12.

[e] Trace mineral premix provided per kg of final diet: 20 mg of copper as copper sulfate, 150 mg of iron as ferrous sulfate, 40 mg of manganese as manganese sulfate, 150 mg of zinc as zinc sulfate, 0.5 mg of iodine as calcium iodine, 0.3 mg of selenium as sodium selenite.

[f] Values were calculated using NRC (1998), and data provided by the manufacturer for nutrient and ME composition of Peptiva®.

Table 3.2. Composition of phase II and III diet in Exp.1

Ingredient	Phase II [a]	Phase III [b]
Ground corn	53.69	66.11
Soybean meal, dehulled	20.00	28.91
Dried whey	15.00	---
Fish meal, menhaden	7.50	---
Soybean oil	1.75	1.75
Dicalcium phosphate	0.95	1.71
Calcium carbonate	0.16	0.72
Salt	0.30	0.35
Synthetic lysine	0.25	0.10
Vitamin premix [c]	0.25	0.20
Trace mineral premix [d]	0.15	0.15
Totals	100.00	100.00
Calculated analysis [e]		
Crude protein, %	20.42	19.23
Total lysine, %	1.43	1.12
Methionine + cystine, %	0.71	0.65
Calcium, %	0.86	0.77
Total phosphorus, %	0.80	0.70
ME, Mcal/kg	3.388	3.384

[a] Phase II diets fed from d 11 through d 20.

[b] Phase III diet fed from d 21 through d 35 (end of nursery period).

[c] In phase II, vitamin premix provided per kg of diet: 12,400 IU of vitamin A as acetate, 2,067 IU of stabilized vitamin D3, 82 IU of vitamin E as DL-tocopheryl acetate, 5.5 mg of vitamin K as menadione sodium bisulfate, 11 mg of riboflavin, 36 mg of D-Pantothenic acid as calcium pantothenate, 63 mg of niacin as nicotinamide, 0.28 mg of D-biotin, 1.65 mg folic acid, 2.07 mg pyridine, 1.25 mg thiamine, and 0.05 mg of vitamin B12. In phase III, vitamin premix

provided per kg of diet: 9,921 IU of vitamin A as acetate, 1,653 IU of stabilized vitamin D3, 66 IU of vitamin E as DL-tocopheryl acetate, 4.4 mg of vitamin K as menadione sodium bisulfate, 8.8 mg of riboflavin, 29 mg of D-Pantothenic acid as calcium pantothenate, 51 mg of niacin as nicotinamide, 0.22 mg of D-biotin, 1.32 mg folic acid, 1.65 mg pyridine, 1.0 mg thiamine, and 0.04 mg of vitamin B12.

[d] Trace mineral premix provided per kg of final diet: 20 mg of copper as copper sulfate, 150 mg of iron as ferrous sulfate, 40 mg of manganese as manganese sulfate, 150 mg of zinc as zinc sulfate, 0.5 mg of iodine as calcium iodine, 0.3 mg of selenium as sodium selenite.

[e] Values were calculated using NRC (1998).

Table 3.3. Effects of dietary supplementation with spray-dried plasma protein (SDPP) or Peptiva® product during Phase I on growth performance in early weanling pigs [a]

	Experimental diet during phase I (d 1-10)					
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	
Criteria	Control	6 % SDPP	3 % SDPP + 3 % Peptiva®	6 % Peptiva®	6 % Peptiva® (adjusted) [b]	SEM [c]
Phase I (d 0-10)						
BW (initial), kg.	6.34	6.38	6.38	6.33	6.41	0.11
BW (d 10), kg.	7.38^{x}	8.01^{y}	7.49^{x}	7.43^{x}	7.48^{x}	0.24
ADG, g.	116^{x}	163^{y}	111^{x}	109^{x}	107^{x}	11
ADFI, g.	221^{x}	295^{y}	228^{x}	231^{x}	205^{x}	13
G:F	0.47	0.54	0.46	0.47	0.49	0.03
Phase II (d 11-20)						
BW (d 20), kg.	11.44	12.24	11.39	11.86	11.97	0.63
ADG, g.	405	423	385	443	449	22
ADFI, g.	655	652	578	658	646	24
G: F	0.61^{x}	$0.65^{x,y}$	$0.67^{x,y}$	$0.67^{x,y}$	0.69^{y}	0.02
Phase III (d 21-35)						
BW (d 35), kg.	20.23	21.00	20.10	20.42	20.57	1.08
ADG, g.	586	584	584	560	573	16
ADFI, g.	1020	1057	1022	1015	1025	26
G: F	0.57	0.55	0.57	0.55	0.56	0.01
Overall (d 1-35)						
ADG, g.	396	418	392	398	405	14
ADFI, g.	684	723	668	689	682	48
G: F	0.58	0.58	0.58	0.57	0.59	0.01

[a] Seven replicate pens of pigs for each treatment, four pigs per pen.

[b] This diet has similar nutrient and energy concentration as diet 1 and 2, with adjustment with soy concentrate, soybean oil, and corn starch.

[c] Standard error of the mean.

[x,y] within a row, means without a common superscript differ ($P < 0.05$).

Table 3.4. Effects of dietary supplementation with spray-dried plasma protein or Peptiva® during Phase I on fecal firmness scores in early weanling pigs [a]

	Experimental diet during phase I (d 1-10)					
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	
Day	Control	6% SDPP	3 % SDPP 3 % Peptiva®	6 % Peptiva®	6% Peptiva® adjusted	SEM [b]
Day 5	2.57	2.43	2.28	2.43	2.57	0.21
Day 10	2.71 [x,y]	2.28 [y]	2.86 [x,y]	3.00[x]	3.00 [x]	0.17
Day 20	2.43	2.71	2.68	2.57	2.85	0.16
Day 35	3.00	3.00	2.85	2.85	3.00	0.15

[a] Fecal firmness score was on a scale of 1 to 5 with a score of 1 indicating exceptionally firm fecal material and 5 indicating very loose, watery feces.

[b] Standard error of the means.

[x,y] Means in the same line without a common superscript differ ($P < 0.05$).

Table 3.5. Effects of dietary supplementation with SDPP or Peptiva® product on digestibility and nitrogen balance in early weanling pigs [a]

Item	Control	6 % SDPP	6 % Peptiva®	SEM [b]	P value
Daily N intake, g	5.61 [c]	5.18 [d]	5.42 [e]	0.04	0.01
Daily fecal N, g	0.61	0.55	0.62	0.03	0.13
Daily urine N, g	0.96	1.04	1.05	0.08	0.71
Daily absorbed N, g	5.00 [c]	4.63 [d]	4.80 [c,d]	0.06	0.01
Daily retained N, g	4.04 [c]	3.59 [d]	3.74 [c,d]	0.11	0.03
Absorbed N, %	89.06	89.34	88.51	0.53	0.55
Retained N, %	72.01	69.33	69.08	1.63	0.39
Biological Value, % [f]	80.92	77.62	78.03	1.78	0.39
Daily intake DM, g	140 [c]	133 [d]	139 [c]	1.11	0.01
Daily fecal DM, g	11.89 [c,d]	10.61 [d]	12.15 [c]	0.38	0.03
DM Digestibility, %	91.51	92.01	91.28	0.29	0.22

[a] Eight replications (cages) per treatment with two pigs per cage.

[b] Standard error of the mean.

[c,d,e] Means in the same line without a common superscript differ ($P<0.05$).

[f] Biological value was calculated as the percentage of absorbed N that was retained.

Chapter IV

Improved growth performance and intestinal morphology with dietary Spray-dried plasma protein and organic copper in weanling pigs reared in sanitary and sub-sanitary pens

ABSTRACT

Weanling pigs (n = 192, 18 $\pm$ 2 d of age, 6.0 $\pm$ 0.2 kg BW) were used to investigate the effects of dietary addition of spray-dried plasma protein (SDPP, 0 or 6 % for 10 d) and Cu from an organic Cu complex (0 or 200 ppm for 35 d) on growth performance and intestinal morphology under sanitary or sub-sanitary conditions. The sub-sanitary condition was created by applying swine manure slurry to all surfaces of the sub-sanitary pens during the wk prior to weaning with the acknowledgement that pen sanitation and nursery room would be confounded. There were four pigs per pen; feed and water were available *ad libitum*. On d 10 one pig per pen was killed and sections of duodenum, jejunum and ileum were fixed, stained and prepared for microscopic assessment of mucosal morphology. By d 10 post-weaning SDPP and Cu supplementation improved ADG and ADFI (P = 0.001), while pigs reared in the sub-sanitary pens had lower ADG (P = 0.05) than those from sanitary pens. Trends for interaction of diet and pen sanitation were observed for G:F with more pronounced response to SDPP (P = 0.07) or Cu (P = 0.11) supplementation in the sub-sanitary pens. By d 35 there were no main or interactive effects of treatment on performance (P = 0.19). In each intestinal segment, shorter villous length and less crypt depth were observed in pigs housed in the sub-sanitary pens (P < 0.05). In the duodenum, reduced crypt depth with Cu supplementation (P < 0.01) and improved villous height with SDPP supplementation (P = 0.09) were observed. Under these experimental conditions, SDPP and Cu supplementation improved pig growth performance during the initial 10 d post-weaning and responses for G:F tended to be greater under sub-sanitary conditions. Poor

sanitation conditions in the pig housing environment appear to have a negative impact on mucosal morphology.

Key Words: Copper, Intestinal morphology, Pigs, Spray-dried plasma protein.

Introduction

Antibacterial feed additives have been used in swine production for decades and are very effective for improvements in growth rate of up to 16 % and feed efficiency of up to 6 % in weanling and starter pigs (Cromwell, 2001). Despite their effectiveness, continued use of antibacterial growth promoters faces an uncertain future because of the potential development of resistant microbes that may compromise the effectiveness of antibiotics when treating human or animal diseases.

Copper is an essential trace element for all classes of swine. It is well established that elevated dietary Cu (100 to 250 ppm) generally results in increased growth of weanling pigs, much like that seen with sub-therapeutic antibiotic feed additives. Spray-dried plasma protein (SDPP) is a high quality protein source for weanling pigs. Most studies indicate that SDPP consistently improves growth performance when included in phase I pig diets (Kats et al., 1994; de Rodas et al., 1995). The mode of action of SDPP is not completely clear but appears related to localized immunological effects in the gastrointestinal tract. With the uncertain future of antibacterial growth promoters, the use of supplemental products such as organic Cu complexes and SDPP may have increasing importance to the swine industry. Moreover, the level of sanitation management may influence the efficiency of such products. Coffey and Cromwell (1995) reported that the increased performance with supplementation of SDPP was more profound when pigs were reared in conventional, on-farm nurseries compared with those reared in optimal experimental conditions. Similar observations have been reported for the response to

antibacterial growth promoters (Cromwell, 2001). However, such studies were to some degree confounded by physical location and facility differences and may not reflect true sanitation or microbial environment differences.

Because the response to SDPP and antibacterial growth promoters are considered additive (Coffey and Cromwell, 1995), the potential exists for organic complex copper supplementation and SDPP supplementation to produce additive responses as well. Considering these factors, the objective of this study was to determine the effects of dietary supplementation of Bioplex® Copper (200 ppm Cu, Alltech Inc., Nicholasville, KY) and SDPP (6 % Appetein®, American Protein Corp., Ankeny, IA) individually and in combination on the growth performance and intestinal morphology of early weaned pigs housed in sanitary or sub-sanitary environmental conditions.

Materials and Methods

Animals and Facilities

Crossbred weanling pigs (n = 192, 18 $\pm$ 2 d of age, BW 6.04 $\pm$ 0.2 kg) produced from terminal line Duroc AI boars and Yorkshire × Landrace sows were randomly assigned to eight treatments in blocks balanced for initial BW, litter of origin and sex across treatments. There were six replicate pens for each treatment with two gilts and two barrows per pen. The eight treatments were designed according to a 2 x 2 x 2 factorial arrangement. The treatment factors included two levels of dietary Bioplex® Copper (0 and 200 ppm), two levels of SDPP (0 and 6 % Appetein®) and two nursery sanitation conditions. The sub-sanitary condition was created by applying swine manure slurry to all surfaces of the sub-sanitary pens three times during the wk prior to weaning. This manure slurry was collected from growing-finishing pig and sow manure pits on the same research farm.

Pigs were housed in two identical, environmentally controlled nursery rooms, with supplemental heat and a negative pressure ventilation system. Room temperature and humidity were recorded twice per d and managed to ensure that the two nursery rooms had similar environments. This experimental design was followed with the acknowledgement that pen sanitation and nursery room were confounded but the rooms were managed to minimize room effects. Pens were 0.9 by 1.2 m in dimension and equipped with triangular bar flooring, a stainless steel feeder and nipple drinker. Feed and water were available *ad libitum*.

Experimental Diets

Complex nursery diets were formulated to have similar nutrients and metabolizable energy concentrations for all treatments. To ensure consistent nutritional formulation across treatments, the common ingredients for each experimental diet were prepared as a single basal diet and each experimental diet was then prepared from this common basal diet. The phase I experimental diets (Table 4.1) were fed for the first 10 d post-weaning. Phase II diets were fed from d 11 to 20 and phase III diets from d 21 to 35 (Table 4.2). The SDPP treatments were discontinued after phase I similar to standard industry practice. The Bioplex® Copper treatments were continued through phases II and III to the conclusion of the 35-d nursery period. Pigs were individually weighed at the end of each diet phase (i.e. d 10, 20, and 35). Also at these time points, pen feed consumption was determined and feed efficiency was calculated.

Plasma Urea Nitrogen and Intestinal Morphology

On d 10, blood samples were taken from each pig for determining plasma urea nitrogen (PUN). Assay of PUN served as an assessment of the efficiency of dietary protein utilization for protein accretion. Samples were collected into vacuum tubes containing sodium heparin as the anti-coagulant by jugular vein puncture, and plasma harvested by centrifugation and stored at -20

° C until assayed. A standard assay kit (Sterling Diagnostics, Inc. Sterling Heights, MI) was used for PUN determination. Briefly the method is based on hydrolysis of urea by urease with production of ammonia and carbon dioxide. The ammonia then reacts with salicylate, nitroferricyanide and an alkaline solution of hypochloride to yield a blue-green chromophore. The chromophore is measured photometrically (Spectronic® Genesys™ 5, Thermo Electron Corporation, San Jose, CA) and is proportional to the amount of urea in the plasma.

A single female pig closest to the average BW within each pen was chosen on d 10 for intestinal morphology assessment. The selected pigs were humanely killed using an iv injection of sodium pentobarbital injection (80 mg/kg BW). The small intestine was dissected free of mesenteric attachments and placed on a smooth surface. Three 10-cm segments at locations of 25, 50, and 75 % of the total intestinal length were removed and represented duodenum, jejunum, and ileum, respectively. The segments were gently rinsed with ice-cold phosphate buffered saline, cut into two 2-cm segments and fixed in neutral buffered formalin. The tissue segments were transported to a commercial laboratory (HISTO Scientific Research Laboratories, Woodstock, VA) for embedding in paraffin, staining and slide preparation. Two cross sections of each intestinal segment were processed in paraffin and stained with hematoxylin and eosin. Villous height and crypt depth were quantified using a digitized board coupled to a video monitor receiving output from a video camera mounted on a binocular microscope (Olympus Polaroid DMC-IE camera, Polaroid Corp, MA). Output from the digitizing board was collected with the program of SigmaScan Pro 5. (SPSS Inc., Chicago, IL). Twelve of the longest well-oriented villi and associate crypts were measured for each intestinal segment, following the procedure similar to Torrallardona et al. (2003). The villous height was measured from tip to base, and then the crypt depth was measured from the base of the villous to the base of the crypt.

Mean villous height, crypt depth, and villous crypt ratio (VCR) for each pig were calculated for statistical analysis.

Statistical Analysis

The data were analyzed using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). The model included the main effects of Bioplex® Copper, SDPP, and sanitation condition and all appropriate two-way interactions. The three-way interaction was removed from the model once it was determined to be non-significant ($P > 0.10$). The pen served as the experimental unit. Response criteria included growth rate, feed consumption, feed efficiency and PUN. Intestinal morphology criteria included villous height, crypt depth and VCR. All the intestinal morphology values were analyzed separately for each intestinal segment.

Results

Growth Performance

During the initial 10 d post-weaning (phase I), there were pronounced improvements in ADG and ADFI as a result of BioPlex® Copper ($P < 0.002$) and SDPP ($P < 0.001$) addition to the diets (Table 4.3 and 4.4). Because gain and feed intake increased concomitantly, the main effects of BioPlex® Copper ($P = 0.14$) or SDPP ($P = 0.22$) were not significant for G:F. Based on the lack of significant interactions between BioPlex® Copper and SDPP for all growth performance traits ($P = 0.31$ to 0.82), the effects of these supplements can be considered independent and additive during the phase I nursery period.

It was presumed that the most sensitive period for a response to pen sanitation conditions would be during the initial post-weaning period (phase I). Indeed a main effect of sanitation conditions on ADG was observed ($P = 0.05$) with pigs reared in the unsanitary pens displaying a

slower growth rate during this period (Table 4.3 and 4.4). This period was also presumed to be the most likely phase for potential sanitation by feed additive interactions. Such a trend was observed for G:F ratio with SDPP supplementation diets tending to have greater G:F improvement under the unsanitary pen conditions (interaction trend, $P = 0.07$, Table 4.3). Pigs fed diets supplemented with BioPlex® Copper also tended to have greater G:F improvement under the unsanitary pen conditions (interaction trend, $P = 0.11$, Table 4.4).

During phase II (d 11 to 20), dietary inclusion of SDPP had been discontinued, but supplementation of BioPlex® Copper continued. During this diet phase no effect of diet treatment or pen sanitation condition was observed for the growth traits measured (Table 4.3 and 4.4, $P = 0.14$). Likewise there were no significant ($P = 0.46$) two-way interactions during phase II. Main effect responses were limited during phase III (d 21 to 35), with an exception of Cu supplementation. Pigs fed diets supplemented with Cu had slightly reduced ($P = 0.04$) ADFI during phase III but, because ADG was not reduced by the same magnitude, Cu supplemented pigs tended to have a better G:F ratio ($P = 0.08$). Pigs fed SDPP supplemented diets during phase I tended ($P = 0.09$) to have greater ADFI during phase III. For all three diet phases combined (d 1 through 35), there were generally no main or interactive effects of diet treatment or pen sanitation conditions on growth performance. Including SDPP in the diet during phase I tended to result in greater ADFI ($P = 0.10$) for the overall trial (Table 4.3).

Plasma Urea Nitrogen

Plasma urea nitrogen concentration has been used to assess the efficiency of dietary protein utilization for protein accretion (Eggum, 1970). The PUN concentrations observed in this study were from 11 to 13 mg /dl, which is within the normal range for pigs of this age (Table 4.3 and 4.4). Neither SDPP, copper supplementation or pen sanitation condition had any main or

interactive effects on PUN ($P = 0.37$ to 0.91), indicating that dietary treatments and pen sanitation conditions had no measurable effect on protein utilization or nitrogen balance and SDPP and copper had no effect on protein utilization or biological value.

Intestinal Morphology

All intestinal morphology measurements were collected and analyzed separately for each segment (duodenum, jejunum, and ileum); no major segment effect nor interaction was determined. The method of selecting the female pig within each pen that was closest to the average pig weight in the pen was chosen to reduce variation in morphology associated with BW variation. No regression relationship ($P = 0.14$) between BW and villous height or BW and crypt depth were detected (data not presented). Therefore, BW was not considered as a covariance factor when analyzing the histology data.

The most striking morphological differences were observed in the duodenum (Table 4.6). Pigs housed in unsanitary pens had significantly lower ($P = 0.01$) villous height and less crypt depth ($P = 0.02$) than those in sanitized pens (Table 4.6, Fig. 4.1). Because both villous height and crypt depth were decreased to a similar extent in the pigs raised in the sub-sanitary environment, the villous to crypt ratio was not affected ($P = 0.60$ to 0.83) by sanitation treatment. Similar results were observed in the jejunum and ileum ($P = 0.003$ to 0.05). Feeding SDPP tended to increase ($P = 0.09$) the villous height in the duodenum (Fig. 4.1), but effects in the jejunum and ileum were not significant. Pigs fed 200 ppm Cu from BioPlex® Copper had numerically higher villous height than the control group in duodenum and jejunum (Table 4.7), and the difference tended to approach significance in the jejunum ($P = 0.10$). Copper supplementation decreased the duodenal crypt depth, and consequently increased ($P < 0.01$)

VCR in the duodenum and jejunum because of the increased villous height and decreased crypt depth.

Two-way interactions were varied with no defined biological pattern apparent. Trends ($P < 0.10$) of SDPP and environment interaction on crypt depth and VCR in duodenum were observed (Table 4.6 and 4.7). SDPP increased crypt depth relative to the control diet in the sub-sanitary pens, but not in the sanitary pens. The pigs fed SDPP and raised in the sanitary pens had the greatest VCR compared to other groups. Significant interaction of SDPP and copper was also observed on crypt depth and VCR along all sites of the small intestine ($P < 0.10$). The pigs fed diets with 6 % SDPP and 200 ppm BioPlex® Copper had the highest VCR compared to other groups. In the ileum, this group had the lowest crypt depth among treatments ($P < 0.01$).

Discussion

The growth performance results agree with most previous studies that SDPP (Augulo and Cubillo, 1998; van Dijk et al., 2001a) and Cu (Harper et al., 2001; Davis et al., 2002) significantly improve ADG and ADFI in phase I but had no effect on G:F. By using a pair-fed strategy, Jiang et al. (2000a) reported that palatability or high feed intake is not the sole cause of improved growth performance, and that SDPP protein is more efficient for use in lean tissue growth than extruded soy protein. Besides palatability, the mode of action of SDPP is not completely clear but appears related to localized immunological effects in the GI tract. Spray-dried plasma protein contains 15 to 20 % of immunoglobulins and 0.8 ng/mg immune-active IGF (de Rodas et al., 1995), which could prevent viruses and bacteria from damaging the gut wall, resulting in a more functional intestinal wall (Coffey and Cromwell, 1995). In this study, SDPP supplementation tended to increase the villous height in the duodenum. Pigs fed SDPP diet appeared to have a more healthy intestinal morphology compared to the control group, although

no intestinal wall damage was observed for the other treatments. The morphology data provides indirect evidence that SDPP may protect duodenal integrity and development. This protective function was lost in the distal part of GI tract after whole immunoglobulins were digested and destroyed by enzymes. In addition, the villous tip is the active absorption area of enterocyte cell. Longer villi indicate a greater surface area and the potential for more efficient assimilation of nutrients. The increased villous height provides another potential growth promoting mechanism of SDPP, even though the PUN data and our previous nitrogen balance experiment (Chapter III) did not provide direct evidence in support of this theory.

The BioPlex® Copper used in this study is a mineral chelate of AA and short-chain peptides from soy protein (Waldroup et al., 2003). We chose the organic copper because there is evidence that the organic BioPlex® Copper may be more bio-available and more effective (Harper et al., 2001) in stimulating growth than copper sulfate. In this study, 200 ppm BioPlex® Copper supplementation significantly improved ADG and ADFI 10 d after weaning, and G:F tended to increase ($P = 0.08$) in phase III (21 to 35 d after weaning). It was originally proposed that the mode of action of copper is through an antibacterial-like action in the gastrointestinal tract (Fuller, 1960). More recent evidence suggests that Cu may act systemically as well (Zhou et al., 1994). The intestinal morphology results of this study support the idea that the growth stimulation effect of Cu is related to its impact on the intestine. The groups of pigs fed Cu had significantly shorter crypt and numerically longer villi along the whole small intestine compared to the control group. Consequently the VCR was significantly increased with BioPlex® Copper supplementation. Crypt depth gives a general indication of the rate of crypt cell production (Hampson, 1986). When crypt cells migrate, they become increasingly differentiated and their absorptive capacity develops. Therefore, measurement of crypt depth gives a general indication

of the maturity and functional capacity of enterocytes of the villi. The increased VCR usually means more absorption area, and less epithelial cells were recruited for cell renewal. The energy required to maintain the GI tract has been estimated to be approximately 24 % of the pig's daily maintenance energy requirements (Yen et al., 1988). Observations of this study may support the hypothesis that high dietary copper may decrease the energy requirements of the GI tract, and consequently decrease the maintenance energy requirement of the pigs, making more energy and nutrients available for growth (Radechi et al., 1992).

The pigs reared in the the unsanitary environment ate less and gained less in phase I compared with those reared under more sanitary conditions. The environmental conditions also impacted intestinal integrity and development. The pigs reared under the sub-sanitary environment had shorter duodenal villi and less crypt depth compared with those reared under sanitary environments. Similar results were observed in jejunum and ileum sections. Because decreased villi and crypt depth were concomitant, the VCR was not affected. It is possible that intestinal development was negatively affected in unsanitary pens, possibly due to sub-clinical exposure to microflora.

Under the condition of this study, the stimulated growth performance by both SDPP and Cu were more profound when pigs were reared under unsanitary conditions. We created the unsanitary condition by applying swine manure slurry to the surfaces of nursery pens the wk prior to the weaning, while the sanitary nursery room was completely cleaned and sterilized. The purpose was to mimic the commercial nursery condition, that the pigs are exposed to bacteria but without causing serious clinical disease symptoms. Based on the result of this study, it is suggested that the growth stimulation effects of SDPP and Cu are related to their beneficial effects on intestinal immune system, because first, the benefits are more profound when pigs

were under sanitation stress, and second both dietary SDPP and Cu affect the intestinal integrity and morphology. Our studies agree with van Dijk et al. (2002) and Torrallardona et al. (2003) in that SDPP may be an alternative to antibiotics for weanling pigs, because it provides a level of protection in the intestine. However, other studies (Touchette et al., 2002; Frank et al., 2003) indicated that feeding SDPP during lipopolysaccaride challenge resulted in increased stress and over-responses of the immune system, which resulted in major damage to the mucosa of intestinal wall. The authors suspected that the differences in immune response to SDPP supplementation may depend on the degree of challenge or stress. SDPP cannot replace antibody utilization when animals are in a disease condition, but may be used as a preventative medicated additive under sub-clinical sanitation stress conditions. In summary, under conditions of this study, SDPP may improve growth by maintaining intestinal health, while Cu may reduces the maintenance energy requirement of the GI tract, and consequently increases nutrient and energy availability for growth. The pigs raised under more sanitary conditions had better performance and healthier intestines compared with those raised in the sub-sanitary environment.

Implication

Both copper and SDPP improved growth performance of weaned pigs 10 d after weaning. The possible mechanisms of action of SDPP and copper are perhaps different; copper may act as an anti-microbial additive and reduces the maintenance energy requirement of the GI tract, while SDPP protects the intestine from bacterial attachment and maintains intestinal health. Pigs reared in sub-sanitary condition had poorer growth performance. Intestinal morphology was also impacted. Dietary SDPP and copper appear to be effective post-weaning growth promotion in general and may be more effective when pigs are raised in sub-optimal sanitation conditions.

Table 4.1. Phase I dietary treatments [a]

Ingredient, %	Control	SDPP	BioPlex® Copper	SDPP + BioPlex® Copper
Ground corn	37.50	37.50	37.50	37.50
Soybean meal, dehulled	15.00	15.00	15.00	15.00
Dried whey	20.00	20.00	20.00	20.00
Lactose	5.00	5.00	5.00	5.00
Fishmeal, menhaden	2.50	2.50	2.50	2.50
SDPP (Appetein®)	---	6.00	---	6.00
BioPlex® Copper [b]	---	---	0.02	0.02
Soy protein concentrate	12.18	4.87	12.18	4.87
Soybean oil	2.53	1.60	2.53	1.60
Dicalcium phosphate	1.47	1.24	1.47	1.24
Calcium carbonate	0.50	0.67	0.50	0.67
Salt	0.25	0.25	0.25	0.25
Synthetic lysine	0.177	0.044	0.177	0.044
D-L-Methionine	0.140	0.076	0.14	0.076
Vitamin premix [c]	0.25	0.25	0.25	0.25
Trace mineral premix [d]	0.15	0.15	0.15	0.15
Corn starch	2.353	4.85	2.333	4.830
Totals:	100.00	100.00	100.00	100.00
Calculated analysis: [e]				
Crude protein, %	22.00	22.00	22.00	22.00
Total lysine, %	1.50	1.50	1.50	1.50
Methionine + cystine, %	0.86	0.86	0.86	0.86
Calcium, %	0.90	0.90	0.90	0.90
Total phosphorus, %	0.80	0.80	0.80	0.80
ME, kcal./kg	3415	3415	3415	3415

[a] Phase I diets were fed for the initial 10 d post-weaning.

[b] For indicated diets, BioPlex® Copper supplemented to provide 200 ppm additional dietary copper.

[c] Vitamin premix provided per kg of diet: 12,400 IU of vitamin A as acetate, 2,067 IU of stabilized vitamin D3, 82 IU of vitamin E as DL-tocopheryl acetate, 5.5 mg of vitamin K as menadione sodium bisulfate, 11 mg of riboflavin, 36 mg of D-Pantothenic acid as calcium pantothenate, 63 mg of niacin as nicotinamide, 0.28 mg of D-biotin, 1.65 mg folic acid, 2.07 mg pyridine, 1.25 mg thiamine, and 0.05 mg of vitamin B12.

[d] Trace mineral premix provided per kg of final diet: 20 mg of copper as copper sulfate, 150 mg of iron as ferrous sulfate, 40 mg of manganese as manganese sulfate, 150 mg of zinc as zinc sulfate, 0.5 mg of iodine as calcium iodine, 0.3 mg of selenium as sodium selenite.

[e] Values were calculated using NRC (1998).

Table 4.2. Phase II and III diets

Ingredient, %	Phase II [a]	Phase III [b]
Ground corn	53.69	66.11
Soybean meal, dehulled	20.00	28.91
Dried whey	15.00	---
Fishmeal, menhaden	7.50	---
Soybean oil	1.75	1.75
Dicalcium phosphate	0.95	1.71
Calcium carbonate	0.16	0.72
Salt	0.30	0.35
Synthetic lysine	0.25	0.10
Vitamin premix [c]	0.25	0.20
Trace mineral premix [d]	0.15	0.15
Totals:	100.00	100.00
Calculated analysis [e]:		
Crude protein, %	20.42	19.23
Total lysine, %	1.43	1.12
Methionine + cystine, %	0.71	0.65
Calcium, %	0.86	0.77
Total phosphorus, %	0.80	0.70
ME, kcal./kg	3388	3384

[a] Phase II diets fed from d11 through d 20.

[b] Phase III diet fed from d 21 through d 35 (end of nursery period). The BioPlex® Copper treatments were continued through phase II and phase III.

[c] In phase II, vitamin premix provided per kg of diet: 12,400 IU of vitamin A as acetate, 2,067 IU of stabilized vitamin D3, 82 IU of vitamin E as DL-tocopheryl acetate, 5.5 mg of vitamin K as menadione sodium bisulfate, 11 mg of riboflavin, 36 mg of D-Pantothenic acid as calcium pantothenate, 63 mg of niacin as nicotinamide, 0.28 mg of D-biotin, 1.65 mg folic acid,

2.07 mg pyridine, 1.25 mg thiamine, and 0.05 mg of vitamin B12. In phase III, vitamin premix provided per kg of diet: 9,921 IU of vitamin A as acetate, 1,653 IU of stabilized vitamin D3, 66 IU of vitamin E as DL-tocopheryl acetate, 4.4 mg of vitamin K as menadione sodium bisulfate, 8.8 mg of riboflavin, 29 mg of D-Pantothenic acid as calcium pantothenate, 51 mg of niacin as nicotinamide, 0.22 mg of D-biotin, 1.32 mg folic acid, 1.65 mg pyridine, 1.0 mg thiamine, and 0.04 mg of vitamin B12.

[d] Trace mineral premix provided per kg of final diet: 20 mg of copper as copper sulfate, 150 mg of iron as ferrous sulfate, 40 mg of manganese as manganese sulfate, 150 mg of zinc as zinc sulfate, 0.5 mg of iodine as calcium iodine, 0.3 mg of selenium as sodium selenite.

[e] Values were calculated using NRC (1998).

Table 4.3. Effects of spray-dried plasma protein on growth performance and PUN in weanling pigs reared in sanitary and sub-sanitary environments [a,b]

	Unsanitary pens		Sanitary pens			P-value		
SDPP level	0 %	6 %	0 %	6 %	SEM	SDPP	Sanitation	Interaction
Phase 1 (d 1-10)								
BW (initial), kg	6.06	6.03	6.05	6.05	0.31	0.96	0.99	0.94
BW (d 10), kg	8.14	8.69	8.37	8.72	0.38	0.24	0.74	0.79
ADG, g	206	264	230	265	10	0.001	0.05	0.25
ADFI, g	279	332	296	346	14	0.001	0.28	0.92
G:F	0.74	0.80	0.78	0.77	0.02	0.22	0.70	0.07
Phase 2 (d 11-20)								
BW (d 20), kg	13.16	13.73	13.81	14.02	0.54	0.47	0.39	0.74
ADG, g	499	502	541	528	23	0.82	0.14	0.72
ADFI, g	780	790	827	834	35	0.81	0.21	0.97
G:F	0.64	0.64	0.66	0.64	0.02	0.64	0.61	0.87
Phase 3 (d 21-35)								
BW (d 35), kg	22.50	23.09	22.64	23.56	0.79	0.34	0.70	0.83
ADG, g	619	620	585	632	19	0.21	0.56	0.23
ADFI, g	1087	1111	1047	1136	32	0.09	0.81	0.32
G:F	0.57	0.56	0.56	0.56	0.01	0.54	0.68	0.59
Overall (d 1-35)								
ADG, g	467	485	472	498	16	0.17	0.59	0.79
ADFI, g	769	797	769	824	24	0.10	0.57	0.59
G:F	0.61	0.61	0.61	0.61	0.01	0.79	0.95	0.84
PUN [c]	12.59	12.79	12.34	11.91	1.08	0.91	0.60	0.77

[a] Each mean represents 12 pens of four pigs per pen. Average initial age and weight of weanling pigs was 18 ± 2 d and 6.02 ± 0.2 kg, respectively.

[b] Spray-dried plasma protein was only supplemented in the phase I diet. The phase I diet was fed during d 1 through 10, the phase II diet from d 11 through d 20 and the phase III diet from d 21 through d 35 (see Tables 4.1 and 4.2 for diet formulations).

[c] PUN: plasma urea nitrogen, mg/dl

Table 4.4. Effects of BioPlex® Copper on growth performance and PUN in weaned pigs reared in sanitary and sub-sanitary environments [a,b]

Item	Unsanitary pens		Sanitary pens			P-value		
Cu (ppm)	0	200	0	200	SEM	Copper	Sanitation	Interaction
Phase 1 (d 1-10)								
BW (initial), kg	6.07	6.02	6.06	6.04	0.31	0.92	0.99	0.96
BW (d 10), kg	8.22	8.61	8.33	8.76	0.38	0.29	0.74	0.95
ADG, g	214	257	225	270	10	0.001	0.05	0.94
ADFI, g	290	321	290	352	14	0.002	0.28	0.28
G:F	0.74	0.80	0.78	0.78	0.02	0.14	0.70	0.11
Phase 2 (d 11-20)								
BW (d 20), kg	13.12	13.77	13.74	14.09	0.54	0.36	0.39	0.79
ADG, g	487	513	539	530	23	0.70	0.14	0.46
ADFI, g	761	810	812	848	35	0.24	0.21	0.86
G:F	0.64	0.64	0.66	0.64	0.02	0.54	0.61	0.61
Phase 3 (d 21-35)								
BW (d 35), kg	22.64	22.95	23.02	23.17	0.79	0.77	0.70	0.92
ADG, g	630	608	615	602	19	0.36	0.56	0.81
ADFI, g	1140	1058	1119	1063	32	0.04	0.81	0.69
G:F	0.55	0.58	0.55	0.57	0.01	0.08	0.68	0.84
Overall (d 1-35)								
ADG, g	471	481	482	487	16	0.63	0.59	0.86
ADFI, g	789	777	795	799	24	0.85	0.57	0.75
G:F	0.60	0.62	0.61	0.61	0.01	0.19	0.95	0.46
PUN [c]	12.38	13.00	12.80	11.46	1.08	0.74	0.60	0.80

[a] Each mean represents 12 pens of four pigs per pen. Average initial age and weight of weanling pigs was 18 ± 2 d and 6.02 ± 0.2 kg, respectively.

[b] Spray-dried plasma protein was only supplemented in the phase I diet. The phase I diet was fed during d 1 through 10, the phase II diet from d 11 through d 20 and the phase III diet from d 21 through d 35 (see Tables 4.1 and 4.2 for diet formulations).

[c] PUN: plasma urine nitrogen, mg/dl

Table 4.5. Growth performance and PUN concentration of weaned pigs supplemented with spray-dried plasma protein and BioPlex® Copper [a,b]

Item	0 % SDPP		6 % SDPP			P-value		
Cu (ppm)	0	200	0	200	SEM	SDPP	Copper	Interaction
Phase 1 (d 1-10)								
BW (initial), kg	6.07	6.03	6.05	6.03	0.31	0.96	0.92	0.97
BW (d 10), kg	8.10	8.41	8.45	8.96	0.38	0.24	0.36	0.80
ADG, g	201	236	238	291	10	0.001	0.001	0.42
ADFI, g	272	304	309	369	14	0.001	0.002	0.31
G:F	0.74	0.78	0.78	0.79	0.02	0.22	0.14	0.55
Phase 2 (d 11-20)								
BW (d 20), kg	13.31	13.65	13.55	14.21	0.54	0.47	0.36	0.77
ADG, g	519	522	508	523	23	0.82	0.70	0.80
ADFI, g	792	816	782	843	35	0.81	0.24	0.61
G:F	0.66	0.65	0.65	0.63	0.02	0.64	0.54	0.82
Phase 3 (d 21-35)								
BW (d 35), kg	22.60	22.54	23.06	23.59	0.79	0.34	0.77	0.71
ADG, g	616	589	631	622	19	0.21	0.36	0.64
ADFI, g	1118	1016	1143	1105	32	0.09	0.04	0.33
G:F	0.55	0.58	0.55	0.57	0.01	0.54	0.08	0.41
Overall (d 1-35)								
ADG, g	470	469	483	500	16	0.17	0.63	0.61
ADFI, g	783	756	801	820	24	0.10	0.85	0.36
G:F, g	0.60	0.62	0.60	0.61	0.01	0.79	0.19	0.59
PUN [c]	12.51	12.43	12.67	12.03	1.08	0.91	0.74	0.80

[a] Each mean represents 12 pens of four pigs per pen. Average initial age and weight of weanling pigs was 18 ± 2 d and 6.02 ± 0.2 kg, respectively.

[b] Spray-dried plasma protein was only supplemented in the phase I diet while copper from BioPlex® Copper was supplemented in all three diet phases. The phase I diet was fed during d 1 through 10, the phase II diet from d 11 through d 20 and the phase III diet from d 21 through d 35 (see Tables 4.1 and 4.2 for diet formulations).

[c] PUN: plasma urine nitrogen, mg/dl

Table 4.6. Small intestinal morphology of weaned pigs supplemented with SDPP in diets and reared in sanitary and sub-sanitary environments [a]

Sanitation	Unsanitary pens		Sanitary pens			P-value		
SDPP	0 %	6 %	0 %	6 %	SEM	Sanitation	SDPP	Interaction
Duodenum								
Villous height, μm	392	422	445	484	19	0.006	0.09	0.80
Crypt depth, μm	258	276	309	296	9	0.001	0.77	0.08
VCR [b]	1.53	1.53	1.44	1.67	0.07	0.70	0.10	0.08
Jejunum								
Villous height, μm	400	407	469	447	21	0.01	0.75	0.47
Crypt depth, μm	277	276	310	302	12	0.02	0.70	0.75
VCR	1.45	1.49	1.52	1.50	0.07	0.59	0.89	0.66
Ileum								
Villous height, μm	389	390	431	431	21	0.05	0.99	0.97
Crypt depth, μm	272	273	314	304	11	0.003	0.68	0.63
VCR	1.45	1.44	1.39	1.46	0.08	0.83	0.77	0.65

[a] Six pigs were killed per each treatment for measurement of intestinal morphology.

[b] VCR = villous height: crypt depth ratio.

Table 4.7. Effects of BioPlex copper (ppm) on small intestinal morphology in weanling pigs reared in sanitary and sub-sanitary environments [a]

Sanitation	Unsanitary pen		Sanitary pen			P-value		
Cu (ppm)	0	200	0	200	SEM	Sanitation	Copper	Interaction
Duodenum								
Villous height, μm	398	416	448	482	19	0.006	0.20	0.68
Crypt depth, μm	278	257	320	287	9	0.0002	0.005	0.50
VCR [b]	1.44	1.62	1.41	1.70	0.07	0.70	0.001	0.40
Jejunum								
Villous height, μm	376	430	449	467	21	0.01	0.10	0.41
Crypt depth, μm	282	271	315	297	12	0.02	0.20	0.78
VCR	1.34	1.61	1.43	1.59	0.07	0.59	0.004	0.41
Ileum								
Villous height, μm	385	394	431	431	21	0.05	0.83	0.84
Crypt depth, μm	279	265	313	305	11	0.003	0.35	0.77
VCR	1.39	1.50	1.40	1.45	0.08	0.83	0.34	0.73

[a] Six pigs were killed per each treatment for measurement of intestinal morphology.

[b] VCR: villous height: crypt depth ratio.

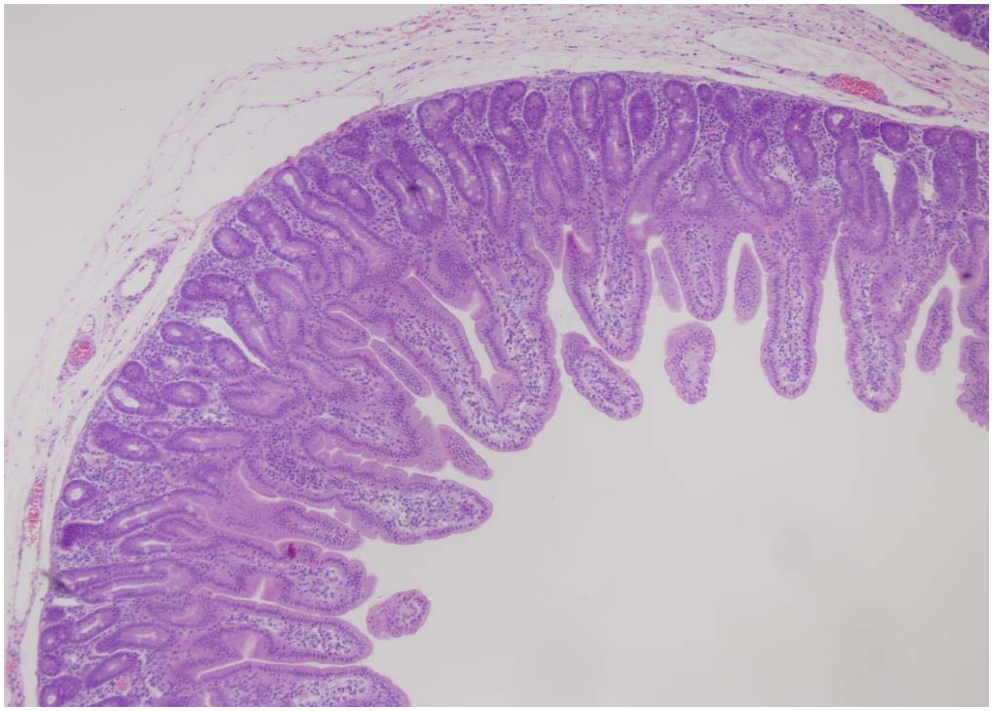
Table 4.8. Small intestinal morphology of weaned pigs supplemented with spray-dried plasma protein and BioPlex® Copper in the diets [a]

Item	0 % SDPP		6 % SDPP			P-value		
Cu (ppm)	0	200	0	200	SEM	SDPP	Copper	Interaction
Duodenum								
Villous height, µm	417	420	429	477	19	0.09	0.20	0.25
Crypt depth, µm	290	277	307	266	9	0.77	0.005	0.13
VCR [b]	1.43	1.53	1.41	1.79	0.07	0.10	0.001	0.03
Jejunum								
Villous height, µm	423	445	402	452	21	0.75	0.10	0.52
Crypt depth, µm	293	294	305	273	12	0.70	0.20	0.16
VCR	1.44	1.53	1.33	1.66	0.07	0.89	0.004	0.09
Ileum								
Villous height, µm	423	397	292	428	21	0.99	0.83	0.14
Crypt depth, µm	287	299	304	271	11	0.68	0.35	0.06
VCR	1.48	1.36	1.30	1.59	0.08	0.77	0.34	0.01

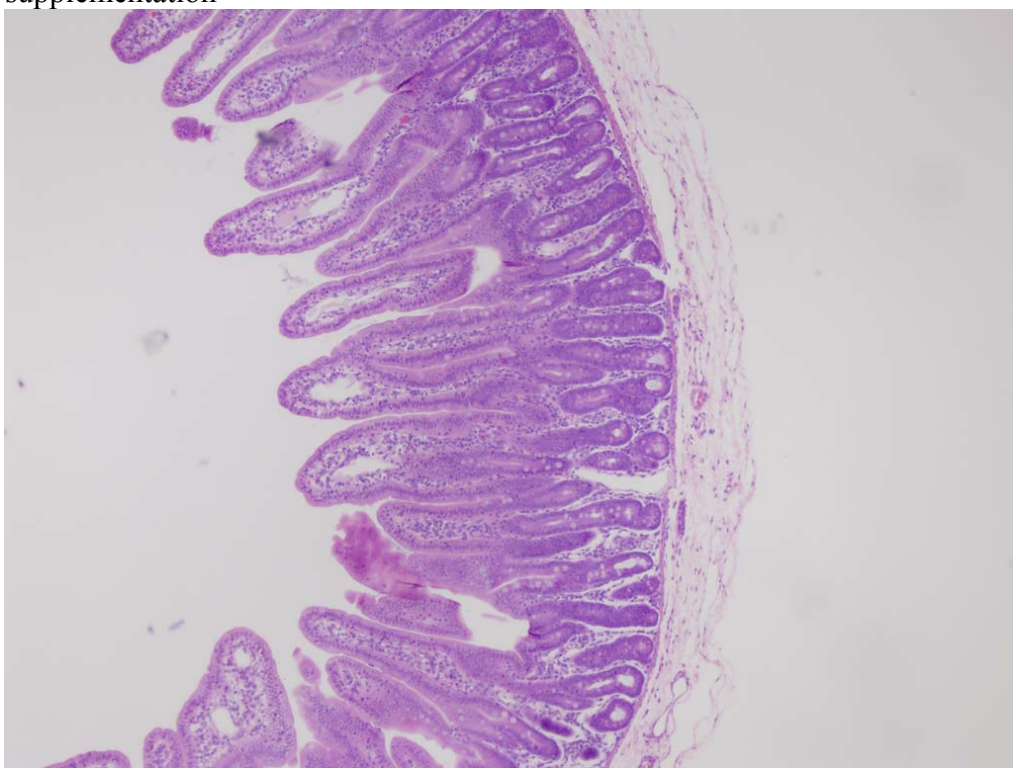
[a] Six pigs were killed per each treatment for measurement of intestinal morphology.

[b] VCR: villous height: crypt depth ratio.

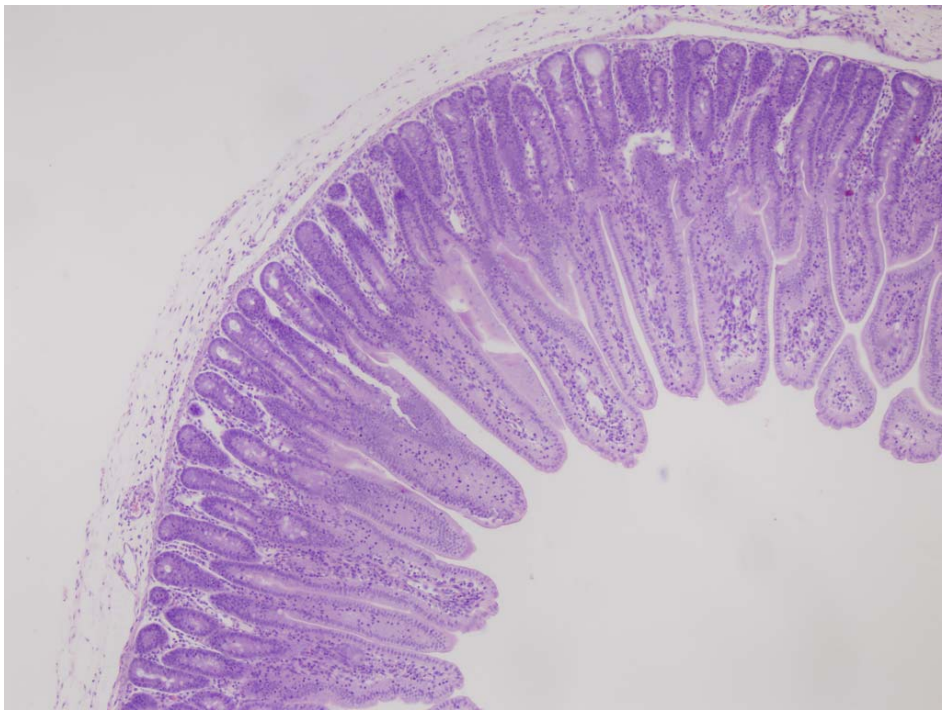
Fig. 4.1. Histological slides of the mucosal epithelium of the small intestine.



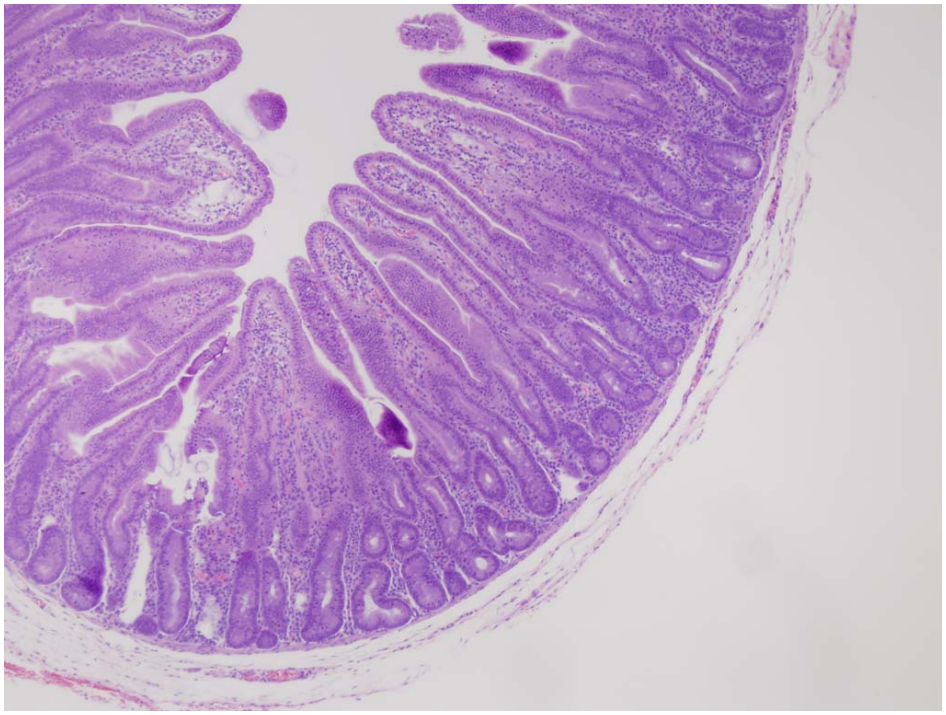
A. Pig reared in sub-sanitary nursery condition without spray-dried plasma protein and copper supplementation



B. Pig reared in sanitary nursery condition without SDPP and BioPlex® Copper supplementation



C. Pig reared in sub-sanitary condition with BioPlex® Copper supplementation



D. Pig reared in sub-sanitary condition with SDPP supplementation in diets

Chapter V

Dietary and ontogenetic regulation of nutrient transporter and digestive enzyme mRNA expression in the small intestine of weanling pigs

ABSTRACT

Dietary and ontogenetic regulation of digestive enzyme mRNA was evaluated in weanling pigs (n = 54, 17 $\pm$ 2 d of age, 6.2 $\pm$ 0.3 kg BW) along the horizontal axis of the small intestine. Experimental diets included a control soy protein diet, a diet containing 6 % spray-dried plasma protein (SDPP), or 0.5 % of a commercial hydrolyzed protein source (Peptiva®, Vitech Biochem, San Fernando, CA). Pigs were housed in double-decker nursery pens with continuous lighting at 29 ^{o}C. Pigs were killed at weaning, and 3 d, and 10 d after weaning for harvest of mucosa from duodenum, jejunum, and ileum. The mRNA levels of digestive enzymes: aminopeptidase A (APA), aminopeptidase N (APN), diepeptidyl peptidase IV (DPPIV), and maltase-glucoamylase (MGA), and nutrient transporters, NBAT, $b^{0,+}$ AT, EAAC1, and PepT1 were measured by northern blot and expressed as relative abundance to the housekeeping gene GAPDH. Significant diet by intestinal segment interactions ($P < 0.05$) were found for APA and APN. Aminopeptidase A was evenly distributed along the small intestine in the Peptiva® group, but decreased dramatically from jejunum to ileum in the other groups. Aminopeptidase N increased from the proximal to distal intestine in the soy protein and SDPP groups, whereas in the Peptiva® group, expression was highest in the jejunum and lowest in the duodenum. Generally, most of the digestive enzymes and nutrient transporters were expressed in the middle and distal part of the small intestine. The mRNA level of MGA was at a higher level in the jejunum compared to duodenum and ileum ($P < 0.01$). Aminopeptidase A decreased from the proximal to distal part ($P < 0.01$), whereas APN, DPPIV and EAAC1 exhibited the opposite (*P*

< 0.01). The mRNA levels of MGA and EAAC1 increased as pigs matured, compared to APA and DPPIV which decreased ($P < 0.05$). The mRNA levels of other genes measured were constant during the entire experimental period. In this study, protein sources altered the expression pattern of APA and APN mRNA. Expression of digestive enzymes and nutrient transporters was differentially regulated along the length of the small intestine and varied as a function of age.

Key Words: Digestive enzyme, mRNA, Peptide, Spray-dried plasma protein, Transporter.

Introduction

Membrane hydrolysis and absorption in the small intestine are two closely related and key steps of nutrient assimilation (Johnson, 1997a). Current knowledge about brushborder membrane hydrolases and nutrient transporters has accumulated dramatically in the last few years. Most previous studies focused on molecular characteristics of specific genes and their functions (Lambeir et al., 2003; Verrey et al., 2004). The regulation of those genes involved in the assimilation process is also being studied (Chen, 2001).

In addition to being substrates for digestion, nutrients also act as signals for regulation of small intestinal function. Intestinal epithelial cells are among the most metabolically active cells; they begin as immature crypt cells and mature when they migrate up the villous axis over a period of 2 to 4 d (Buddington et al., 2001). The small intestine has the ability to adjust its absorption capacities to achieve physiological needs, and displays fast and dramatic modification with change in the luminal environment. Significant research has been conducted on glucose and carbohydrate regulation of gene expression involved in glucose digestion and absorption (Ferraris, 1989). Less information is available for dietary protein regulation of enzymes and

transporters that are involved in protein digestion and absorption. This may be due to the fact that more than 20 AA, 400 dipeptides, and 8,000 tripeptides are involved in this process.

Considering the pivotal role of protein and the value of regulatory information of hydrolases and transporters, the objectives of this study were to investigate the effects of different dietary protein sources on regulation of enzyme and transporter mRNA abundance in the brushborder membrane and the ontogenetic regulation and distribution of these genes along the small intestine. The protein sources were the same as reported in previous chapters, a commonly used commercial soy protein, spray-dried plasma protein (SDPP), and a peptide protein source, Peptiva® (Vitech Biochem, San Fernando, CA). Improving our understanding of ontogenetic and dietary protein regulation of gene expression and distribution in the small intestine will enrich our understanding of the relationship between nutrients and gene expression.

Materials and Methods

Chemicals

All chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise stated. TriReagent was purchased from Molecular Research Center (Cincinnati, OH). ^{32}P dNTP was purchased from PerkinElmer (Boston, MA). DNA polymerase I/DNAse I was purchased from Invitrogen Life Technologies (Carlsbad, CA).

Animals and Diets

All the animal procedures were approved by the Virginia Tech Animal Care Committee. A total of 54 crossbred (Yorkshinre × Landrace ×NPD Hamline®) male weanling pigs (17 $\pm$ 2 d, 6.16 $\pm$ 0.34 kg BW) were randomly distributed among four dietary treatments and three sampling times (at weaning, 3 d, and 10 d after weaning) from outcome groups based on BW at the day of weaning. Littermates were balanced across treatments. The pigs were transported from Virginia

Tech Tidewater Agricultural Research and Extension Center Swine Unit in Suffolk, VA to the Blacksburg campus (5 h transport time) in a covered truck on the first day of the experiment. Three complex nursery diets (Table 5.1) were formulated to meet the NRC (1998) requirements and to have similar nutrient and metabolizable energy concentrations for all treatments. The four dietary treatments were: 1) a soy protein based control diet; 2) a diet supplemented with 6 % SDPP (AP920®, American Proteins Corp., Ames, IA), 3) a diet supplemented with 0.5 % Peptiva® (Vitech Biochem, San Fernando, CA). These three groups of pigs had *ad libitum* access to feed. The fourth group of pigs (SDPP-PF) was fed the 6 % SDPP diet, but the feed intake was controlled to the same level as the feed consumption of the previous day of the control group. Pigs were placed in double-decker nursery pens (0.6 × 0.9 m) with plastic coated, expanded metal floors and a baffle between decks. Each pen was equipped with a nipple waterer and a stainless steel feeder. Pens were located in two environmentally controlled rooms with continuous lighting and a temperature of 27 ^{o}C with recommended air ventilation rates (Murphy et al., 1990). Six pigs were killed on the day of weaning to provide baseline data. The others were killed 3 d or 10 d after weaning. Feed consumption for all pigs was recorded daily and BW was obtained when pigs were removed for tissue sampling.

Sampling and Tissue Preparation

Pigs were euthanized with an overdose injection of 10 % sodium pentobarbital before sampling. The entire small intestine was then removed and dissected free of mesenteric attachments and placed on a smooth, cold surface. The duodenum, jejunum, and ileum were separated into equal lengths. The intestinal lumen was opened lengthwise following the mesentery line and washed with ice-cold PBS (NaH_2PO_4, 1.47 m*M*; Na_2HPO_4, 8.09 m*M*, NaCl, 0.145 *M*). The epithelium was scraped from the surface using glass slides; approximately 0.25 g

of sample were wrapped in aluminum foil and immediately frozen in liquid nitrogen and stored at – 80 ^{o}C for later analysis.

cDNA Probes for Nutrient Transporters and Digestive Enzymes

The PepT1 probe was the whole cDNA sequence of pig PepT1 (AY180903) cloned in our laboratory. Primers of other genes for PCR were designed based on published nucleic acid sequences (Table 5.2). One-step RT-polymerase chain reactions (Qiagen, Valencia, CA) were run according to the manufacturer's instructions to amplify the desired template. Briefly, 1 μg of total RNA was added with a set of gene specific primers and amplified under thermal cycle conditions of 50 °C for 30 min (reverse transcription), 95 °C for 15 min (initial PCR activation), 35 cycles of: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and final extension step of 72 °C for 10 min. The sequences of the sense and anti-sense primers [Aminopeptidase A (APA), aminopeptidase N (APN), dipeptidyl peptidase IV (DPP IV), maltase-glucoamylase (MGA), $b^{0,+}$AT, NBAT, PepT1, and EAAC1] are listed in Table 5.2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an endogenous control. The PCR products were separated on a 1 % agarose gel and a fragment of the expected size was excised for purification using the Qiagen Quick-Gel Extraction Kit (Valencia, CA). The products were subsequently cloned into a pGEM® –T easy vector system (Promega, Madison, WI) following the manufacturer's protocol, and transformed into *Escherichia coli* competent cells using a BTX-Harvard Apparatus ECM Electro Cell Manipulation System (Hollistan, MA). Clones containing the correct insert were screened by cutting the plasmids with endonucleases, and were sequenced by dideoxy termination sequencing. The plasmid containing the confirmed fragment (> 99 % homology with the designed gene sequence) was then cultured, and purified by Qiaprep Spin Miniprep kit (Valencia, CA) and labeled by nick translation for northern blotting.

Quantification of mRNA by Northern Blot

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc, Cincinnati, OH) following the procedures provided by the company with minor modifications (RNA percipitation was at – 20 °C for 2 h instead of at room temperature for 10 min). Absorbance at 260 and 280nm (Model U-2000, Hitachi Instrument Inc, Tokyo, Japan) was used to quantify the isolated RNA. For northern blot, 10 μg of total RNA were denatured and size-fractionated on 1 % agarose gel in 2.2 mol/L formaldehyde, and transferred onto a Nylon membrane overnight and cross-linked with UV light (BIOS Bioslink UV Crosslinker, New Haven, CT) at 0.30 J/cm^2 . Following pre-hybridization for 2 h in a solution containing 50 % formamide, 5X Denhardt's solution, 6X SSPE, 0.5 % SDS and 10 mg/L tRNA, RNA blots were hybridized for 16 to 18 h with the addition of labeled cDNA probe. The probes were labeled with [α-^{32}P] dCTP by nick translation using DNA polymerase I/DNase I and purified by Sephadex G-50 spin column chromatography (Roche, Pleasanton, CA).

Post-hybridization washing was under high-stringency conditions, and was adjusted to achieve the best final signal. Washed filters were exposed to phosphorimaging screens and then scanned using a FX laser scanner with PDQUEST software (Bio-Rad Laboratories, Hercules, CA). To correct for differences in RNA loading onto gels or RNA transfer to membranes, GAPDH was measured as an endogenous control. The density of hybridization bands was quantified using volume tools of Quantity One Quantification Software (Bio-Rad Laboratories), and reported in relative units.

Statistical Analysis

All data were analyzed using the PROC MIXED procedure of SAS (SAS Institute, Cary, NC). The pig served as the experimental unit. Response criteria for growth performance

included ADG, ADFI, and G:F. For mRNA expression, the model included the main effects of dietary protein source, intestinal segment, age, and all appropriate two-way interactions. The three-way interaction was removed from the model once it was determined to be non-significant ($P > 0.10$). Data at weaning served as a baseline, and was analyzed separately because no diet was applied. Single contrasts were used to compare the 3 d after weaning with baseline and 10 d after weaning with baseline.

Results

Growth Performance

In this experiment, the growth performance was measured for two phases. The first group of pigs was killed 3 d after weaning and growth performance was measured for 3 d (Phase I). The second group of pigs was killed 10 d after weaning, and their performance was measured for 10 d (Phase II). Because of the weaning and transportation stress (5 h transport time), all pigs had low feed intake and lost weight during the first 3 d after weaning (Table 5.3). Most of the ADG and G:F were negative during this period, even though the pigs fed SDPP still ate more feed than the control and SDPP-PF groups ($P < 0.10$). In addition, the ADFI of Peptiva® group was also higher than the control and SDPP-PF groups ($P < 0.10$). During phase II, the pigs fed SDPP diet had numerically higher ADG, ADFI, and G:F compared to the control group. But the differences were not statistically significant ($P > 0.10$). The pair-fed group ate about 40 g less per day than the control group, and the 6 % SDPP *ad libitum* group ate about 40 g more per day than the control group (Table 5.3).

Distribution of mRNA in the Small Intestine at Weaning (Baseline)

The data for intestinal distribution of enzyme and transporter mRNA at weaning are shown in Table 5.4. The mRNA abundance of APN and DPP IV increased ($P < 0.05$) from the

proximal to the distal part of the small intestine. The APN and DPP IV mRNA abundance in the ileum was more than 1.5 times as much as in the duodenum. In contrast, the abundance of APA mRNA in the duodenum was 33 % higher than the ileum ($P < 0.05$). The greatest abundance of the glycohydrase MGA mRNA was observed in the jejunum, and the abundance was 40 % higher than in the duodenum ($P < 0.05$). The mRNA abundance in the ileum was similar to the jejunum. For the excitatory amino acid transporter, EAAC1, the mRNA levels were increased from the proximal to the distal part of the small intestine ($P < 0.05$). Compared to the duodenum, the mRNA abundance of EAAC1 was threefold greater in the ileum. Similar to APN and DPP IV, the mRNA for PepT1 was mainly found in the middle and distal part of the small intestine, while the duodenum had the lowest mRNA abundance. No significant difference was observed for the heterodimeric transporter $b^{0,+}$AT and NBAT. In general, mRNA abundance of the brushborder hydrolases, AA and small peptide transporters were greater in the middle and distal regions of the small intestine. The intestinal distribution pattern did not change as animals matured (Table 5.5), except mRNA abundance of MGA in the ileum decreased at 3 d and 10 d after weaning, and was significantly lower compared to the abundance in the jejunum ($P < 0.05$; Table 5.5).

Aminopeptidases

There was no effect of diets on relative abundance of mRNA for APA, APN, or DPP IV. There was an effect of segments on APA mRNA abundance ($P < 0.05$), but there was also a diet by segment interaction ($P < 0.02$). Aminopeptidase A mRNA abundance was evenly distributed along the small intestine in the Peptiva® group (Figure 5.1), while for the other three dietary treatments, APA mRNA decreased dramatically from the jejunum to ileum, and was similar in the duodenum and jejunum (Figure 5.1). Aminopeptidase A mRNA abundance decreased as

animals matured from 3 to10 d postweaning. Abundance at 3 d postweaning was higher ($P < 0.05$) compared to 10 d postweaning (Table 5.5). Between weaning and 3 d postweaning, APA mRNA abundance did not differ, but abundance was lower ($P < 0.02$) at 10 d postweaning compared to weaning (Table 5.6).

Again, there was an effect of segment on APN mRNA abundance ($P < 0.05$), but a diet by segment interaction ($P < 0.03$) was also observed. Aminopeptidase N mRNA generally increased from the proximal to the distal intestine in the control and SDPP fed groups (Figure 5.2). In contrast, for the group fed Peptiva®, APN mRNA abundance in the ileum was not higher than the jejunum. For the SDPP-PF group, the increased gradient was more dramatic from jejunum to ileum; as a consequence, this group had the highest level of APN mRNA abundance in the ileum compared to other dietary treatments. Abundance of APN mRNA at weaning did not differ from abundance at 3 and 10 d postweaning (Table 5.6).

Similar to APN, DPP IV mRNA abundance increased from the proximal to the distal part of the small intestine ($P < 0.05$, Table 5.5). The mRNA abundance in the ileum was about 2.3 times higher than the duodenum, and 1.6 times higher than the jejunum. The DPP IV mRNA abundance decreased as pigs matured ($P = 0.06$). The mRNA abundance was 1.70, 1.58, and 1.28 at weaning, and 3 d, and 10 d postweaning, respectively.

Glycohydrolase

The MGA mRNA was greatest ($P < 0.05$) in the jejunum (Table 5.5). The duodenum and ileum had similar lower abundance of MGA mRNA. This result was slightly different from the distribution pattern at weaning, in that mRNA abundance was similar in the jejunum and ileum, and was also significantly higher than the abundance in the duodenum (Table 5.4). The mRNA abundance of MGA, which is responsible for maltose and amylose digestion, increased

dramatically from weaning to 10 d postweaning ($P < 0.04$, Table 5.6). The mRNA abundance increased five- and tenfold at 3 d and 10 d postweaning, respectively, compared to the abundance at weaning. No dietary effect was detected.

Nutrient Transporters

For the two subunits of the heterodimeric transport system, NBAT and $b^{0,+}AT$, neither diet nor segment nor age influenced the mRNA abundance (Table 5.5 and 5.6). The EAAC1 expression was increased from the proximal to the distal part of the small intestine ($P < 0.05$, Table 5.5). The mRNA abundance at the duodenum, jejunum, and ileum level was 0.32, 0.58, and 0.91, respectively. The mRNA abundance at the ileum was three times higher than the duodenum.

Most of the PepT1 mRNA abundance was observed in the mid to the distal part of the small intestine. The mRNA abundance in the jejunum and ileum was higher than in the duodenum ($P < 0.05$). The PepT1 mRNA abundance was also increased as pigs matured, but the difference was not significant ($P = 0.37$). No major dietary influence was detected.

Discussion

It is well established that both macro and micro nutrients can be regulators of gene expression at the transcription and translation levels in vitro (Fafournoux et al., 2000; Paoloni-Giacobino et al., 2003). Less information is available for in vivo situations, especially for domestic animals because many other factors (e.g. endocrine and nervous systems) are involved. More attention has been focused on nutrigenetic science, but most previous studies focused on extreme nutritional conditions such as fasting, starving, or over-nutrition (Ferraris and Carey, 2000). This study focused more on an applied view and all animals were in a normal nutrient

condition. The original objectives were to investigate the effect of special protein sources and peptide structure and profiles on the expression of genes involved in the assimilation process.

Soy protein is a high quality feed ingredient and commonly used protein source in swine diet formulation, but young weanling pigs do not adapt well to this protein source because of the presence of antigen and immaturity of the GI system (Li et al., 1990). Spray-dried plasma protein is a high quality but very expensive protein source. It has been showen to be an excellent protein source for young weanling animals because of its growth promotion effects and benefits on the immune system. Peptiva® is a peptide protein source hydrolyzed from marine products. A previous study indicated that animals cannot survive with a diet of free AA but can survive with a peptide diet (Zambonino infante et al., 1997). Results from in vitro studies also indicated that small peptide absorption is faster and more efficient than free AA (Leibach and Ganapathy, 1996; Ganapathy and Leibach, 1999; Zhao et al., 1997). In this study, the Peptiva® diet changed the aminopeptidase distribution pattern in the small intestine; it increased APA expression in the ileum and APN in the jejunum. Neither SDPP nor limited feeding changed gene expression in the small intestine. As mentioned before, the luminal environment is very complicated, and limited information is available related to the influence of different peptide structures. Our hypothesis is that the digestion procedure for different peptide chains could be different, including the rate of hydrolysis, end products of AA and small peptide profile, as well as the rate of assimilation and the site of absorption. All these factors may impact gene expression along the small intestine, which then affect animal performance. Our results suggest that dietary peptide can influence gene expression, but limited information about the peptide profiles and the absence of a precise model to monitor the assimilation process in the small intestine makes it very difficult to interpret the results precisely. Only 0.5 % Peptiva® was added in the diet, which

accouts for only a small amount of dietary protein supplementation. The manufacturer prefers to market Peptiva® as a functional ingredient rather than a protein source. Not enough information is available yet to interprete the impact of Peptiva® on aminopeptidase distribution in the small intestine. As to SDPP, the beneficial response in performance for this study did not appear related to the mRNA expression of hydrolysis or nutrient transporters in the small intestine.

In general, most of the digestive enzymes and nutrient transporters were distributed in the middle and distal segments of the small intestine, and the distribution pattern along the small intestine did not change as animals matured. These results were in agreement with Erickson et al. (1995). The authors suspected that the distribution patterns are related to the physiological function. For example, most of the nutrients are digested and absorbed in the middle segment, so it is reasonable that most of the digestion enzymes and nutrient transporters are expressed in the middle part of the small intestine. The high affinity glutamate transporter, EAAC1, is responsible for glutamate uptake, the major fuel for the intestine. The small intestine accounts for 90 % of the glutamate expense (Stoll et al., 1998). It may be more efficient for the body to absorb the glutamate in the distal part of the small intestine, so other AA can be absorbed in the middle. Another concern of this study is that even though the small intestine is physiologically divided into three segments, duodenum, jejunum and ileum, there is still the possibility that the mRNA levels are different within each segment. More knowledge of the function of each gene is needed to further understand the precise pattern of distribution.

Most of the ontogenetic effects observed were related to the hydrolysases. Peptidase expression level decreased, while the glycohydrases increased as pigs matured. No large changes were found for nutrient transporters, except for EAAC1, which increased dramatically with age. These changes are more or less related with the diet change from a high protein milk-based diet

to a high carbohydrate solid feed diet. More enzymes are needed for starch and carbohydrate digestion, and less for protein digestion. The other consideration is that we measured the relative amount of mRNA per gram, and did not account for the total capacity of digestion and absorption. Obvious enlargement of the small intestine and thicker intestinal tissue were noticed at 10 d compared to 3 d postweaning. As previous indicated, the intestine accounts for 90 % of the total glutamate utilization of the body, as the total mass of the small intestine grows, more and more glutamate is required for maintenance of small intestinal function. This may be responsible for the dramatically increased EAAC1 transporters. The other interesting thing is that the baseline data fits very well in the whole story; there is no significant peak for any gene at 3 d after weanling. This result is in disagreement with other results from our laboratory (unpublished data). That work indicated an obvious peak one d after weaning in pigs. Also previous studies indicated that weaning usually impacts gene expression, especially during 0 to 3 d after weaning (Toloza et al., 1992; Ferraris, 2001). For this study the authors are not sure of the basis if the smooth change from weaning to d 3 and 10 after weaning was due to the fact that the pigs had already recovered after 3 d or if other factors are involved.

This study is among the few that investigated different hydrolase and nutrient transporter distribution along the small intestine, and the ontogenetic and dietary protein regulation of those genes in the brushborder membrane. Our results increased our knowledge of nutrigenetic science but further research is needed on several areas: 1) more detailed information about the distribution along the small intestine is necessary; the intestine could be divided into more than three physiological segments, 2) it is very difficult to monitor the digestion procedure of peptides in the GI tract, even though the general process of digestion has been known for a long time; more detailed information about the process of digestion and absorption of different peptide

chains will be very helpful, 3) information about protein expression of each gene and enzyme activity are also valuable, and 4) combining information about ability per se and total capacity of the whole small intestine is important to fully understand ontogenetic regulation.

Table 5.1. Ingredient composition and calculated analysis of experimental diets [a]

Item	Soy protein	6 % SDPP	0.5 % Peptiva®
Ingredient			
Ground corn	37.50	37.50	37.50
Soybean meal, dehulled	15.00	15.00	15.00
Dried whey	20.00	20.00	20.00
Lactose	5.00	5.00	5.00
Fish meal, menhaden	2.50	2.50	2.50
SDPP (AP920) [b]	---	6.00	---
Peptiva® [c]	---	---	0.50
Soy protein concentrate	12.18	4.87	11.44
Soybean oil	2.53	1.60	2.58
Dicalcium phosphate	1.47	1.24	1.49
Calcium carbonate	0.50	0.67	0.52
Salt	0.25	0.25	0.25
Synthetic lysine	0.177	0.044	0.20
D-L-Methionine	0.14	0.076	0.14
Vitamin premix [d]	0.25	0.25	0.25
Trace mineral premix [e]	0.15	0.15	0.15
Corn starch	2.35	4.85	2.48
Totals	100.00	100.00	100.00
Calculated analysis [f]			
Crude protein, %	22.00	22.00	22.00
Total lysine, %	1.50	1.50	1.50
Methionine + cystine, %	0.86	0.86	0.86
Calcium, %	0.90	0.90	0.90
Total phosphorus, %	0.80	0.80	0.80
ME, Mcal/kg	3.415	3.415	3.415

[a] Diets were fed for the whole experimental period (d 0 to d 10 d after weaning).

[b] SDPP: spray-dried plasma protein, provided by American Protein Corp, Ames, IA

[c] Peptiva®: provided by Vitech Biochem, San Fernando, CA

[d] Vitamin premix provided per kg of diet: 12,400 IU of vitamin A as acetate, 2,067 IU of stabilized vitamin D3, 82 IU of vitamin E as DL-tocopheryl acetate, 5.5 mg of vitamin K as menadione sodium bisulfate, 11 mg of riboflavin, 36 mg of D-Pantothenic acid as calcium pantothenate, 63 mg of niacin as nicotinamide, 0.28 mg of D-biotin, 1.65 mg folic acid, 2.07 mg pyridine, 1.25 mg thiamine, and 0.05 mg of vitamin B12.

[c] Trace mineral premix provided per kg of final diet: 20 mg of copper as copper sulfate, 150 mg of iron as ferrous sulfate, 40 mg of manganese as manganese sulfate, 150 mg of zinc as zinc sulfate, 0.5 mg of iodine as calcium iodine, 0.3 mg of selenium as sodium selenite.

[d] Values were calculated using NRC (1998) data, and data supplied by manufacturer for SDPP and Peptiva®.

Table 5.2. Primer sequences for synthesis of cDNA probes by one-step PCR [a]

Gene name [b,c]	Primer sequence	Genebank ID
MGA sense	CAGAAGCTGCCAAGACTGTG	NM-004668
MGA anti-sense	AGAACTGTTGGTGCACATCC	
APA sense	GTCTCTACCACCTGACGAT	U66371
APA anti-sense	CTCTGTAAGTGATGAGTCC	
APN sense	ACATCACTCTCATCCACCCT	Z29522
APN anti-sense	GCAATCACAGTGACAACTCG	
DPP4 sense	CCTCCGGCGTCTGTGTTA	X73277
DPP4 anti-sense	TGGATTCAGCTCACAGCT	
$b^{0,+}$AT sense	AGACTTGTTTATGTGGCGGG	Degenerate [d]
$b^{0,+}$AT anti-sense	GGAGGCTGAGCTTGTTACTC	
NBAT sense	ATGCCCAAGGAGGTGCTGTT	Degenerate [d]
NBAT anti-sense	GGTGTGGTTGGGGATGAAATC	
EAAC1 sense	GGGAAGATCAT(ACT)GA(AG)GT(AGCT)	AY195622
EAAC1 anti-sense	AACCGGTCCAG(AGCT)A(AG)CCA(AG)TC	
PepT1 sense	GTCTTGAACTTCCCCAGCCA	AY180903
PepT1 anti-sense	AAGCATCTTCTTCATCGTGGTCAATG	
GAPDH sense	ATGCCTCCTGTACCACCAAC	AF017079
GAPDH anti-sense	CACAACCTGGTGCTCAGTGT	

[a] All the primers were synthesized by MWG-Biotech (High Point, NC).

[b] MGA: maltase-glucoamylase; APA: aminopeptidase A; APN: aminopeptidase N; DPP IV: dipeptidyl peptidase IV; $b^{0,+}$AT: sodium independent cationic and zwiterionic AA

transporters; NBAT: represents the heavy chain of a heterodimeric transporter involved in the heteroexchange of cationic and neutral AA corresponding to the $b^{0,+}$ transport system; EAAC1: excitatory amino acid carrier 1; PepT1: peptide transporter 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase .

[c] The primers of APA, APN, MGA, and DPP IV were adopted from Petersen et al. (2002).

[d] The primers were designed using the degenerate method from gene sequences including the human, mouse, and rabbit.

Table 5.3. Effects of dietary supplementation with spray-dried plasma protein or Peptiva® on growth performance of early-weanling pigs [a]

Item	Experimental dietary treatments				
	Control	6 % SDPP	0.5 % Peptiva®	SDPP-pair fed [d]	SEM
Phase I (d 0 to 3) [b]					
BW (initial), kg.	6.16	6.16	6.16	6.16	0.34
ADG, g	-13	-31	-11	-73	63
ADFI, g	60 [y]	130 [x]	114 [x]	40 [y]	20
G:F	-2.15	-1.46	-1.16	-5.91	2.41
Phase II (d 0 to 10) [c]					
ADG, g	282	309	273	270	30
ADFI, g	401	440	439	367	26
G:F	0.69	0.70	0.62	0.74	0.05

[a] A total of 48 weanling pigs were used. There were six observations per treatment.

[b] This group of pigs was killed 3 d after weaning, performance was measured from d 0 to 3 after weaning.

[c] This group of pigs was killed 10 d after weaning, performance was measured from d 0 to 10 after weaning.

[d] The SDPP-PF group pigs were fed 6 % spray-dried plasma protein diet, and feed intake level was controlled to be the same as the contol group.

[x,y] Within a row, means without a common superscript differ ($P < 0.10$).

Table 5.4. Distribution of mRNA abundance in the small intestine at the day of weaning [a]

Item [b]	Intestinal segments			SEM
	Duodenum	Jejunum	Ileum	
APA	0.69^{x}	0.66^{x}	0.46^{y}	0.04
APN	2.73^{x}	3.72^{y}	4.40^{y}	0.21
DPP IV	0.92^{x}	1.48^{y}	1.98^{z}	0.10
MGA	0.41^{x}	0.79^{y}	0.75$^{x, y}$	0.11
EAAC1	0.31^{x}	0.55^{y}	0.91^{z}	0.04
NBAT	0.35	0.29	0.37	0.10
$b^{0,+}$AT	0.43	0.53	0.76	0.15
PepT1	0.97	1.15	1.10	0.08

[a] All the means were expressed as relative abundance to the housekeeping gene GAPDH. Six pigs were killed at weaning to provide the baseline data.

[b] APA: aminopeptidase A; APN: aminopeptidase N ;DPP4: dipeptidyl peptidase IV; MAG: maltase-glucoamylase ; $b^{0,+}$AT: sodium independent cationic and zwiterionic AA transport; NBAT: represents the heavy chain of a heterodimeric transporter involved in the hteroexchange of cationic and neutral AA corresponding to the $b^{0,+}$ transport system; PEPT1: peptide transporter in small intestine; EAAC1: excitatory amino acid carrier; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

[x, y, z] Within a row, means without a common superscript differ ($P < 0.05$).

Table 5.5. Dietary protein and developmental regulation of mRNA relative abundance in the small intestine of weanling piglets

Item		Gene [a] APA	APN	DPP IV	MGA	$b^{0,+}AT$	NBAT	EAAC1	PepT1
mRNA relative abundance [b]									
Diet [c]	Control	0.55	3.13	1.64	0.76	0.29	0.47	0.57	1.14
	SDPP	0.57	3.47	1.25	0.64	0.49	0.95	0.61	1.00
	Peptiva	0.62	3.61	1.46	0.85	0.27	0.49	0.55	1.03
	SDPP-PF	0.53	4.00	1.37	0.68	0.30	0.46	0.69	1.15
	SEM	0.07	0.36	0.15	0.21	0.13	0.24	0.05	0.14
	P-value	0.78	0.73	0.36	0.90	0.64	0.42	0.27	0.83
Segment	Duodenum	0.65^{x}	2.70^{y}	0.86^{y}	0.46^{y}	0.37	0.43	0.32^{z}	0.94^{y}
	Jejunum	0.63^{x}	3.73^{x}	1.48^{x}	0.89^{x}	0.29	0.54	0.58^{y}	1.17^{x}
	Ileum	0.43^{y}	4.22^{x}	1.94^{x}	0.44^{y}	0.35	0.81	0.91^{x}	1.13^{x}
	SEM	0.04	0.22	0.10	0.11	0.11	0.17	0.04	0.09
	P-value	0.001	0.001	0.001	0.001	0.87	0.16	0.001	0.02
Sampling time	At weaning	0.61	3.94	1.70	0.10	0.34	0.44	0.47	0.97
	3 d	0.66^{x}	3.40	1.58	0.49^{y}	0.42	0.76	0.57	1.02
	10 d	0.48^{y}	3.70	1.28	0.97^{x}	0.26	0.43	0.64	1.14
	SEM	0.05	0.25	0.11	0.15	0.10	0.17	0.04	0.10
	P-value	0.02	0.40	0.06	0.03	0.24	0.18	0.15	0.37
Interaction *P*-value, $P < 0.05$ significant									
Interaction [d]	D*S	0.02	0.03	0.14	0.74	0.32	0.65	0.12	0.30
	D*T	0.95	0.62	0.81	0.60	0.45	0.58	0.61	0.98
	S*T	0.13	0.23	0.09	0.54	0.50	0.24	0.38	0.10

[a] APA: aminopeptidase A; APN: aminopeptidase N ;DPP4: dipeptidyl peptidase IV; MAG: maltase-glucoamylase ; $b^{0,+}AT$: sodium independent cationic and zwiterionic AA transport; NBAT: represents the heavy chain of a heterodimeric transporter involved in the

heteroexchange of cationic and neutral AA corresponding to the $b^{0,+}$ transport system; PEPT1: peptide transporter; EAAC1: excitatory amino acid carrier.

[b] The mRNA abundance was expressed relative to the house keeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

[c] A total of 48 pigs was used. There were six experimental units per treatment. Control: soy protein diet; SDPP: 6 % spray-dried plasma protein diet; Peptiva: 0.5 % Peptiva diet; SDPP-PF: pigs were fed the SDPP diet, but feed intake level was controlled to be the same as the control diet.

[d] For the interaction, D, S, and T represent the main effects of diet, intestinal segment, and sampling time, respectively.

[x, y, z] Within a separated column, means without a common superscript differ ($P < 0.05$).

Table 5.6. Single contrast comparison of d 3 and 10 after weaning with baseline at weaning [a, b]

	APA	APN	DPPIV	MGA	$b^{0,+}$AT	NBAT	EAAC1	PepT1
3 d	0.05	-0.20	-0.16	0.12	0.19	0.08	-0.02	-0.06
P-value	0.30	0.43	0.28	0.28	0.28	0.41	0.55	0.58
SEM	0.05	0.25	0.11	0.15	0.17	0.10	0.04	0.10
10 d	-0.13	0.10	-0.19	0.32	-0.15	-0.08	0.05	0.07
P-value	0.02	0.68	0.10	0.04	0.40	0.41	0.16	0.49
SEM	0.05	0.25	0.11	0.15	0.17	0.10	0.04	0.10

[a] APA: aminopeptidase A; APN: aminopeptidase N ;DPP4: dipeptidyl peptidase IV; MAG: maltase-glucoamylase ; $b^{0,+}$AT: sodium independent cationic and zwiterionic AA transport; NBAT: represents the heavy chain of a heterodimeric transporter involved in the heteroexchange of cationic and neutral AA corresponding to the $b^{0,+}$ transport system; PEPT1: peptide transporter in small intestine; EAAC1: excitatory amino acid carrier; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

[b] The means were expressed as the relative abundance mRNA levels at 3 d and 10 d after weaning minus the relative abundance at weaning. Six pigs were killed at weaning, and 24 pigs were killed at 3 d and 10 d postweaning.

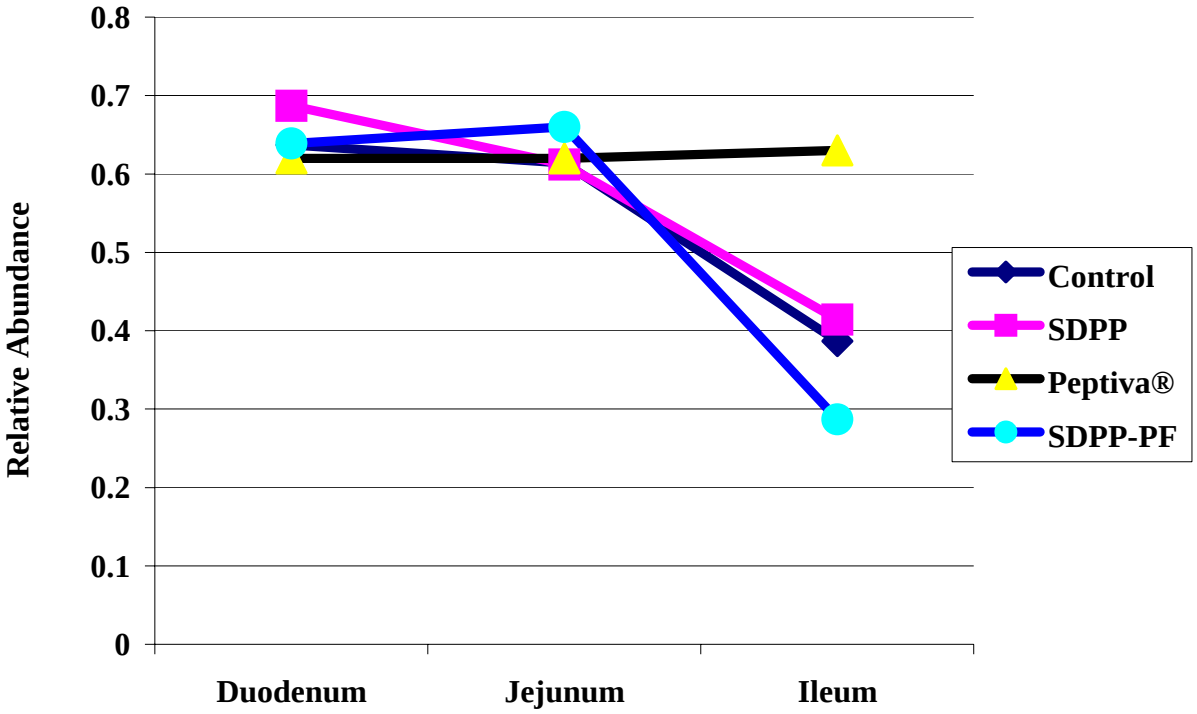


Fig. 5.1. Two-way interaction of dietary protein source and intestinal segment on aminopeptidase A. The means were expressed as relative abundance to house keeping gene GAPDH. SEM = 0.08. A total of 48 pigs were used. There were six pigs per treatment.

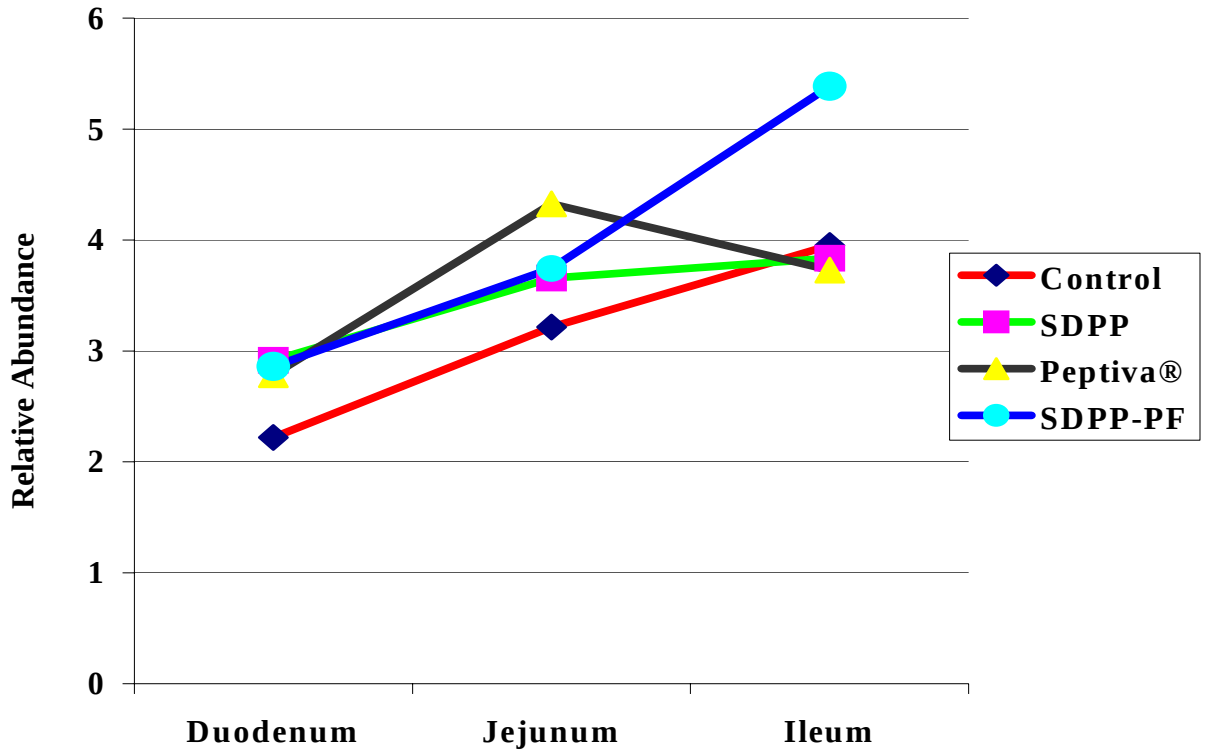


Fig. 5.2. Two-way interaction of dietary protein source and intestinal segment on aminopeptidase N. The means were expressed as relative abundance to house keeping gene GAPDH. SEM = 0.44. A total of 48 pigs were used. There were six pigs per treatment.

Chapter VI

Dietary protein and developmental regulation of cytokine mRNA expression in the brushborder membrane of the small intestine in weanling pigs

ABSTRACT

Cytokines play a central role in the mucosal immune response and are involved in regulation of nutrient absorption, metabolism and animal growth. Many factors affect cytokine production in the intestinal epithelial cells. This study investigated the effects of special dietary protein sources and restricted feeding on cytokine mRNA expression in the brushborder membrane of weanling pigs, and how pro-inflammatory and inflammatory regulatory cytokines changed as pigs matured. Real-Time PCR was performed and the comparative ΔΔ Ct method was used for calculation of changes in cytokine mRNA abundance relative to the housekeeping gene GAPDH. A total of 48 weanling pigs (17 $\pm$ 2 d, 6.16 $\pm$ 0.34 kg BW) were fed different supplemental protein source diets (soy protein control diet, 6 % spray-dried plasma protein (SDPP), and 0.5 % Peptiva®) and was slaughtered at 3 d and 10 d postweaning. A fourth treatment group of pigs (SDPP-PF) was fed the 6 % SDPP, but the feed intake level was controlled to be the same as the soy control diet. At 3 d postweaning, pigs fed the SDPP consumed more feed than the other groups ($P < 0.05$). The ADFI of the SDPP-PF group was 30 % and 80 % of the level of the SDPP group in the first 3 and 10 d postweaning. All pigs lost weight during the first 3 d postweaning, and there was no significant difference in growth performance 10 d after weaning. Spray-dried plasma protein tended to decrease the proinflammatory cytokine IL-1-β and TNF-α mRNA relative abundance, while IL-6 mRNA abundance was greater in the SDPP fed group compared to other groups. The limited feeding group exhibited an increased TNF-α mRNA abundance compared to the *ab libitum* SDPP group

($P < 0.10$). The TNF-α and IL-10 mRNA abundance increased from the proximal to the distal part of the intestine, and the mRNA abundance was higher ($P < 0.01$) in the ileum than in the duodenum and jejunum. The mRNA levels of IL-1-β and IL-6 showed similar trends but the differences were not significant. Higher mRNA levels of cytokines in the ileum may indicate an increased microbial load in the distal part of the intestine. The cytokines IL-1-β, IL-10 and TNF-α mRNA abundance also increased as the animals matured ($P < 0.01$). In summary, SDPP tended to decrease proinflammatoy cytokine expression, while limited feeding increased the TNF-α mRNA level in the small intestine. The mRNA abundance of most measured cytokines increased from the proximal to the distal part of the small intestine, and increased as the animals matured.

Key Words: Cytokines, Dietary protein, Real-Time PCR, Weanling pigs.

Introduction

The epithelial cells that line the small intestine have two major functions: one is to facilitate the controlled and selective absorption of nutrients from the lumen; the other is to restrict the passage of potentially harmful microorganisms from the intestinal tract into the surrounding tissue (Pitman and Blumberg, 2000). Cytokines are a group of factors exerting profound influence on the functional state of immune cell populations as well as regulation of nutrient absorption and metabolism (Spurlock, 1997). Several studies confirm that the intestinal epithelium can synthesize cytokines (McGee et al., 1993; Rogler and Andus, 1998; Wittig and Zeitz, 2003), and then mediate local immune function. The results from the previous study reported in Chapter V indicated that both limited feeding and dietary protein sources changed the distribution of aminopeptidase gene expression along the small intestine. Manipulation of the luminal constituents through dietary means could be a potential therapy of intestinal related

diseases. Few studies have been conducted to elucidate the mechanisms underlying the pathway of how luminal nutrients alter cytokine production, and how cytokines target the immune system.

Spray-dried plasma protein (SDPP) is a commercially available, high quality protein source for weanling pigs. Results described in previous chapters of this dissertation indicated that SDPP improved growth performance and the intestinal integrity of weanling pigs. The high concentration of immunoglobulins in SDPP may be partly responsible for the benefits it has on intestinal health and growth stimulation (Thomson et al., 1994). Furthermore, the fact that weanling pigs do not adapt to the soy proteins as well may be related to the antigen presented and potential immune reaction (Li et al., 1991). Considering that the intestine is the first organ tissue exposed to the diet, the objectives of this study were to investigate the effect of protein sources on cytokine mRNA expression in the brushborder membrane and the distribution of cytokine mRNA in the small intestine.

Materials and Methods

Animals, Diets, Housing, Sampling, and RNA Extration

A total of 48 male weanling pigs (17 $\pm$ 2 d, 6.16 $\pm$ 0.34 kg BW) was used in this study. These were the same animals used in the study reported in Chapter V. Diets, animal management, housing, tissue sampling, and RNA extractions were the same as reported in Chapter V.

Reverse Transcription and Real-Time PCR SYBR Green Detection

The mRNA abundance of two pro-inflammatory factors (IL-1-β and TNF-α) and two anti-inflammatory factors (IL-6 and IL-10) was measured by Real-Time PCR. All the primers were designed based on the published NCBI gene sequences, by using the Primer Express software (NIH, Bethesda, MD). The primer sequences are listed in Table 6.1.

Total RNA was extracted from intestinal mucosa and qualified as described in Chapter V. All samples were then normalized to 0.1 μg/μL with DEPC water. Reverse transcription (RT) was performed using TaqMan Reverse Transcription reagents (Roche Molecular System Inc., Branchburg, NJ). The reverse transcription mixture consisted of 1X TaqMan RT buffer, 5.5 mM manganese chloride, 500 μM each dNTP, 2.5 μM random hexamers, 0.4 U/μL RNAse inhibitor, 1.25 U/μL MultiScribe Reverse Transcriptase, 2 μL 0.1 μg/μL diluted total RNA template, made up to 10 μL with RNAse-free water. The RT was performed using the PCT ^{TM}-200 system (MJ Research, Ramsey, MN) with the following thermal cycling profile: incubation 10 min at 25 °C, reverse transcription 60 min at 45 °C, reverse transcriptase inactivation for 5 min at 95 °C.

Relative quantitative PCR was performed using a model 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green PCR master mix. Each reaction contained: 12.5 μL 2X SYBR Green PCR master mix, 0.5 μL 5 u*M* forward and reverse primer each for each specific gene, 400 ng cDNA, and water to a final volume of 25 μL. Amplification was performed in MicroAmp® optical 96-well reaction plates (Applied Biosystems). PCR parameters were as follows: incubation at 50 °C for 2 min, initial denature at 95 °C for 10 min to activate AmliTaq Gold DNA polymerase followed by 40 cycles of 15 s at 95°C, 1 min at 60 °C. Following the final cycle, melting curve analysis was performed to examine the specificity in each reaction well for all tested samples in a given run. The dissociation step thermal cycles included a step of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. A single melt peak for each reaction confirmed a single PCR product without non-specific amplification or primer-dimers.

Comparative ΔΔCt Method of Real-Time PCR Data Analysis

The Comparative $\Delta\Delta C_t$ method is based on the principle that the difference in threshold cycles (ΔC_t) between the target gene and housekeeping gene is proportional to the relative

expression level of the target gene (Livak and Schmittgen, 2001; Pfaffl, 2001). To ensure the accuracy of the obtained data, the reaction efficiencies of the two assays should be similar and close to 100 %. The amplification efficiency was determined by serial dilutions (4 ng/μL, 0.8 ng/μL, 0.4 ng/μL, 0.08 ng/μL, and 0.008 ng/μL) of positive control cDNA and amplified by Real-Time PCR using gene specific primers. A plot of the log cDNA dilution versus the C_t (threshold cycles) was made, and the amplification efficiencies were calculated and are listed in Table 6.2. For each amount of RNA tested, triplicate C_t values were obtained and averaged. The threshold was adjusted for each plate in a PCR run and was close to 0.20. The fold change of each target gene was normalized to the housekeeping gene, and expressed relative to the duodenal segment of the control diet at d 20 of age (calibrator) using the following formula:

Fold change = $2^{-\Delta\Delta Ct}$

Where $\Delta\Delta C_t = (C_{t\ target} - C_{t\ housekeeping})_{treatment} - (C_{t\ target} - C_{t\ housekeeping})_{calibrator}$

Using this analysis, if the level of the target gene was not affected by diet or age, the values of the mean fold change for each diet and time point should be very close to 1 (since $2^0 = 1$).

Statistical Analysis

Before the statistical analysis was performed, all $2^{-\Delta\Delta Ct}$ data were logarithmically transformed to obtain normally distributed data. The 10 based logarithmically transformed data were analyzed using the PROC MIXED procedure of SAS (SAS Institute, Cary, NC). For the mRNA abundance, the model included the main effects of dietary protein sources, intestinal segments, age, and all appropriate two-way interactions. The three-way interaction was removed from the model once it was determined to be non-significant ($P > 0.10$). The changes in mRNA expression were tested using ANOVA followed by the Tukey test for multiple comparisons with

unequal sample sizes. Significance was set at $P < 0.10$. Single contrasts were used to compare the difference between SDPP and the control group, the SDPP and SDPP-PF group.

Results

Amplification Efficiency

The efficiency standard curve for each target gene is listed in Table 6.2. The amplification efficiency was calculated by plotting the log of the input template versus the ΔCt of each reaction curve. According to the equation of $E = 10^{(-1/slope)} - 1$, a slope of -3.33 demonstrates a 100 % efficiency of amplication. This means the PCR products were duplicated after each cycle. From Table 6.2, the amplication efficiency for each gene is within the acceptable range of the manufacturer's recommendation. High linear regression coefficients (> 0.99) R^2 indicated good quality of each standard curve.

Proinflammatory Cytokines

A two-way interaction ($P < 0.01$) of age and intestinal segment was observed for IL-1-β (Table 6.3). The IL-1-β mRNA abundance increased two- to threefold in the jejunum and ileum from 3 to 10 d after weaning, while the mRNA abundance decreased slightly in the duodenum (Figure 6.1). The pigs fed the SDPP *ad libitum* had numerically lower IL-1-β mRNA fold change compared to other dietary treatments. The mRNA abundance of IL-1-β in SDPP *ad libitum* group was 2.5 times less than the control group, but the difference was not significant ($P = 0.29$). The IL-1-β mRNA abundance increased ($P < 0.10$) as animals matured (Table 6.3). The IL-1-β mRNA abundance at 10 d postweaning was twice as much as the abundance at 3 d postweaning.

The pigs fed the SDPP diet had numerically lower relative abundance of of TNF-α than the control and Peptiva® groups and a lower abundance than the SDPP-PF group ($P < 0.10$;

Table 6.3). The abundance of TNF-α mRNA for the SDPP-PF and SDPP *ad libitum* group was 1.88 and 0.82, respectively. The TNF-α mRNA abundance increased from the proximal to the distal small intestine ($P < 0.10$). The mRNA abundance in the duodenum, jejunum, and ileum was 0.96, 1.14, and 1.78, respectively. Similar to IL-1-β, TNF-α mRNA abundance increased ($P < 0.10$) as the pigs matured. The TNF-α mRNA abundance was 0.93 and 1.67 at 3 and 10 d postweaning, respectively.

Anti-inflammatory Cytokines

No dietary effect was detected for IL-10 mRNA abundance. The IL-10 mRNA abundance increased dramatically from the duodenum and jejunum to the ileum ($P < 0.10$; Table 6.3). The mRNA abundance in the ileum was 3.5 times higher than in the duodenum, and 2.0 times higher than in the jejunum. As animals matured, IL-10 mRNA abundance increased more than doubled (3.26 to 8.28) from 3 to 10 d postweaning ($P < 0.10$). Interleukin 6 mRNA abundance was not influenced by diet, intestinal segment or age.

Discussion

The intestinal epithelium acts as a barrier to the external environment contained within the gut lumen (Sanderson, 2001), but also allows macromolecules to be transferred from the lumen into the blood. The intestinal mucosal immune system must balance two opposing functions: mounting an immune response to pathogens, while maintaining tolerance to antigens derived from commensal bacteria and food (Lindsay and Hodgson, 2001). This balance is regulated by both cellular interactions and the release of soluble mediator cytokines. It has been shown that cytokines can be altered by dietary factors (Sanderson, 2001; Gil, 2002). This has stimulated huge medical interests in manipulating the immune system by adjusting dietary composition, to prevent or cure diseases such as Crohn's disease. Results of previous studies

were inconsistent and the impacts of dietary factors were different from study to study. For example, dietary nucleotides have been shown to enhance the expression of IL-6 and IL-8 in fetal small intestinal explants (Gil, 2002). Dietary n-3 polyunsaturated fatty acid decreased IL-1-β, IL-2, IL-6, and TNF-α in the human peripheral blood mononuclear cells compared with a control diet (Blok et al., 1996). In contrast, Matheson et al. (2003) found no change of IL-4 and IL-10 expression in the small intestine by immune-enhancing diets (L-arginine, ω-3 fatty acid and RNA fragments), even though the blood flow in the distal small intestine increased in the rat. Currently there are very few studies on the dietary protein regulation of cytokine expression in the small intestine.

The three supplemental protein sources used in this study were SDPP, Peptiva® and soy protein. Previous studies indicated that oral infusion of soy protein reduced villous height, and increased anti-soy IgG titers and skin-fold thickness (Li et al., 1990; 1991). The SDPP is a high quality protein source for young pigs, and part of its benefits may be due to the impact of high immunoglobulin concentration on the immune system. A previous study (Touchette et al., 2002) indicated that SDPP reduced mRNA expression of TNF-α, IL-1-β, and IL-6 expression in the liver, spleen, and pituitary glands in pigs. Our study is in agreement with Touchette et al. (2002) in that SDPP tended to reduce the pro-inflammtory cytokines IL-1-β and TNF-a in the small intestine. Both IL-1-β and TNF-α are the principal cytokines that mediate acute inflammation and act on endothelial cells to stimulate inflammation and the coagulation pathway. Activation of the immune system is associated with reduced feed intake and growth (Coffey and Cromwell, 1995). The low immune activation of reduced pro-inflammatory cytokines by SDPP supplementation suggested another possible pathway of SDPP benefit in pigs, even though in this study, the pigs that were fed SDPP did not have a better growth performance compared to

other treatments. Both Frank et al. (2003) and Touchette et al. (2002) indicated that SDPP supplementation resulted in over-activation of the immune system or increased stress when pigs were challenged with lipopolysaccharide. These results may indicate that pigs fed SDPP are more susceptible to pathogenic infection.

Reduced feed intake has been suggested as a cause for reduction in villous height (Kelly et al., 1991; Li et al., 1991). A strong relationship (R = 0.82) between total DM intake and villous height was established in pigs fed cow's milk for 5 d after weaning (Pluske et al., 1996a). It is well known that limited feed intake is one of the most important factors affecting weanling pig performance and intestinal morphology. Consuming enough feed usually solves most of the problems that occur during the weaning period. The feed intake level of the SDPP-PF group was controlled to be similar to the soy control group. The ADFI of the SDPP-PF group was only 30 % and 80 % of the SDPP group for 3 and 10 d postweaning, respectively, and was slightly lower than the control group. The limited feed intake increased TNF-α expression, and the mRNA abundance was threefold lower than the SDPP group. It is suspected that the pair-fed pigs were in a higher stress condition compared to other groups because of inadequate supply of nutrients presented to the intestine for normal function.

Weaning usually results in both physiologically and immunologically stressed pigs. Pie et al. (2004) indicated that weaning resulted in the upregulation of inflammatory cytokines IL-1, IL-6 and TNF and IL-12 gene expression in the gut. Most of these changes occurred within 2 d after weaning and then levels returned to pre-weaning levels and remained constant for the entire experimental period (3 to 8 d after weaning). In the present study, most of the cytokines measured increased as animals grew from 3 to 10 d after weaning. The weaning stress was not considered, and an insufficient number of time points were taken to evaluate the effect of stress

on cytokines. However, based on most previous studies, the critical point for weaning stress is 2 to 3 d after weaning, and pigs usually then recover soon after, thus cytokines were not considered to be affected by weaning stress in this study. The results of developmental regulation were inconsistent with Pie et al. (2004) in that IL-1, IL-10, and TNF mRNA expression increased from 3 to 10 d after weaning. This difference may be due to the age of the pigs that were used. In the present study, the pigs were much younger than those used in the Pie et al. study (17 d vs. 28 d), and the immune system may not have matured. The increased cytokines may indicate a maturing process of the immune system.

There is limited information available for the distribution of cytokines in the small intestine. In this study, the ileum had the highest level of cytokines, which may indicate a more profound loading of microorganisms in the distal part of the small intestine. However, Pie et al. (2004) showed that the distribution of IL-6, IL-8, IL-18, and IL-12p40 along the small intestine was not different. Not enough information is available to explain the different pattern of distribution between these two studies.

In conclusion, limited feeding significantly increased the TNF- β mRNA expression. Most of the cytokines were distributed in the distal part of the small intestine, and they increased from d 3 to 10 post-weaning. This suggests that the distal part of the small intestine has a heavier load of microflora, and weanling pigs at 20 d of age may not have a mature mucosal immune system.

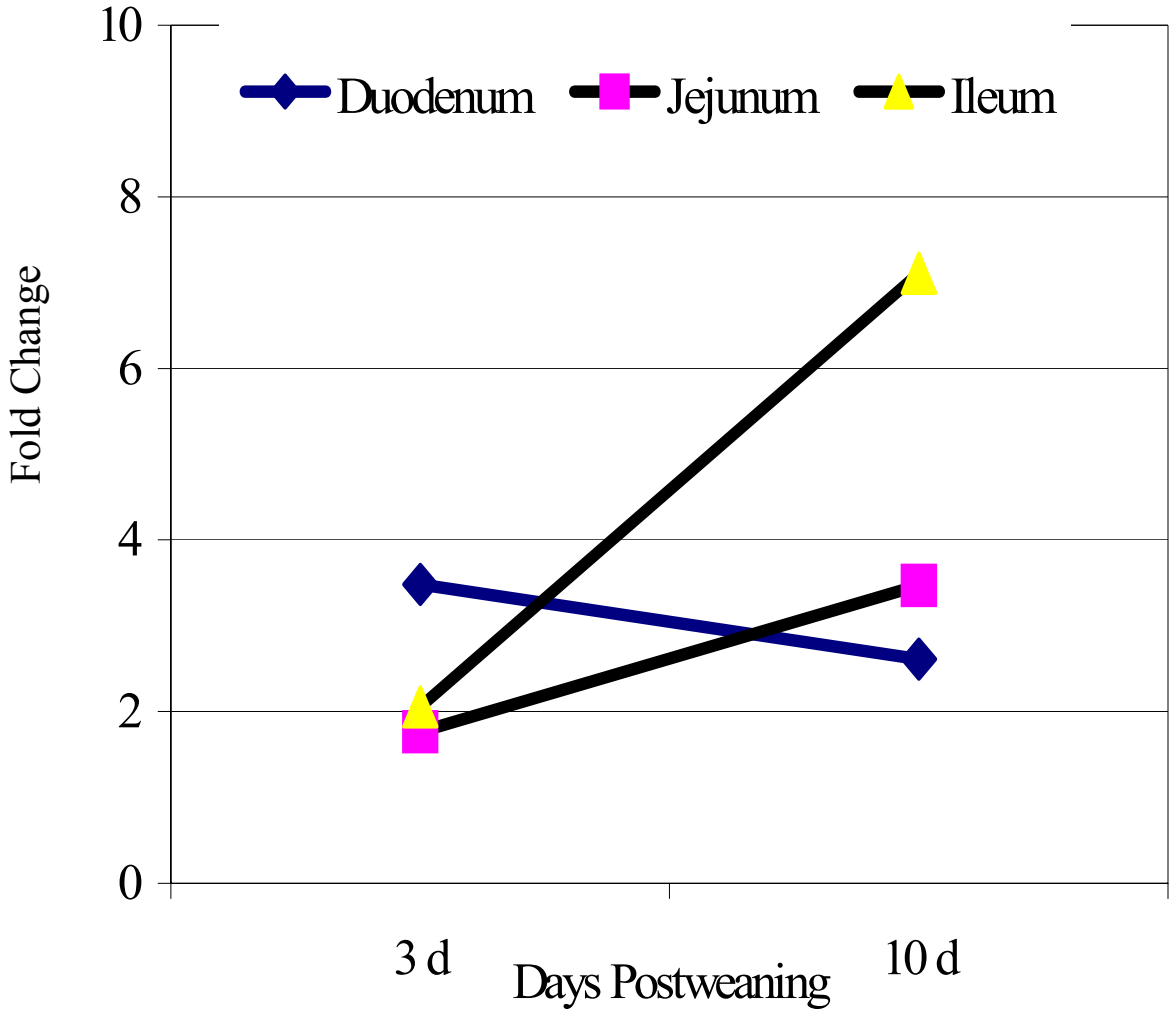


Fig. 6.1. Interaction of developmental regulation on IL-1 mRNA distribution along the small intestine. There were 24 replicates for each point. 95 % of two-side confidence limits are 2.43 to 4.05.

Table 6.1. Real-time PCR primer sequences

Target		Primer sequences [a]	Size	Acc. no.
GAPDH [b]	Forward	TGATGCCCCCATGTTTGTG	77	AF017079
	Reverse	CAGGAGGCATTGCTGATGATC		
TNF-α	Forward	CTGGCCCCTTGAGCATCA	69	X57321
	Reverse	ACGGGCTTATCTGAGGTTTGAG		
IL-1	Forward	ATCGACCATCTCTCTCTGAATCAGA	73	X52731
	Reverse	ATGCCGTCCCCAGGAAGT		
IL-6	Forward	CGCAGCCTTGAGGATTTCC	80	M80258
	Reverse	GGACGGCATCAATCTCAGGT		
IL-10	Forward	TCAGGAGCCAACTGCAGCTT	80	L20001
	Reverse	AGTGGGAGGCAGTCAGGACA		

[a] All the primers were designed using Primer Express software, and synthesized by MWG-Biotech (High Point, NC).

[b] GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is the house keeping gene.

Table 6.2. Efficiency of amplification

Target gene/reference gene	Slope	Efficiency [a]	R^2
GAPDH [b]	-3.19	106 %	0.99
IL-1-β	-3.02	110 %	0.98
IL-6	-2.94	118 %	0.99
IL-10	-3.20	105 %	0.99
TNF-α	-3.33	100 %	0.97

[a] The efficiency was measured by plotting the log10 of the input template versus the ΔC_t; a slope of –3.33 demonstrates that the efficiency is 100%

[b] GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was the house keeping gene

Table 6.3. Dietary protein and developmental regulation of cytokines relative mRNA abundance in the small intestine of young pigs [a,b,c]

Item		IL-1-β	TNF-α	IL-6	IL-10
Dietary treatments [d]	Control	4.02	1.07 [x, y]	2.09	3.46
	SDPP	1.61	0.82 [x]	4.12	4.06
	Peptiva®	3.46	1.47 [x, y]	2.23	6.53
	SDPP-PF	3.08	1.88 [y]	3.65	7.93
	P-value	0.29	0.10	0.44	0.46
Segments	Duodenum	3.02	0.96 [x]	2.29	2.82 [x]
	Jejunum	2.47	1.14 [x]	2.41	4.97 [x]
	Ileum	3.83	1.78 [y]	4.41	10.00 [y]
	P-value	0.16	0.005	0.16	0.0002
Sampling time	3 d	2.33 [x]	0.93 [x]	2.58	3.26 [x]
	10 d	4.01 [y]	1.67 [y]	3.25	8.28 [y]
	P-value	0.04	0.02	0.52	0.03
Confidence limits [e]	Lower	2.43	0.98	2.06	3.72
	Upper	4.05	1.61	4.15	8.17
----------Interaction *P*-value, $P < 0.05$ significant----------					
Interaction [f]	D*S	0.43	0.46	0.46	0.38
	D*T	0.30	0.53	0.27	0.64
	S*T	0.01	0.21	0.62	0.62

[a] Six pigs from each diet were sampled on d 3 and d 10 postweaning.

[b] All the data were acquired using Real-time PCR. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was the housekeeping gene, and duodenal segments of the pigs that were fed the control diet and killed on 3 d postweaning was the calibrator sample.

[c] All the data were transformed to log10 based to obtain a normal distribution prior to statistical analysis.

[d] Control: soy protein diet, SDPP: 6 % spray-dried plasma protein diet, Peptiva: 0.5 % Peptiva diet, SDPP-PF: pigs were fed the SDPP diet, but feed intake level was controlled to be the same as the control diet.

[e] 95 % upper and lower confidence intervals.

[f] Two-way interactions, D: diet; S: intestinal segment; T: sampling time.

[x,y] Means within a separated column lacking common superscripts differ ($P < 0.10$).

Chapter VII

Epilogue

Results of this study indicated that the growth stimulation effects of spray-dried plasma protein (SDPP) were unrelated to digestibility and nitrogen balance, but were potentially related to the impact on intestinal morphology and cytokine expression in the intestinal mucosa. One explanation is that the high levels of immunoglobulins in the SDPP may assist in preventing bacterial attachment, and by doing that, protect intestinal integrity. However, there is no well known method available to quantify the luminal microfloral population. The traditional culture method can only recover part of the bacteria population because of the lack of proper culture media and the difficulty of mimicking the intestinal environment in vitro. More modern techniques using bacterial gene studies have enabled scientists to identify species in situ, which should allow for much faster identification of the organisms as well as indication of the presence of species that might not have survived laboratory culture. Because of the huge bacterial population in the lumen and many unknown factors, complete and accurate characterization of the microflora population in the intestinal lumen is currently impossible. Future research should attempt to alleviate this problem and address how special ingredients like SDPP impact gut microflora.

In addition to the total population of the luminal microflora, the amount and the type of bacteria that adhere to the mucosa are other concerns. In order to infect, a bacterium must adhere to a surface and form a colony of actively growing cells. However, there is no good method to quantify and categorize the attached pathologic bacteria in the mucosa. A new method uses confocal laser scanning microscopy to categorize the attached bacteria in a fixed tissue sample, and then confirms the microscopy results by PCR and proteomics (Kleessen et al.,

2005). The successful utilization of this kind of molecular technology may further enhance our understanding of the relationship of the host and luminal bacteria. Without an appropriate method to identify and quantify the attached luminal bacteria in this study, we are not sure if benefits of SDPP supplementation on intestinal integrity are due to preventing bacterial attachment, or to other unknown benefits beneath mucus levels.

The relationship of nutrients and immune function has become a hot topic in science. Using dietary changes to manipulate the immune system sounds like an ideal way to reduce or eliminate the need for feed additive antibiotics. However, the immune system is very complicated and is also highly regulated. Immune function consists of a huge network; many immune organs and cells as well as regulatory factors are involved. The most important issue of studying nutrient immunity interaction is to find the target organs and cells of certain nutrient factors. Due to the high cost and unknown gene sequences, we only chose four well studied cytokines. It is possible that we overlooked some of the important cytokines that are regulated by diet and the bioactive molecules in SDPP.

Since the identification of the peptide transporter in the small intestine in 1994, peptide absorption has become widely accepted. Studies indicated that peptide absorption in the intestine is more efficient and quicker than free AA absorption. The end-products of protein digestion absorbed as peptides may be critical in young animals because of the high expression level and activity of the peptide transporter during the first few days after birth. From a nutritional point of view, dietary peptide supplementation or protein hydrolysis may benefit young animal growth and health. Our results indicated that Peptiva® increased feed intake during the first 3 d postweaning, but had no effect on growth performance. This was in

agreement with other studies in that peptide stimulated feed intake in weanling pigs and sows, but no obvious explanation was available for this result.

Most of the commercially available peptide products are manufactured by hydrolysis of certain intact proteins, such as marine products, porcine intestinal mucosa, or microbiological tissues. They are marketed as functional proteins, but no one really knows what the functional components are, and the associated mechanism or special function. From an industry view, there are two factors suggesting that peptides could be a potential ingredient in livestock diets. First, most high quality protein sources used in diets for young weanling pigs are very expensive. Spray-dried plasma protein has another issue in that consumers do not like the idea of feeding animal products back to animals. So peptides can be a potential substitute for SDPP if a good quality peptide product can be manufactured. Small peptides have several physical advantages (high solubility, stability, bioavailability) compared to synthesized crystalline free AA. Utilization of synthesized small peptides to provide glutamate has been widely accepted in medical science. Using small peptides could be an efficient way of balancing AA profiles in diet formulation.

Even though utilization of peptides in diet formulation is theoretically proven to be advantageous, there are several considerations nutritionist need to address before peptides can be practically used in the animal industry:

1) The lack of a simple and economical method to detect the peptide profile of a protein is a hindrance to utilizing peptides in diet formulation. Consumers need a guaranteed analysis to understand all components of a manufactured product.

2) We know that the peptide transporter PepT1 has a different affinity for different N-terminus peptides. This may allow for choosing the right intact protein source to be

manufactured with the right hydrolysis conditions (such as pH, time, moisture et al.) to produce the most efficacious peptide mixture. Another practical way is to synthesize certain di- or tripeptides to satisfy limiting AA requirements, such as lysine and methionine requirements in weanling pigs.

3) Peptides are considered as a protein source in the general industry, but it may be important to balance the ratio of protein:peptides and peptides:free AA. It is well known that intact protein is necessary for normal animal function because animals are unable to survive on diets based solely on free AA. The relationship between protein, small peptides, and AA may provide new hints of protein quality and quantity in nutrition.

4) Small peptides have a different route of absorption compared to AA. It is possible that peptide metabolism also differs from free AA. It would be valuable to study peptide metabolism in greater detail.

One component of this dissertation was to investigate the dietary and ontogenetic regulation of gene expression in the small intestine. The results indicated that protein sources influenced aminopeptidase distribution along the small intestine. It is important to point out that we only measured the mRNA abundance. From a molecular biology view, the regulation of gene expression involves both transcriptional factors and translational and protein processing control, which is expressed as a variation in mRNA abundance and the number of functional proteins. These regulations result in changes in overall transporter protein function (V_{max} and K_m). Because mRNA abundance is not always in equilibrium with the number of proteins and physiological activity, it is important to include protein level and physiological activity in future studies.

Certain patterns of mRNA abundance were observed for hydrolysis enzymes as the animals matured. For example, the aminopeptidase mRNA abundance decreased while the maltase-glucoamylase mRNA abundance increased as animals matured. We suspect that these results are partly related to the diet change from a high-protein milk to a high-carbohydrate solid diet. More enzymes are needed in digestion of starch and carbohydrates, while less is required for protein digestion. In addition to lack of protein and activity data, another disadvantage of this research is that we did not record the total mass of the small intestine. We expressed the mRNA abundance as unit per g of tissue. Because of the dramatic enlargement of intestinal mass during early development of the animal, the total activity as well as the number of proteins per se may also differ as animals matured. Previous studies suggested that generally, the activity of transporter per cell decreases while the absorptive capacity of the small intestine increases due to the increased total mass. Generally, it is well accepted that only mature epithelial cells on the villous tip exhibit transport activity. Changing the ratio of mature to immature epithelial cells along the villous is another mechanism of regulation. In-situ hybridization and immunohistochemistry can provide this kind of cell type-specific information.

A gene generally has its own regulatory regions (promoter), and these regions are also where the regulatory binding occurs and has impact. Most gene regulation at the molecular level was conducted in vitro by using certain cell lines. The cell culture method has several advantages (i.e., easy to control and very straightforward). However, cell culture cannot mimic all physiological situations. Combining the regulatory information at both the cellular and animal level may enrich our understanding of gene regulation and function.

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