Gastrointestinal Health and Function in Weaned Pigs: The Role of Low Dietary Crude Protein

by

Florence Omobola Opapeju

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

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Department of Animal Science

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ABSTRACT

Post weaning diarrhea (**PWD**) is a major global threat to the swine industry and this disease has been managed in the past by supplementing starter diets with subtherapeutic levels of antibiotics. With the ban of in-feed antibiotics in the European Union and increasing interest to eliminate their usage in livestock diets around the world, the swine industry faces a major challenge of finding effective alternatives to in-feed antibiotics. A series of experiments were conducted to determine the effectiveness of low crude protein (**CP**), amino acid (**AA**)-supplemented diets as a nutritional strategy of enhancing gut health and mitigating PWD in piglets. The first experiment investigated the performance and gut health benefits of feeding low CP, AA-supplemented diets to piglets. Low CP diets reduced ammonia N concentration in cecal digesta, small intestine weight and crypt hypertrophy in piglets compared with the high CP diet. In the second experiment, the effect of dietary CP level on performance, immunological response and gut ecology of weaned pigs challenged with enterotoxigenic *Escherichia coli* (**ETEC**) K88 was evaluated. Compared with the high CP diet, the low CP diet reduced the impact of ETEC infection on growth performance, minimized the incidence of diarrhea, reduced ETEC count in the luminal content of the small and large intestine, increased the prevalence of butyrate producing bacteria in colonic digesta and protected against inflammatory-associated responses induced by ETEC. The third experiment was conducted to determine the effects of dietary CP level on intestinal development. The low CP diet did not impair jejunal brush border enzyme development of weaned pigs. The fourth experiment determined the effect of dietary CP level on intestinal response of

piglet to ETEC K88 infection. The high CP diet increased the number of adherent ETEC and the expression of sodium-coupled glucose transporter 1 in jejunum of piglets compared with the low CP diet. Overall, the results demonstrated that low CP diets supplemented with crystalline AA according to the ideal protein pattern could be used as a dietary tool for enhancing gastrointestinal health and function in piglets. In addition, the results revealed evidence of novel mechanisms underlying gut health benefits associated with feeding low CP diets supplemented with AA to piglets.

DEDICATION

This thesis is dedicated to my husband, Dr. Ademola Opapeju and daughter, Olutomisin Opapeju. Words can not express how grateful I am for having you both in my life!

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All the glory, honor. power and adoration belong to the almighty God for ever and ever!

FOREWORD

This thesis was prepared in a manuscript format and it is composed of five manuscripts. All manuscripts were written according to the format of the Journal of Animal Science. Manuscripts 1, 2 and 3 have been published in Animal, Journal of Animal Science and Archives of Animal Nutrition, respectively. Manuscript 4 has been accepted for publication in Livestock Science and Manuscript 5 is in under review (Comparative Immunology, Microbiology and Infectious Disease).

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LIST OF ABBREVIATIONS

AA	Amino acid
ADG	Average daily gain
ADFI	Average daily feed intake
ALP	Alkaline phosphatase
APA	Aminopeptidase A
APN	Aminopeptidase N
APP	Acute phase proteins
ARISA	Automated ribosomal intergenic spacer analysis
BW	Body weight
BSA	Bovine serum albumin
CD	Crypt depth
CD2	Cluster of differentiation 2
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CFA	Colonization factor antigen
CP	Crude protein
DDP	Dipeptidyl peptidase
DM	Dry matter
DNA	Deoxyribonucleic acid
EAA	Essential amino acids
EAST1	Enteroaggregative *E. coli* heat stable toxins

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
FC	Fecal consistency
FOS	Fructooligosaccharides
G:F	Gain-feed ratio
GIT	Gastrointestinal tract
GTP	γ-glutamyl transpeptidase
Hp	Haptoglobin
ICE	Incidence-based coverage estimator
IgA	Immunoglobulin A
IL-1β	Interleukin 1-beta
IL-6	Interleukin 6
LAP	Leucine aminopeptidase
LI	Large intestine
LPS	Lipopolysaccharide
LT	Heat labile enterotoxins
ME	Metabolizable energy
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MM	Michaelis-Menten
mRNA	messenger ribonucleic acid

NE	Net energy
NEAA	Non-essential amino acids
NSP	Non-starch polysaccharides
PBS	Phosphate buffered-saline
PCR	Polymerase chain reaction
PUN	Plasma urea nitrogen
PW	Post-weaning
PWD	Post-weaning diarrhea
PWC	Post-weaning colibacillosis
rDNA	Recombinant deoxyribonucleic acid
RISA	Ribosomal intergenic spacer analysis
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SBM	Soybean meal
SD	Standard deviation
SEM	Standard error of means
SGLT1	Sodium-coupled glucose cotransporter 1
SI	Small intestine
STa	Heat stable enterotoxin a
STb	Heat stable enterotoxin b
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TNF-α	Tumor necrosis factor-alpha

TOS	Trans-galactooligosaccharides
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
UV	Ultraviolet
VFA	Volatile fatty acids
VH	Villus height
ZI	Zone of inhibition

CHAPTER 1

GENERAL INTRODUCTION

Weaning imposes a tremendous amount of stress on piglets and is usually accompanied by changes in gut physiology, microbiology and immunology (Hampson, 1986; Pluske et al., 1997). Due to these changes, the period following weaning is usually marked with high incidence of intestinal disturbances such as diarrhea and depression of growth performance in piglets. Poor growth performance associated with weaning in pigs is a result of multi-factorial stressors – environmental, nutritional, and psychological stressors (Williams, 2003; Lalles et al., 2004). Not only does the weaned pigs have to deal with the abrupt interruption in the established social interaction with sow and litter mates but also with the stress of adapting to a new environment (Lalles et al., 2007a). In addition, the piglet has to cope with the sudden withdrawal of sow milk and adapt to less digestible, plant-based dry diets containing complex protein and carbohydrate and at times anti-nutritional factors (Wilson and Leibholz, 1981; Cranwell, 1995; Lalles et al., 2007a). Hence, there is always a sharp reduction in feed intake immediately after weaning in piglets (Pluske et al., 1997). While about 50% of weaned pigs consume their first feed within 24 h post-weaning, weaning anorexia persists up till 48 h in about 10% (Brooks et al., 2001).

Antibiotics and minerals, especially ZnO and $CuSO_4$, are often included in the diets for weaned pigs to control post-weaning diarrhea (**PWD**) and optimize growth performance (Verstegen and Williams, 2002). Post-weaning diarrhea is often caused by

enterotoxigenic *Escherichia coli* (**ETEC**) which has been described as an opportunist bacteria that quickly establishes itself in the gastrointestinal tract (**GIT**) of weaned pigs under suitable conditions (Fairbrother et al., 2005; Nagy and Fekete, 2005). Due to the possible contribution of in-feed antibiotics to the development of antibiotic resistant strains of bacteria (Amezcua et al., 2002), the European Union (**EU**) implemented a full ban on in-feed antibiotics usage in livestock diets in January, 2006 (Wellock et al., 2008b). There is also an ongoing interest to minimize or completely eliminate the inclusion of in-feed antibiotics in livestock diets in other parts of the world (Lusk et al., 2006). Hence, it is logical to speculate that there is going to be more demand for pork produced without in-feed antibiotics at the international market. Based on the EU experience, a ban in the usage of in-feed antibiotics is usually accompanied by serious economic and production consequences such as increase in weaning age and a reduction in the number of piglets weaned per sow (Stein, 2002; Hayes et al., 2002). There are also concerns about environmental accumulation of minerals resulting from high dietary levels of inorganic Zn and Cu. To keep the swine industry profitable, it is imperative to find alternatives to in-feed antibiotics that are effective in reducing the incidence and severity of digestive problems associated with the period immediately after weaning.

A number of nutritional strategies, including low CP, AA-supplemented diet, have been proposed as potential alternatives to in-feed antibiotics for weaner pigs (Stein and Kil, 2006; Wellock et al., 2006b). Typical starter diets contain high levels of dietary protein, 21 – 25% CP (Pluske et al., 2002). Feeding starter diets containing high levels of dietary CP to piglets will leave a substantial amount of undigested protein in the gut due to the immaturity of their digestive system and this could serve as a substrate for

proliferation of pathogenic bacteria in the absence of in-feed antibiotics (Nollet et al., 1999; Pluske et al., 2002; Hedemann et al., 2003). Furthermore, a shift in gut microflora towards pathogenic bacteria may promote proteolytic fermentation (Macfarlane, 1995). Products of proteolytic fermentation such as amines and ammonia are toxic (Lin and Visek, 1991; Williams et al., 2001) and may induce local and systemic inflammatory responses. Stimulation of immunological responses promotes partitioning of absorbed nutrient towards production of immune protein instead of being used for tissue accretion (Johnson, 1997). In fact, it was recently demonstrated by Houdijk et al. (2007) that pigs (weaned on d 28) fed a low, 13% CP diet had lower plasma haptoglobin concentrations compared to those fed a high, 23% CP diet.

There is a body of convincing evidence in the literature that low CP, AA-supplemented diets improve indices of enteric health such as low pH, increased lactobacilli:coliform ratio and decreased ammonia N concentration in piglets (Wellock et al., 2007; Heo et al., 2008). Low dietary CP content decreased intestinal luminal ammonia N concentration, coliform count, *E. coli* count and the incidence of diarrhea, and increased intestinal *Bifidobacteria* counts (Nyachoti et al., 2006; Pierce et al., 2007; Wellock et al., 2008a). However, the performance of pigs fed low CP diets supplemented with AA has not been consistent across studies. For example, some studies (Le Bellego and Noblet, 2002; Heo et al., 2008) showed that dietary CP level had no effect on performance of weaned pigs but others did not (Nyachoti et al., 2006; Wellock et al., 2008b). With the use of crystalline AA, it is possible to formulate starter diets with low CP content but with adequate amounts of AA recommended for growth and development (Stein and Kil, 2006). Such diets are expected to support optimal performance of weaned

pigs. Most studies that investigated the potential benefits of low CP diet for piglets were conducted in relatively clean research facilities. Thus, additional research is required to investigate gut health benefits of low CP, AA supplemented diet under conditions similar to those in a commercial production setting.

The general hypothesis of the research described in this thesis was that feeding a low CP diet supplemented with crystalline AA based on the ideal protein ratio to weaned pigs will reduce the amount of undigested protein in the small intestine and thereby reduce ETEC proliferation, minimize proteolytic fermention, improve the health status of weaned pigs and minimize the impact of infection on growth performance.

CHAPTER 2

LITERATURE REVIEW

THE WEANING PROCESS AND ITS INFLUENCE ON PIGLET PERFORMANCE

Young pigs have the potential for extremely rapid growth as a result of advances in animal genetics. For example, Hodge et al. (1974) demonstrated that piglets fed a reconstituted cow milk diet from 10 to 30 d of age had about 600 g/d average daily gain (**ADG**) in body weight. Likewise, Williams et al. (1997) showed that segregated early weaned and individually housed pigs had a growth rate of about 550 and 800 g/d at 10 and 17 kg BW, respectively. However, under practical circumstances, this potential is usually under-expressed (Williams, 2003). Under-expression of the potential for growth performance is further amplified at weaning which is a stressful period for pigs.

In the wild, weaning is a gradual process for pigs occurring between 8 and 20 wk of age (Boe, 1991; Mavromichalis, 2006), which allows for gradual adjustment to solid food (Castillo et al., 2007). On the contrary, weaning is an abrupt process in the modern day swine production occurring at about 2 to 5 wk of age and imposing a number of stressors on piglets (Lesniewska et al., 2000; Brooks and Tsourgiannis, 2003). Prior to weaning, the piglet depends on sow milk as its source of nutrition. Sow milk is readily digestible and offers some protection against diseases through its immunoglobulin (Bourne and Curtis, 1973; Wilson, 1974; Deprez et al., 1986) and other bioactive peptides

(Schanbacher et al., 1997; Rooke and Bland, 2002) components. At weaning, however, the piglet has to adapt to a dry, plant-based diet which is less digestible (Hays et al., 1959; Owsley et al., 1986). In addition, pigs are physically separated from the sow at weaning and mixed together with other litters. As a result, they have to deal with changes in the environment and psychological stress associated with weaning (Pluske et al., 1995).

The production penalty of the abrupt weaning process is a period of growth check immediately after weaning (Carroll et al., 1998; Le Dividich and Seve, 2000; Lalles et al., 2004). This period of growth check is closely associated with post-weaning anorexia (Figure 1). A study by Bruininx et al. (2001) showed that in pigs weaned at 27 d of age, only 54% of the group had taken their first meal within the first 28 h after weaning. In addition, during the first wk after weaning, the quantity of feed consumed by piglets is far below the amount required for growth. For example, Williams et al. (2003) reported that a pig growing at the rate of 250 g/d would need to consume 300 g of DM of a high quality creep feed per day. Also, combining data from different studies, Le Dividich et al. (2000) reported that ME intake in weaned pigs accounts for only 60 to 70% of the pre-weaning milk ME intake. Hence, implementation of management and nutritional measures to improve feed intake during the period immediately after weaning is critical in maintaining post-weaning performance. This is even more important considering the fact that poor growth performance during the first week after weaning usually result in a carry over effect on subsequent performance (Pluske et al., 1995; Dunshea et al., 1999). Tokach et al. (1992) reported that pigs with higher ADG during the first week after weaning reached market weight earlier than their slow-growing counterparts (Figure 2).

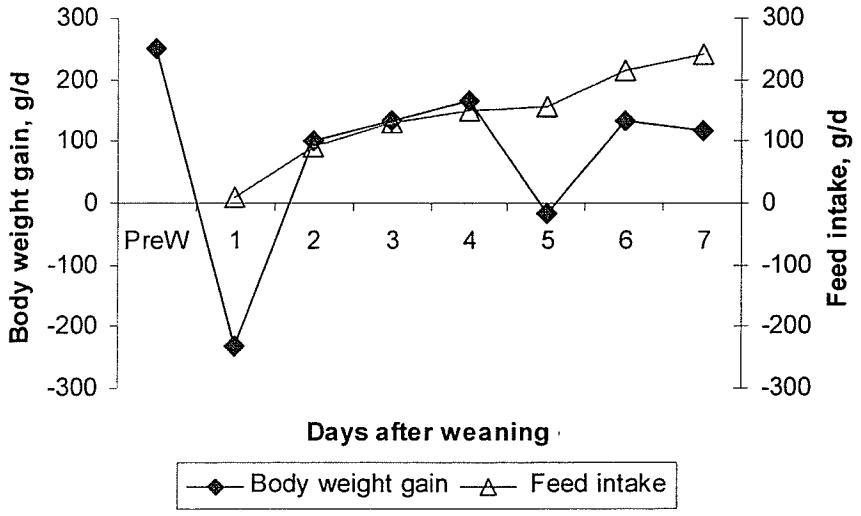

Figure 1. Effects of weaning on body weight gain and feed intake in pigs during the first 7 d after weaning.

Pigs were weaned at 21 d of age and had access to feed *ad libitum*. Adapted from Bark et al. (1986)

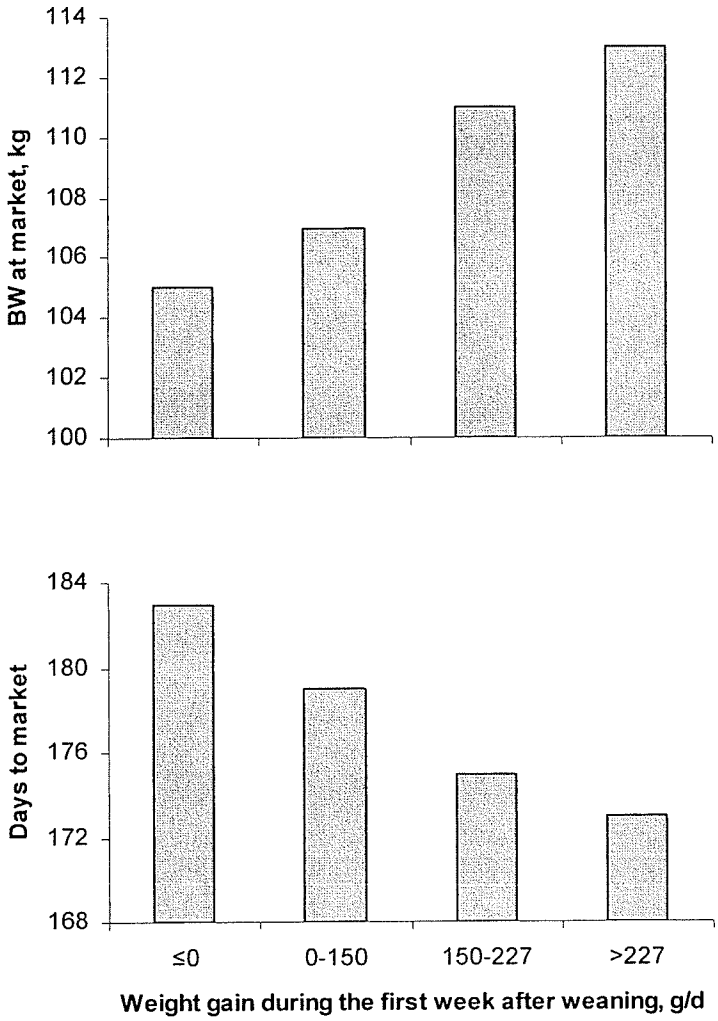

Figure 2. The influence of piglets' performance during the first wk after weaning on their subsequent growth performance.

Pigs were weaned at 21 d of age. Adapted from Tokach et al. (1992)

CHANGES IN PHYSIOLOGY AND FUNCTION OF GIT AROUND WEANING

Stomach

The functions of stomach include feed mixing and motility, partial digestion of feed and serving as a barrier against external environment (Barrow et al., 1977; Zhang and Xu, 2003). To achieve the digestive function, the stomach is endowed with acid (HCl) secreting cells which help to keep its pH low (Yen, 2000). This is because the zymogens of all the gastric proteases require low pH for their conversion into active enzymes (Khan et al., 1999). In addition, the optimal pH for digestion in the stomach is 3.0 (Prohaszka and Baron, 1980). The effect of weaning on gastric enzymes is not consistent in the literature. In a study by Hedemann et al. (2004), weaning resulted in a decline in pepsin activity in the stomach mucosa but had no effect on the activity of lipase. In contrast, other studies reported an increase in the activities of pepsin and lipase in stomach mucosa after weaning (Cranwell, 1985; Jensen et al., 1997). The discrepancies between studies have been attributed to differences in experimental and statistical methodologies and animal variation (Hedemann and Jensen, 2004).

Low pH (3.0 – 4.0) is also considered bactericidal for many pathogenic bacteria, *E. coli* inclusive (Prohaszka and Baron, 1980; Modler et al., 1990; Yen, 2000). Hence, in addition to its influence on nutrient digestion, maintenance of low gastric pH is essential for a healthy gut. Low gastric pH may help to reduce the passage of pathogenic bacteria from stomach into the small intestine (**SI**). Compared with the sow-reared pigs, weaned pigs have higher gastric pH and this may be due to low acid secretory capacity of the SI

at weaning (Manners, 1976; Efird et al., 1982). The high gastric pH may contribute, in part, to the piglet susceptibility to enteric infections after weaning.

Furthermore, weaning has an effect on gastric motility. Snoeck et al. (2004) reported a reduction in stomach emptying rate in pigs on d 3 and wk 2 after weaning compared with the suckling pigs (Figure 3). Given the high gastric pH that is usually observed post-weaning, gastric stasis may contribute to development of PWD in piglets by promoting proliferation of pathogenic bacteria. Indeed, gastric stasis has been documented in early-weaned pigs with diarrhea (White et al., 1969; Barrow et al., 1977). In addition, a stress gene, corticotrophin-releasing factor receptor 2, whose activation has been implicated with inhibition of gastric motility (Martinez et al., 2004), was up-regulated in the jejunum of weaned pigs (Moeser et al., 2007). Although, corticotrophin-releasing factor receptor 2 is yet to be determined in the stomach of the weaned pig, changes in gastric emptying rate could be modulated by intestinal feedback (Boudry et al., 2004a; Lalles et al., 2007b). Other factors which can be involved in gastric emptying rate are feed intake and composition of the diet (Rydning et al., 1985; Shi et al., 1997; Lalles et al., 2007b). For example, switching pigs abruptly from a milk-based diet to a wheat-based diet at 5 wk after weaning resulted in a transient increase in gastric emptying rate (Boudry et al., 2004a).

Small Intestine

Significant changes occur in the SI structure and functions during the immediate post-weaning period (Hopwood and Hampson, 2003). Although some of the changes are temporal, they are connected to the observed growth check and enteric disorders in

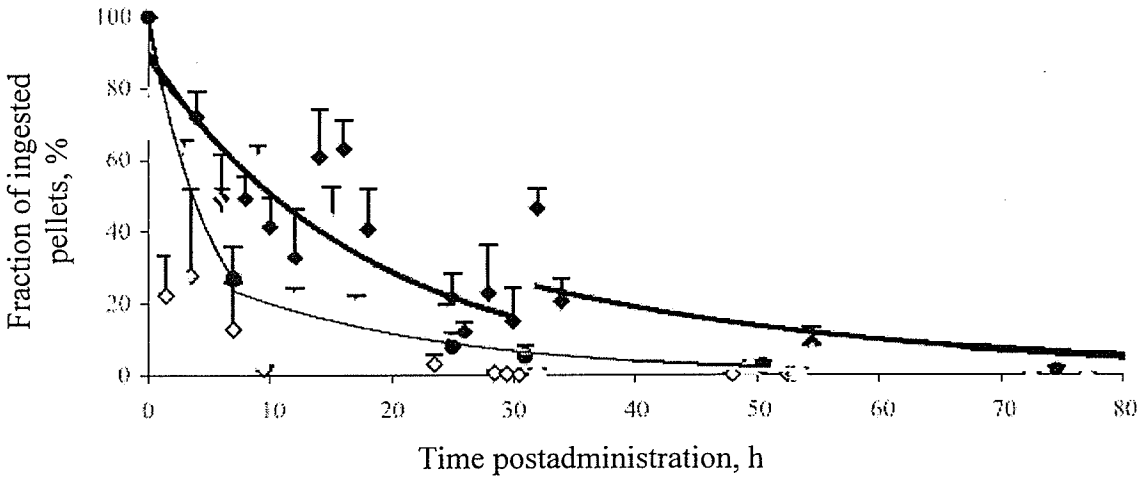

Figure 3. Effects of weaning on gastric emptying rate. Adapted from Snoeck et al. (2004).

Fraction of ingested pellets (%) present in the stomach of the suckling piglet —◊—, the piglet 3 d post-weaning —♦—, the piglet 2 wk post-weaning —▲—, and the piglet 3 wk post-weaning —●— at different hours after administration.

weaned pigs. In the present review, effect of weaning on the architecture and functions of SI in pigs will be described. Although the SI performs a lot of physiological functions, emphasis will be placed on functions related to nutrient digestion as well as fluid and electrolytes secretion and absorption.

Morphology. There are clear indications in the literature that weaning causes tremendous structural changes to the pig intestine (Hampson, 1986). Epithelial lining of the SI has finger-like projections known as villi which help to increase its surface area for digestion and absorption processes (Zhang and Xu, 2003). In addition, the mucosal surface of the SI has tubular glands that open into the intestinal lumen at the base of the villi known as crypts. Crypts contain epithelial stem cells required for repopulation of epithelial cells (Zhang and Xu, 2003; Llyod and Gabe, 2008). For optimal function of SI, long villi are desirable. However, there is a period of transient villus atrophy and crypt hyperplasia after weaning, and post-weaning anorexia has been suggested to be the main etiological factor for these changes (Pluske et al., 1997). According to McCracken et al. (1999), weaning anorexia correlates with crypt hypertrophy and local inflammatory responses. In fact, McCracken et al. (1995) suggested that the compromised jejunum epithelial architecture in pigs fed a diet based on soybean meal compared with those fed a milk replacer diet was due to reduced feed intake and not the diet composition.

Apart from a reduction in feed intake after weaning, other factors are partly responsible for villus atrophy. For example, Kelly et al. (1991b) reported a reduction in villous height (**VH**) and an increase in crypt depth (**CD**) within the first 3 d post-weaning in pigs that were continuously fed via gastric intubation. The authors suggested that morphological changes to gut architecture will occur after weaning even in the presence

of continuous nutrient supply. Although no particular underlying mechanism for these changes was presented by the author, it was probably due to weaning stress. For example, concentration of blood glucagon, a hormone associated with stress, was elevated between d 2 and 5 after weaning in piglets compared with the pre-weaning level (van Beers-Schreurs et al., 1998). Glucagon is a catabolic hormone that helps in mobilizing stored energy substrates and subsequent conversion of the substrates to glucose. van Beers-Schreurs et al. (1998) concluded that stress associated with the separation of piglets from sow and their transportation to pen partly contributed to the alteration to intestinal architecture after weaning. Hence, it appears that the effect of weaning on gut architecture is caused by a combination of different weaning stressors. A summary of recent published articles on changes to intestinal architecture during the immediate post-weaning period is presented in Table 1. It is rather difficult to compare data on intestinal morphology from different experiments due to differences in the age, breed, diets and experimental conditions.

Digestive Function. The brush-border surface of enterocytes performs digestive actions in the SI. Enterocytes account for about 90% and 95% of the epithelial cells in the crypt and villus, respectively, and they are responsible for secreting digestive enzymes (Cheng and Leblond, 1974). These enzymes are mainly mucosa-based and can be easily distinguished from pancreatic enzymes that act mainly on the luminal content of the intestine (Adeola and King, 2006). Activities of the brush border enzymes in weaned pigs have been used as indicators of maturation and digestive capacity of the SI (Henning, 1985; Hampson and Kidder, 1986).

A reduction in lactase activity is usually observed after weaning and this is partly

Table 1. Morphological changes in the small intestine of pigs around weaning[1]

Reference	Weaning age (d)	Results	Intestinal Section
Van Beers-Schreurs (1998)	28	↓ VH 3 d PW ↑ CD 3 d PW	Small intestine
Tang et al. (1999)	21	↓ VH 3 d PW ↑ CD 3 d PW ↓ VH:CD 3 d PW	Duodenum, Jejunum and Ileum
McCracken et al. (1999)	21	↓ VH 2 d PW ↓ CD 2 d PW	Jejunum
Hedemann et al. (2003)	29	↓ VH 3 d PW ↑ CD 5 d PW	10% of small intestine
Boudry et al. (2004b)	21	↓ VH 2 d PW ↑ CD 5 d PW	Jejunum
Verdonk et al. (2007)	26	↓ VH 3 d PW ↑ CD 3 d PW	Proximal and mid-small intestine

[1]VH = villus height, CD = crypt depth, PW = post-weaning

related to ontogenic decline in brush-border lactase activity (Montgomery et al., 1981; Kelly et al., 1991a; Motohashi et al., 1997). However, the effects of weaning on the activities of other brush border dissacharidases were not consistent in the literature. For example, weaning caused an increase in the activity of sucrase, maltase and glycoamylase activities during the first wk after weaning in some studies (Kelly et al., 1991b) but others reported a decrease in sucrase and maltase activities (Miller et al., 1986; Hampson and Kidder, 1986). Discrepancies between studies are probably a result of multiple variations such as experimental design, experimental diets, age of the animals, analytical and statistical methodologies and days post-weaning at which measurements were taken.

There was a decrease in the activities of dipeptidylpeptidase IV, aminopeptidase N, and alkaline phosphatase 3 d after weaning (Tang et al., 1999; Hedemann et al., 2003). However, in an early weaning model where pigs were weaned at 7 d of age, Marion et al. (2005) reported that jejunal peptidase activities increased 3 d after weaning. This observation has little or no practical significance because pigs are rarely weaned below 2 weeks of age. Weaning had no effect on tripeptidases (Collington et al., 1990; Hedemann et al., 2003). Starvation, due to a reduction in feed intake, and the presence of immature enterocytes, due to villus atrophy, could play a role in the decline in brush border peptidase activities around weaning (Kim et al., 1973; Hedemann et al., 2003).

Jejunal phosphatase gene expression decreased dramatically after weaning in piglets (Li et al., 2007). In studies where transient reduction in brush-border enzyme activities have been reported, enzyme activities usually reach minimum levels between 3 to 5 d post-weaning after which the enzyme activities usually increase. The increase in brush border enzyme activities after the first 5 d post-weaning is probably due to an

increase in substrate availability as daily feed intake increases (Pluske et al., 1997). For instance, activities of maltase and glycoamylase were higher in pigs receiving continuous supply of nutrients compared with those receiving restricted nutrient supply (Kelly et al., 1991c). Table 2 summarizes recent research on the effects of weaning on brush-border enzymes activities.

Secretory and Absorptive Function (Fluid and Electrolytes). Secretion of fluids and electrolytes from crypt cells and nutrient absorption from intestinal lumen are parts of the primary functions of SI (Pacha, 2000; Xu, 2003). Small intestinal secretion is a natural physiological phenomenon and it is essential for nutrient digestion and absorption (Kaunitz et al., 1995; Pacha, 2000; Wapnir and Teichberg, 2002). However, a net secretory condition occurs when fluid and electrolytes influx into the gut lumen exceeds its exflux into blood and this may serve as a predisposing factor to secretory diarrhea (Pacha, 2000; Wapnir and Teichberg, 2002). Weaning results in a reduction in the net absorption of fluid and electrolytes, and malabsorption of nutrients in the SI of piglets (Nabuurs et al., 1994; Miller and Skadhauge, 1997).

Changes in the absorptive and secretory function of the SI after weaning are segment-dependent. For example, Boudry et al. (2004) reported an increase in Na^+-dependent glucose absorption in the jejunum of weaned pigs but the opposite was the case in the ileum. Likewise, basal short-circuit current, which is a measure of ion transport, was increased in the jejunum. The authors, however, called for caution in the interpretation of increased jejunal absorptive capacity as this was accompanied by villus atrophy and decreased enzymatic activities in the jejunum. Hence, the increased jejunal absorptive capacity might have little or no biological significance. The low ileal

Table 2. Effects of weaning on the intestinal digestive enzyme activities

Reference	Weaning age (d)	Results	Intestinal Section
Tang et al. (1999)	21	↓ Lactase 3 d PW ↓ ALP 3 d PW	Duodenum and ileum
Fan et al. (2002)	28	↓ ALP 7 d PW ↑ APN 7 d PW ↑ Sucrase 7 d PW	Jejunum
Pluske et al. (2003)	14	↑ Glucoamylase 14 d PW ↓ Lactase 14 d PW ↑ Sucrase 14 d PW ↑ Maltase 14 d PW	Jejunum
Hedemann et al. (2003)	29	↓ DDP IV 3 d PW ↓ APN 3 d PW No effect on GTP	75% of small intestine
Boudry et al. (2004b)	21	↓ Lactase 2 d PW No effect on maltase	Jejunum and ileum
Marion et al. (2005)	7	↑ DDP IV 3 d PW ↑ APN 3 d PW No effect on APA ↓ Lactase 3 d PW ↑ Sucrase 7 d PW ↑ Maltase 3 d PW	Jejunum

[1]DDP IV = dipeptidyl peptidase IV, APA = aminopeptidase A, APN = aminopeptidase N, GTP = γ-glutamyl transpeptidase, ALP = alkaline phosphatase, PW = post-weaning

absorptive capacity shortly after weaning could contribute to high incidences of osmotic diarrhea in piglets by increasing the amount of nutrients in the hindgut.

Large Intestine

The three components of large intestine (**LI**) are the cecum, colon and rectum (Zhang and Xu, 2003). Physiological functions of the LI include fluid and electrolyte absorption, and provision of a physical barrier against microbial invasion (Williams et al., 2001; Zhang and Xu, 2003). Hence, alteration in these functions may play a role in physio-pathogenesis of PWD. The mucosal surface of the LI is lined with crypts but unlike SI, it lacks villi projections. Weaning decreased the crypt density and increased mitotic index in the cecum of piglets (Castillo et al., 2007). Weaning caused a transient reduction in the absorption capacity of the colon as indicated by basal short-circuit current at 2 d after weaning (Boudry et al., 2004b). It has been documented that excessive fluid loss in the SI will only result in PWD when the absorption capacity of the LI is exceeded. The effect of weaning on maturity and absorptive capacity of the LI has not been investigated extensively. Hence, the role of the LI in development of diarrhea in piglets after weaning is not clear yet. However, based on the available data, it appears that a combination of alteration in structure and absorptive function of the LI could contribute to the increased incidence of PWD in piglets (van Beers-Schreurs et al., 1992; Hopwood and Hampson, 2003). Nabuurs (1998) reported that simulated halving of the absorption capacity of the LI increased the adverse effects of ETEC in the SI of piglets. Future research should investigate changes in absorption capacity of the LI in piglets after weaning.

CHANGES IN MICROBIAL ECOLOGY OF GIT AROUND WEANING

Colonization of the Pig GIT from Birth to Weaning

Prior to birth, the GIT of pig is free of germ (Maxwell and Stewart, 1995). However, the gut is rapidly colonized by microbes shortly after birth via exposure to microorganisms in the birth canal, maternal feces and the immediate environment (Maxwell and Stewart, 1995; Pluske et al., 2002). During lactation, bacteria adapted to utilize milk as a substrate, lactobacilli and streptococci, are the dominant species in the stomach and SI (Hopwood and Hampson, 2003). The LI on the other hand contains a large and diverse selection of micro-organisms due to increased transit time of digesta (Pluske et al., 2002). Strict anaerobes including *Bacteroides, Eubacterium, Bifidobacterium, Propionibacterium, Fusobacterium and Clostridium* predominate in the LI (Gaskins, 2001; Hopwood and Hampson, 2003). After the initial colonization, the gut microflora remains relatively stable until weaning when sow milk is no longer available.

Effects of Weaning

The desired outcome of colonization of GIT is to have a stable, normal floral that offers protection against intestinal colonization by non-indigenous organisms including pathogens (Maxwell and Stewart, 1995; Gaskins, 2001). After weaning, a brief period of anorexia and an abrupt change in diet result in alteration to gut architecture and the amount and type of substrate available along the GIT (Maxwell and Stewart, 1995; Hopwood and Hampson, 2003). Hence, the microbiota in the GIT is unstable during the

first wk after weaning (Figure 4) compared with the pre-weaning period (Kenworthy and Crabb, 1963; Jensen, 1998; Konstantinov et al., 2006). In addition, piglets are deprived of protection from maternal antibodies in sow's milk at weaning (Deprez et al., 1986; van Beers-Schreurs et al., 1992). Combination of all these factors increases the vulnerability of weaned pigs to enteric diseases.

During the first wk after weaning, the pre-weaning predominant lactobacilli population was decreased and the population of coliform was increased in the SI of piglets (Jensen, 1998; Konstantinov et al., 2006). Weaning led to an increase in the number of hemolytic *E. coli* and rotavirus in the SI of piglets (Mcallister et al., 1979; Hampson et al., 1985). Similarly, molecular analysis of microbial ecology of the GIT of weaned and suckling pigs showed that weaning promoted an increase in the ratio between Enterobacteria and Lactobacilli (Castillo et al., 2007) and increased the abundance of clones showing high similarity to *Clostridiuo spp* and *E. coli* (Konstantinov et al., 2006). The impact of weaning on gut microbiology was greater in pigs weaned at 17 d of age compared with those weaned at 24 d of age (Franklin et al., 2002) indicating that early weaning may increase the susceptibility of piglets to enteric diseases. This was shown to be the case by Wellock et al. (2008a) who reported that ETEC excretion persisted longer in pigs weaned at 4 wk of age than those weaned at 6 wk of age. In general, a shift in microbial population from beneficial to pathogenic ones and instability in gut microbiota may partly be responsible for intestinal problems observed in pigs during the immediate post-weaning period (Konstantinov et al., 2006).

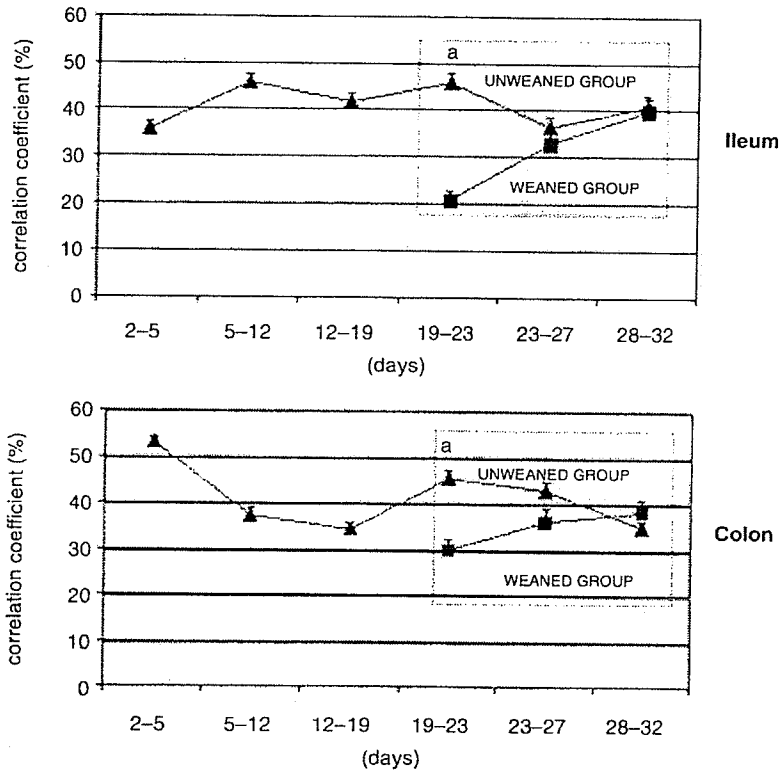

Figure 4. Porcine microbiota stability in weaned and unweaned pigs.

The correlation is between microbiota stability and age (days).
Source: Konstantinov et al. (2006).

Techniques for Enumeration of Porcine GIT Microbiota

Traditionally, the microbial composition of the pig GIT is studied using culture-dependent microbial methods. These methods are time-consuming and can only quantify culturable bacteria (Pryde et al., 1999; Leser et al., 2002). Given the fact that a larger percentage of gut microflora are not culturable, these methods may give a biased view of the composition and diversity of the GIT microbiota (Pryde et al., 1999; Zoetendal et al., 2004). In addition, some culturable bacteria have similar nutrient requirements and grow under similar environmental conditions, thereby limiting the adequacy of the culture-based methods (Soto et al., 2009). Molecular-based/culture-independent techniques are becoming increasingly available for the analysis of microbial communities. These techniques are more sensitive and, therefore, provide powerful tools for revealing the true phylogenic diversity of microbes within an ecosystem (Pryde et al., 1999). Majority of the molecular-based methods are based on polymerase chain reaction (PCR) amplification of genes of interest (Kitts, 2001). Hence, the culture-independent techniques based on PCR of the DNA or RNA extracted from gut samples will be discussed in this review. The detection and quantification of bacterial species in the pig GIT is based on the sequence comparison of the 16S rRNA gene (Tannock, 2001; Zoetendal et al., 2004). This method is very reliable because these genes are highly conserved and are present in large numbers of copies within each bacterial cell (Ventura et al., 2001; Zoetendal et al., 2004).

Sequencing of smaller subunit rRNA clone libraries: This technique utilizes smaller subunit rRNA clone libraries. Sequencing of the 16S rRNA has become a

standadrd procedure in the identification of bacterial isolates (Zoetendal et al., 2004). This approach involves sequencing of the 16S rRNA from 16S rDNA clone libraries (Zoetendal et al., 2004). Basically, the 16S rDNA are amplified, cloned and sequenced randomly within the clone library (Dorigo et al., 2005). The sequences of the cloned amplicons are compared to the sequences available in the smaller subunit rDNA database to identify the bacteria. The sequence information can be used to compare species richness, which is an indicator of the number of distinct species present, and diversity, which is a weighting abundance of distinct species, in different samples (Dorigo et al., 2005). Sequencing of 16S rRNA is not without limitations. It is laborious, subject to PCR biases and the data are not quantitative (Zoetendal et al., 2004; Dorigo et al., 2005). Several steps to minimize PCR bias have been suggested (Polz and Cavanaugh, 1998). Thus, the 16S smaller subunit DNA clone libraries are still very useful in analyzing pig GIT microbial composition and diversity.

Community fingerprinting techniques: Fingerprinting of the smaller subunit 16S rRNA, for example, 16S rRNA, has be suggested to be ideal for monitoring community shift and comparing different communities (Zoetendal et al., 2004). There are several fingerprinting techniques including denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis and temporal temperature gradient gel electrophoresis which are based on sequence-specific melting behavior of amplicons (Zoetendal et al., 2004). Additional techniques are single strand conformational polymorphism which detects sequence variations among DNA fragments, ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA). The RISA and ARISA

techniques involve PCR amplification of the spacer region located between the small (16S) and large (23S) subunit rRNA genes in the rRNA operon (Dorigo et al., 2005).

Another fingerprinting technique is terminal-restriction fragment length polymorphism (T-RFLP) analysis which is based on the restriction digest of double-stranded fluorescently end-labeled PCR fragments (Liu et al., 1997). Briefly, following an extraction of DNA from samples, a portion of the 16S rRNA gene is amplified and digested to produce a pattern of different length fragments (Kitts, 2001). The labeled fragments are then separated by gel electrophoresis in non-denaturing polyacrylamide gels or by capillary electrophoresis and detected with appropriate fragment software in the form of electrophoregrams, in which the peaks represent fragments differing in size, and the areas under the peaks indicate the relative proportions of the fragments (Dorigo et al., 2005). This technique is able to detect rarer members of a microbial community. The sizes of the terminal restriction fragment can be used for phylogenetic assignments by searching web-based databases for matching sequences that might identify individual bacteria in the community profile (Kitts, 2001).

The major limitation of this technique is the formation of pseudo-terminal restriction fragments, which can result in the overestimation of microbial diversity. The choice of the primers and restriction enzymes are important for obtaining an accurate evaluation of the microbial diversity (Engebretson and Moyer, 2003). Like other community analysis methods, the use and utilization of T-RF patterns is subjected to DNA and PCR biases. However, T-RFLP technique is rapid, reproducible and has high throughput (Kitts, 2001; Dorigo et al., 2005). It is very useful for monitoring microbial community dynamics.

CHANGES IN GIT IMMUNE SYSTEM AROUND WEANING

Mucosal Immune System – Birth to Weaning

At birth, the GIT mucosa of the piglet is exposed to a vast array of antigens including harmless antigens from food and dangerous antigens from pathogens (Stokes et al., 1994; King et al., 2003). Hence, piglet survival depends on a functional GIT immune system that is capable of tolerating dietary antigens and normal micro-flora, and mounting immunological responses against pathogens (Stokes et al., 1994). The intestinal tract plays a critical role in this regard by allowing for the absorption of nutrients and at the same time serving as a physical barrier against most luminal antigens (King et al., 2003).

The newborn pig is immunodeficient and, thus, it depends on passive immunity from maternal antibodies until it can develop its own active immune system (Rooke and Bland, 2002; Stokes et al., 2004). Immunoglobulins, of which immunoglobulin G is the predominant, in the sow colostrum are absorbed intact within the first 48 h after birth when the gut is still 'open'(Komuves and Heath, 1992; Gaskins and Kelley, 1995). Thereafter, immunoglobulin A becomes the major isotype immunoglobulin in sow milk and acts locally to provide enteric protection (Gaskins and Kelley, 1995; Stokes and Bailey, 2000).

Cells and structures involved in the mucosal immune system are almost absent at birth in piglets (Bailey et al., 2001; Stokes et al., 2004). The pig intestine is rapidly colonized with lymphoid cells expressing the CD2 surface marker but lacking CD4 and CD8 during the first 2 wk of life (Stokes et al., 2004). The $CD4^+$ T cells appears between

2 to 4 wk of age whereas $CD8^+$ T cells and IgA^+ B cells only appear at 5 wk of age (Stokes et al., 2004). Since weaning usually occur between 2 to 4 wk of age under commercial conditions (King et al., 2003), the above data indicate that the mucosal immune system is relatively immature at weaning.

Effect of Weaning on Intestinal Immunity

Piglets are prone to enteric diseases immediately after weaning. Immaturity of the intestinal immune system and removal of IgA and other bioactive compounds derived from sow milk could contribute to their susceptibility to these diseases (Bailey et al., 1992; Stokes et al., 2004; Bailey et al., 2005). Immaturity of the intestinal immune system may fault the ability of the weaned pig to mount appropriate immunological response to pathogens and/or its ability to tolerate dietary antigens. Weaning caused a transient reduction in the ability of intraepithelial lymphocytes to respond to mitogens (Bailey et al., 2005) and a transient hypersensitivity to dietary soy protein (Li et al., 1990).

Weaning has also been shown to activate intestinal inflammatory responses. In piglets (weaned at 21 d of age) fed a soy-based or a milk-based diet, McCracken et al. (1999) reported a decrease in the jejunal expression of major histocompatibility complex (**MHC**) class 1 mRNA and an increase in $CD4^+$ T cells in jejunal villi at 1 and 2 d after weaning, respectively. However, pre-weaning values of $CD8^+$ T cells and MHC class 1 mRNA were achieved upon resumption of feed intake. The authors also reported an increase in $CD8^+$ T cells in pigs fed a soy-based diet at 2 d after weaning and suggested that soy-induced inflammation is secondary to local inflammation caused by anorexia.

Hence, post-weaning anorexia coupled with its consequences on gut morphology was reported to be the major contributor to local intestinal inflammation during the immediate post-weaning period (McCracken et al., 1999). Likewise, Vega-Lopez et al. (1995) reported an increase in $CD2^+$ T cells in jejunal villi of piglets at 4 d after weaning. Weaning was also associated with up-regulation of pro-inflammatory cytokines interleukin 1 beta, tumor necrosis alpha and interleukin 6.

POST-WEANING DIARRHEA IN PIGLETS

Etiology of PWD

Post-weaning diarrhea, also known as post-weaning collibacillosis (**PWC**), is a major source of economic loss to the swine industry via its role in post-weaning morbidity and mortality (Pluske et al., 2002; Fairbrother et al., 2005). Post-weaning diarrhea is a complex and multifactorial disease. However, beta-hemolytic, enterotoxin-producing strains of *E. coli* are considered to be the major etiological bacteria (Pluske et al., 2002; Amezcua et al., 2002). Although strains of ETEC expressing F4 and F18 fimbriae (also known as pilli or adhesins) have been implicated with PWD, those expressing F4 (K88) fimbriae are the predominant causative organisms (Pluske et al., 2002; Fairbrother and Gyles, 2006). Also, the presence of rotavirus could exacerbate the severity of PWD (Nabuurs, 1998; Hopwood and Hampson, 2003). Other factors that could contribute to the development of PWD includes the abrupt removal of a maternal source of passive immunity provided via milk, immature immune system/depressed

immune response, low level of digestive enzyme, gut stasis, change in diet and/or form of diet, cold stress, and poor hygiene (Shimizu and Terashima, 1982; van Beers-Schreurs et al., 1992; Pluske et al., 2002).

Furthermore, the act of weaning itself is a major contributory factor. Evidence in the literature suggests that weaning stress contributes significantly to the development of PWD and this is believed to be mediated by stress signaling pathways. Moeser et al. (2007) demonstrated an up-regulation of intestinal corticotrophin-releasing factor receptors 1 in weaned pigs. Corticotrophin-releasing factor receptor 1 has been implicated with a series of stress-induced intestinal disorders such as hypersecretion, increased colonic motility and visceral hypersensitivity in rodents. In addition, the authors showed that weaning activated prostaglandin pathways. Postaglandins are important mediators of inflammatory responses and fluid and Cl^- secretion (Rachmilewitz, 1980; Roze et al., 1998; Sharkey and Mawe, 2002).

Pathogenesis of Postweaning E. coli Diarrhea

Post-weaning *E. coli* diarrhea involves ingestion and proliferation of ETEC in sufficient amounts in the gut followed by bacterial attachment to the receptors on intestinal epithelium via specific fimbriae (Fairbrother and Gyles, 2006). Apart from allowing ETEC to attach firmly onto SI microvilli and/or mucus covering the villi (Pluske et al., 2002; Nagy and Fekete, 2005), fimbriae also enables the ETEC to evade the natural cleansing mechanism of SI by peristalsis (Kenworthy, 1976). Upon successful adherence to the host cells, ETEC multiply rapidly to attain massive intestinal population

of 10^9 cfu/mL (Williams and Jones, 1963; Fairbrother and Gyles, 2006). Thus, development of PWD depends on the degree of intestinal colonization by ETEC. Colonization of the intestinal surface by ETEC is followed by elaboration of enterotoxins that act locally on enterocytes to induce diarrhea. Three major enterotoxins are produced by ETEC: heat labile toxins, enteroaggregative *E. coli* heat stable toxins (**EAST1**) and heat stable toxins (Moeser and Blikslager, 2007). Enterotoxigenic *E. coli* may produce one or two or a combination of all toxins (Nataro and Kaper, 1998). Fimbriae and enterotoxins are important virulence attributes of ETEC (Chen et al., 2004; Fairbrother et al., 2005; Turner et al., 2006).

Heat labile toxin binds to receptor on the surface of intestinal mucosal cells and activates water and electrolyte (Na^+, Cl^- and HCO_3^-) secretion into the gut lumen via cyclic adenosine monophosphate pathway (Spangler, 1992). Heat stable toxins have 2 unrelated, functionally distinct sub-classes, STa and STb (Turner et al., 2006). The STa stimulates Cl^- secretion and/or inhibits NaCl absorption by increasing the intracellular level of cyclic guanosine monophosphate (Currie et al., 1992). Mechanism by which STb induce diarrhea is not well understood (Nagy and Fekete, 2005). However, it is known that STb mechanism does not involve cyclic nucleotides (Nataro and Kaper, 1998). Heat stable enterotoxin STb induced the release of prostaglandin E2 and serotonin, which have both been shown to stimulate intestinal cell secretion via enteric nervous system (Hitotsubashi et al., 1992; Fujii et al., 1995). The STb also induces morphological damage such as villus atrophy to SI and stimulates secretion of HCO_3^- (Sears and Kaper, 1996; Nataro and Kaper, 1998). The EAST1 toxin was originally isolated from enteroaggressive *E. coli* but it has been identified in other strains of *E. coli* including

ETEC (Yamamoto and Nakazawa, 1997; Veilleux and Dubreuil, 2006). The role of EAST1 in mediating diarrhea is controversial but it is known to activate cyclic guanosine monophosphate in a manner similar to STa (Turner et al., 2006).

Animal factor is also important in pathogenesis of PWD. Some pigs have no/weak brush border receptors for F4 and F18 and are thus considered resistant to PWD {Chandler, 1994 297 /id;Frydendahl, 2003 298 /id}. In general, the basic principles of ETEC infection in animals are: 1) adherence and colonization of enteric mucosal surface, 2) evasion of host defense mechanisms and damage to the host, and 3) multiplication within host (Nataro and Kaper, 1998).

ANTIBIOTICS IN WEANER PIG NUTRITION: USAGE AND PROBLEMS

'Antibiotics are substances that can deleteriously affect bacteria either by interfering with their growth, metabolism or actually killing them in situ' (Walton, 2001). Majority of the antibiotics are usually produced naturally by yeast, moulds and bacteria but some are man-made (Walton, 2001; Li et al., 2003). There are 2 major classes of antibiotic usage in livestock production: 1) therapeutic usage for treating microbial infection and 2) inclusion in feed as additive at sub-therapeutic level for prophylaxis and growth promotion (Walton, 2001). Usage of in-feed antibiotic in pig diets is the most effective approach in managing enteric diseases and growth depression associated with weaning. About 70 to 80% of pig starter diets are supplemented with antibiotics in the US (Cromwell, 2002). Inclusion of antibiotics in diets of young pigs improves growth performance (Figure 5). Growth and feed efficiency were increased by 16% and 7%,

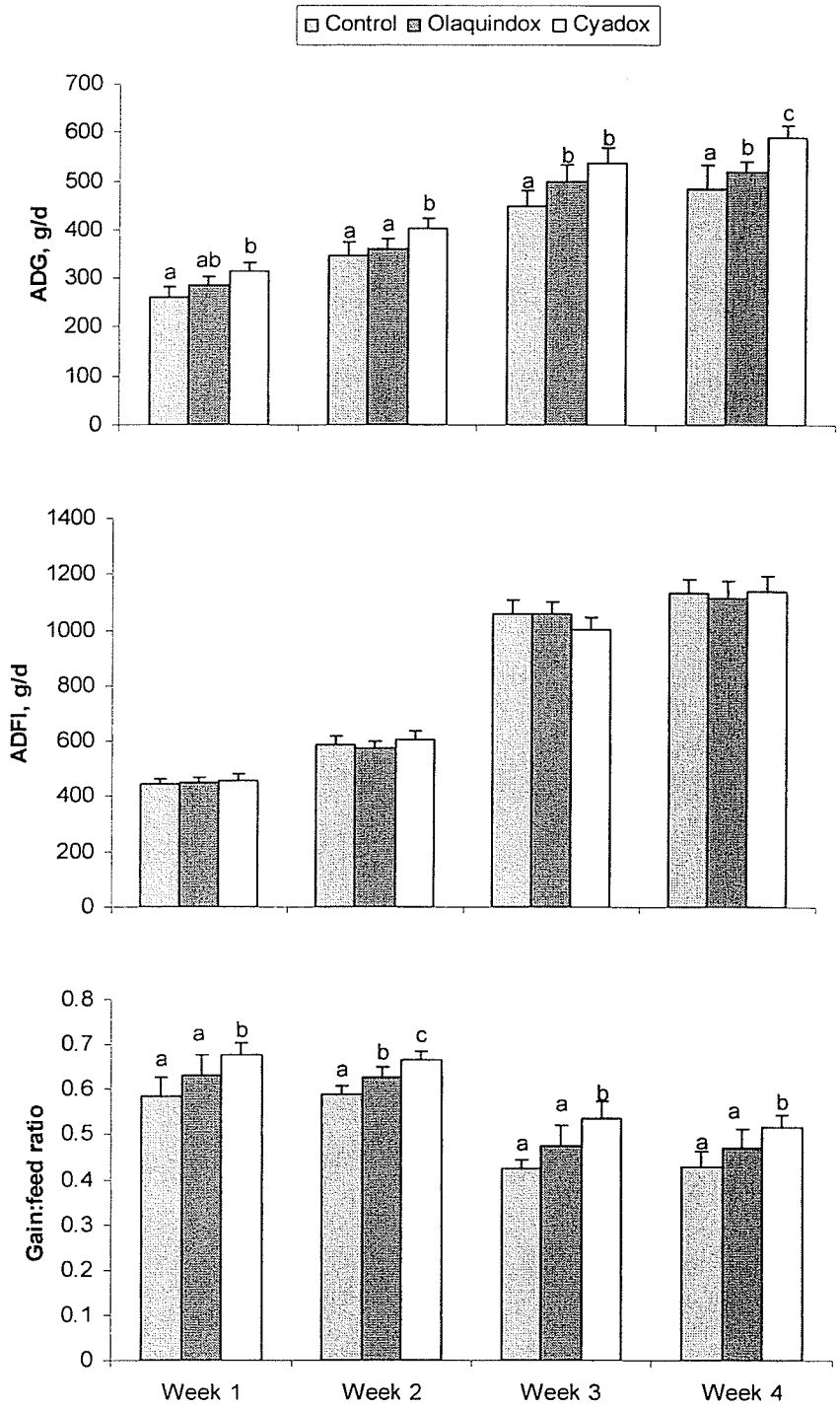

Figure 5. Effect of antibiotics on growth performance of weanling pigs.

Within a panel, labeled means without a common letter differ, $P < 0.05$. Adapted from Wang et al. (2005).

respectively, under experimental conditions and by 28% and 15%, respectively, at the farm level (Cromwell, 2002).

The mechanisms by which antibiotics promote animal growth has not been completely elucidated. However, a few modes of action have been proposed. First, antibiotics usage inhibits sub-clinical infection (Visek, 1978; Walton, 2001). Second, antibiotics exert a direct suppressive effect on microbial growth and subsequently reduce microbial use of nutrients, microbial degradation of bile products and concentration of toxic and growth depressing microbial metabolites such as ammonia, aromatic phenol and (Visek, 1984; Jensen, 1998; Gaskins et al., 2002). Third, antibiotics may enhance nutrient absorption from SI by reducing intestinal wall thickness and rate of feed passage (Ravindran et al., 1984).

Microbial activities in the LI are mostly beneficial to the host except for the production of toxic fermentation metabolites (Figure 6). Bacterial activities in the SI have the biggest impact on nutrient utilization in pigs because it is the major site of nutrient absorption (Gaskins et al., 2002). Antibiotics reduced microbial fermentation of carbohydrates in the SI (Vervaeke et al., 1979). Microbial fermentation could result in as much as 6% loss in net energy (Hedde and Lindsey, 1986). Carbadox supplementation improved the ileal digestibility of AA in growing pigs fed a high fibre diet (Partanen et al., 2001). Wang et al. (2005) reported that cyadox and olaquindox supplementation increased apparent fecal digestibility of DM, CP, crude fibre, ether extract, energy, Ca, P, Cu and Mn in weanling pigs.

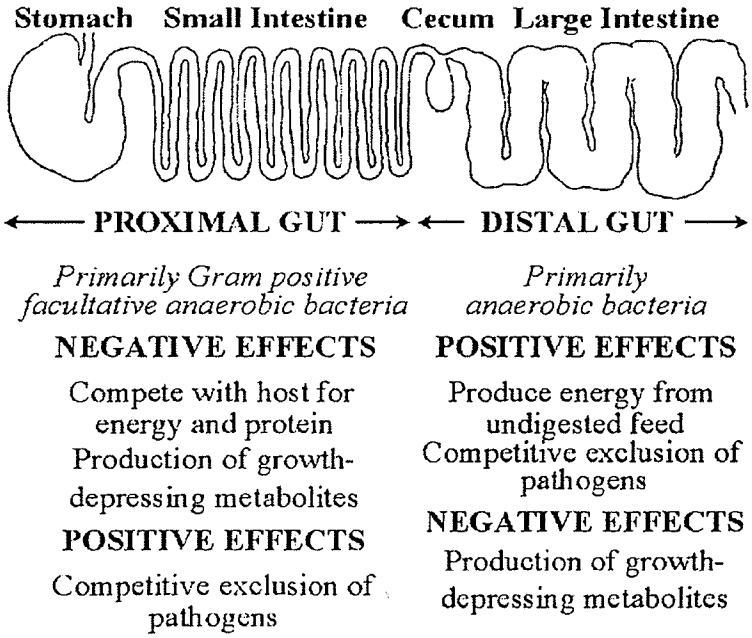

Figure 6. The positive and negative effect of microbiota in the proximal and distal gut.

Source: Gaskins et al. (2002)

There are growing concerns over the use of subtherapeutic level of antibiotics in feed due to the possible development of antibiotic-resistant bacteria and the subsequent potential health risk to humans (Stobberingh and van den Bogaard, 2000; van den Bogaard and Stobberingh, 2000). Gellin et al. (1989) reported that prevalence of antibiotic resistant strains of bacteria was higher in pig herds that were exposed to therapeutic and/or sub-therapeutic doses of antibiotic compared with the herd that was not exposed to antibiotics for 154 months. Similar observation has been made by Mathew et al. (1998b). In 2006, the EU banned the use of sub-therapeutic antibiotics in livestock diets. However, removal of prophylactic antibiotics from swine diet is not without production penalty. Following the ban of in-feed antibiotics in Sweden in 1986 and Denmark in 1999 (Table 3), hog production cost and incidence of health problems and the associated usage of therapeutic antibiotics increased (Hayes et al., 2002; Hayes and Jensen, 2003). Furthermore, productivity of sows was also negatively affected by antibiotic removal in a study conducted in the US (Table 4). If the prices received by hog producers are held constant, implementation of antibiotic ban in the US will make many producers unprofitable and would likely lead to an exit from the industry and/or an increase in prices (Hogberg, 2005). On the other hand, implementation of policy to limit sub-therapeutic usage of antibiotics in swine diets could offer access to niche market around the globe. As a result of the production and economical consequences associated with the ban of in-feed antibiotics, there is an ongoing search for effective alternatives to their usage.

Table 3. Production impact of antibiotic removal from hog diets in Europe.

Item	Sweden	Denmark
Age at weaning	+1 week	*
Days from weaning to reach 25 kg	+5 days	*
Feed efficiency from 23 to 113 kg	-1.5%	-1.5%
Piglet mortality	+1.5%	*
Fattening-finishing mortality	+0.04%	+0.04%
Piglets per sow	-4.82%	-4.82%
Veterinary and therapeutic cost (per pig) net of cost for feed-grade antibiotics		+$0.25
Lawsonia vaccine		$0.75

*These costs totaled $1.25 per animal and were not broken down to specific productivity impacts. Source Hayes et al. (2003)

Table 4. Production impact of antibiotic removal from hog diets in North America[1]

Item	Antibiotics (1963-1972)	No antibiotics (1972-1985)
Number of litters	398	688
Conception rate, %	91.4	82.6
Number of pigs born per litter	10.8	10.2
Live pig born per litter	9.8	9.3
Average birth weight, kg	1.29	1.38
Number of pigs weaned, 21 d	8.8	7.5
Average weaning weight, kg	5.67	5.37
Survival of live born, %	89.7	80.9
Incidence of mastitis, metritis, agalactia	<10	66 (1972-1975)[2]

[1]Closed, specific-pathogen-free herd at the University of Kentucky. Antibiotics were used in breeding, lactation, starting, growing and finishing diet from 1963-1972. Antibiotics were not used in the feed or for treatment purposes from 1972-1985.

[2]For the period 1972-1975 only.

Source: Cromwell et al. (2002)

ALTERNATIVES TO SUBTHERAPEUTIC USAGE OF ANTIBIOTICS IN PIGLET DIET: NUTRITIONAL STRATEGIES

In the absence of in-feed antibiotics, a variety of management and nutritional strategies may be required to secure adequate post-weaning performance in piglets (Goransson, 2001; Stein, 2002). In this section, some of the nutritional strategies of enhancing gut health and/or promoting post-weaning growth performance, specifically probiotics, prebiotics, organic acids, trace minerals and dietary protein source and level, will be discussed. Other suggested nutritional strategies include essential oils, yeast cell wall products, nucleotides and nucleosides, immune egg products, and fermented liquid feeding (Pettigrew, 2006; Stein and Kil, 2006). The nutritional strategies include addition of non-antibiotic products that may improve animal health and performance to the diets and/or manipulation of feed composition (Pettigrew, 2006; Stein and Kil, 2006).

Probiotics

Probiotics are live microbial feed supplements. According to Schrezenmeir et al. (2001), probiotics can be defined as "a preparation or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host, and by that exert beneficial health effects on the host". Microorganisms to be used as probiotics should be able to survive in the gastric acidic environment and bile salts. The three categories of organisms that are commonly referred to as probiotics are bacillus, yeast, and lactic-acid producing

bacteria such as *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* (Stein and Kil, 2006). The results of animal experiments evaluating the efficacy of probiotics as alternatives to antibiotics are variable. For example, lactic-acid producing bacteria as probiotics produced positive responses in some studies (Lessard and Brisson, 1987; Shu et al., 2001; Taras et al., 2006) but not in others (Walsh et al., 2007). Likewise, inclusion of yeast culture in weanling pigs' diets yielded positive responses in some studies (Mathew et al., 1998a; Bontempo et al., 2006; van der Peet-Schwering et al., 2007) but not in others (Kornegay et al., 1995; van Heugten et al., 2003). Inconsistency in the results has been attributed to differences in dosage and type of strain of probiotic, sanitary environment and diet type (Bontempo et al., 2006). Future research should be focused on identification of probiotics as well as doses that can consistently promote enteric health and performance in piglets after weaning.

Although mechanisms by which probiotics confer performance and enteric health benefits to piglets are not completely understood, a few have been proposed. Probiotics inhibit pathogen adhesion by steric hinderance or competitive exclusion (Li et al., 2003; Roselli et al., 2005). Jin et al. {Jin, 2000 422 /id /d} reported that a strain of *Enterococcus faecium* inhibited the adhesion of ETEC K88 to the SI mucosa in piglets. In a similar study, Blomberg et al. (1993) reported that *Lactobacillus fermentum* strain 104R reduced the adhesion of ETEC K88 to ileal mucus by approximately 50% in an *in vitro* study.

Beneficial effects of probiotics are also mediated via production of microbicidal substances such as bacteriosins and organic acids against pathogens (Juven et al., 1991; Gibson and Wang, 1994b; Li et al., 2003). Setia et al. (2009) reported that colicin producing *E. coli* exhibited inhibitory activities against ETEC in an in vitro assay.

Probiotic bacteria increased the production of short chain fatty acids in an *in vitro* study (Sakata et al., 2003). Increase in short chain fatty acids production may help to reduce digesta pH and subsequently depress the growth of pathogenic bacteria (Gibson, 1999).

Modulation of host immune system is another possible mode of action of probiotics (Bontempo et al., 2006; Roselli et al., 2007; Sauerwein et al., 2007). Supplementation of weaned pig diet with *Bifidobacterium lactis* HN019 resulted in higher blood leukocyte phagocytic and T-lymphocytes proliferative responses, and higher GIT pathogen-specific antibody titre (Shu et al., 2001). *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG prevented inflammation-associated response caused by ETEC by inhibiting neutrophil transmigration and preventing ETEC-induced expression of TNF-α and IL-1β (Roselli et al., 2006).

Prebiotics

The concept of prebiosis was introduced by Gibson and Roberfroid (1995) and was defined as "a selectively fermented ingredient that allows specific changes, both in composition and /or activity in the gastrointestinal microflora that confers benefits upon host well-being and health". Prebiotic has now been redefined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of microflora, that confer benefits upon host well-being and health" (Gibson et al., 2004). Only fructooligosaccharides (**FOS**) and trans-galactooligosaccharides (**TOS**) qualify as prebiotics based on the prebiotic selection criteria: 1) resistance to gastric acidity, to hydrolysis by mammalian enzymes and to GIT absorption; 2) fermentation by intestinal microflora; and 3) selective stimulation of the growth and/or activity of intestinal bacteria

associated with health and well-being (Gibson, 1999; Roberfroid, 2007). Other authors suggested potential prebiotic roles of dietary fiber and resistant starch (Bird, 1999; Verstegen and Williams, 2002; Topping et al., 2003). Supplementation of a starter diet with NSP hydrolysis products reduced the severity of enteritis in piglets challenged with ETEC K88 (Kiarie et al., 2008a). Likewise, Bhandari et al. (2009) demonstrated that the fecal consistency score of pigs fed diets containing raw potato starch and those fed a diet supplemented with antibiotics was similar. Using a piglet small intestinal perfusion method, Kiarie et al. (2008b) reported that NSP hydrolysis product of soybean and canola meal protected against ETEC-induced fluid and electrolyte losses.

Beneficial effects of prebiotics are thought to be mediated predominantly through their selective stimulation of the proliferation and activities of bacteria associated with healthy gut such as bifidobacteria, lactobacilli and Eubacteria. Fructooligosaccharides stimulated the proliferation of *Bifidobacterium* species in an *in vitro* study (Gibson and Wang, 1994a) and increased the ratio between the fecal level of FOS-degrading bacteria and saccharolytic bacteria *in vivo* in piglets (Mikkelsen et al., 2003). However based on 16S RNA gene sequencing, Mikkelsen et al. (2004) reported that FOS and TOS had no effect on *Bifidobacterium* concentration. Houdjik et al. (2002) reported that FOS and TOS reduced the pH and aerobic bacterial counts in weaned pigs at the ileal level but not at the fecal level and, hence, suggested that FOS and TOS has pre-cecal prebiotic effects. Similarly, FOS and TOS had no effect on fecal pH, organic acid concentration and culturable bacteria and on growth performance. On the other hand, yeast concentration in the SI and LI (Mikkelsen et al., 2003; Mikkelsen and Jensen, 2004), butyric acid concentration in the LI (Shim et al., 2005), growth, nutrient digestibility and SI

morphology were improved with oligosaccharide supplementation to weaned pigs diet (Liu et al., 2008). Discrepancies between experiments might be due to differences in techniques used in evaluating intestinal microbes, type of microorganism used as response criteria, type and dosage of oligosaccharides, age of the animals, and experimental diets.

Synergetic effects of pre- and probiotics in a relationship termed symbiosis has been reported (Bird, 1999). Combination of FOS and *Bifidobacterium animalis* increased the expression of toll-like receptor 2 in the ileocecal lymph nodes of weaned pigs and thus may play a role in enhancing innate immune response (Trevisi et al., 2008). In another study, Bhandari et al. (2007) reported that colicin producing *E. coli* and raw potato starch acted synergistically to improve growth performance and reduce incidence of scours in piglets infected with ETEC K88.

Organic acids

At weaning, natural acidification of the stomach via HCl secretion is reduced due to immaturity of the digestive system and sudden change in diet from milk to solid diets. Compared with the mature pig with a pH range of 2 to 3, the gastric pH in suckling and weanling piglets ranged between 4 and 5 (Ravindran and Kornegay, 1993). However, suckling piglets maintain gastric acidity by microbial fermentation of milk lactose to lactic acids (Cranwell et al., 1976). It is important to maintain a low gastric pH in order to optimize nutrient digestion and prevent pathogen overgrowth. Dietary addition of organic acids such as citric, fumaric, lactic and formic acids to weaned pig diets has been associated with growth performance and health benefits (Giesting and Easter, 1985;

Edmonds et al., 1985; Tsiloyiannis et al., 2001). Suggested underlying mechanisms include reduction of stomach pH and conferment of bactericidal effects (Roselli et al., 2005; Pettigrew, 2006).

Formic and lactic acid supplementation reduced gastric pH in piglets (Thomlinson and Lawrence, 1981; Bolduan et al., 1988). Acidic gastric environment is required for the conversion of zymogens of pepsin A and pepsin C into their active forms (Fan, 2003). Furthermore, low gastric pH does not support the growth of pathogenic bacteria such as *E. coli* but enhances the growth of beneficial, lactic acid producing bacteria (Smith and Jones, 1963; Fuller, 1977). Hence, it has been suggested that a reduction in gastric pH may improve nutrient digestion and alter microbial population in the stomach and other parts of GIT (Pettigrew, 2006). Fumaric acid supplementation had no effect on apparent ileal nutrient digestibility in piglets (Giesting and Easter, 1991). However, Blank et al. (1999) reported an increase in ileal digestibily of energy, CP and AA with supplementation of fumaric acid to a diet with low buffering capacity but not with diet with high buffering capacity. This observation confirms the fact that factors such as the buffering capacity of the diet, age of the animal, and type and level of organic acid used affect the efficacy of diet acidifiers in promoting growth and enteric health of animals (Ravindran and Kornegay, 1993).

Addition of free organic acid (75% formic and 25% propionic acid) reduced counts of *Samonella* and *E. coli* in the stomach of piglets experimentally infected with *Samonella* or *E. coli* (Taube et al., 2009). In another study, organic acid tended to decrease *E. coli* population and increased Lactobacilli count without having any effects on the GIT pH in pigs infected with ETEC (Li et al., 2008). It thus appears that reduction

of gastric pH is not the primary mechanism of modulating intestinal microbial population and that organic acids do have their own direct antibacterial activities (Cherrington et al., 1991; Risley et al., 1992). For example, organic acids are capable of diffusing across the cell membrane of bacteria and once inside the bacteria, they will dissociate to produce proton (H^+) and anion ($RCOO^-$) and disrupt the pH and the anion pool of the cytoplasm (Warnecke and Gill, 2005). Elevated cell cytoplasmic pH has lethal consequences on the cell by affecting the integrity of purine bases and by denaturing essential enzymes. High concentration of anion within the bacterial cell could also reduce growth and viability of the cell by increasing the turgor pressure within the cell (Warnecke and Gill, 2005).

Trace Minerals: Zinc and Copper

Addition of copper to the diet of nursery pigs beyond the requirement amount enhanced their growth performance (Windisch et al., 2001) and inhibited the activity of coliforms and lactobacilli (Jensen, 1998). Likewise, supplementation of pharmacological levels of Zn to piglets diets has been reported to improve growth performance (Li et al., 2003). Zinc supplementation minimized incidences of diarrhea, maintained intestinal microflora stability and increased diversity of coliforms (Katouli et al., 1999).

Growth and gut health benefits of Zn and Cu have been attributed to their effects on intestinal microflora (Jensen, 1998; Katouli et al., 1999). In addition, Zn supplementation increased the activities of pancreatic digestive enzymes and the mucin staining areas of the LI (Hedemann et al., 2006). Dietary supplementation with high levels of inorganic Cu and Zn, as is usually done under commercial production, leads to excretion of excessive metal ions in feces resulting in environmental accumulation of

these metals (Li et al., 2003). This concern has led to the development of organic sources of Zn and Cu that may have growth and health promoting effects as their inorganic counterparts. Supplementation of piglets diets with proteinates of Zn and Cu increased their levels in the liver compared with Zn and Cu sulphates indicating more bioavailability and possibly a reduced excretion of these metals (Schiavon et al., 2000). However, most studies showed that organic sources of Zn and Cu was not as efficacious in improving growth performance as ZnO (Hill et al., 2000; Hollis et al., 2005). Further studies should explore potentials of organic sources of Zn and Cu in enhancing growth performance and enteric health in piglets.

Dietary Protein

Both sources and level of dietary CP are known to influence enteric health in piglets (Pluske et al., 2002). This section will review the effect of dietary CP source and level on growth and GIT health in piglets.

Sources. Certain dietary components, for instance, leguminous plant proteins, are known to have a negative impact on growth and health of piglets during the period immediately after weaning. Diets based on legume (soybean meal (**SBM**), peas, faba beans, or blue lupin) reduced duodenal activities of most intestinal enzymes and total tract digestibility of energy and N compared with a diet based on casein (Salgado et al., 2002). Compared with dried skim milk, SBM resulted in transient hypersensitivity in early weaned pigs (Li et al., 1990). In that study, pigs fed the SBM diet had a lower rate of gain, shorter villi and a higher immunoglobulin titre to SBM indicating antigenic property of SBM. Other studies showed that SBM reduced chymotrypsin activities in

jejunal digesta (Makkink et al., 1994) and increased the incidence of diarrhea and appearance of ETEC in piglets (Shimizu and Terashima, 1982) compared with skim milk powder. On the contrary, other authors reported no differences in ETEC excretion and other indices of enteric health between pigs fed diets based on SBM and those fed diets based on dried skim milk (Pouteaux et al., 1982; Wellock et al., 2008a).

Processing of the plant protein sources has been shown to improve their nutritive values for piglets. For example, processed soybean products such as microbial-fermented SBM improved the growth performance, increased the number of intestinal lactobacilli, decreased the number of intestinal enterobacteria and increased VH and VH:CD ratio in piglets after weaning compared with conventional SBM (Kim et al., 2006; Wang et al., 2007). In another study, feeding extruded peas to piglets stimulated the activities of amylase, chymotrypsin and carboxypeptidase A in the pancreatic tissue and improved the apparent ileal digestibility of N and starch compared with raw peas {Freire, 1991 445 /id}. Other soybean products such as soybean protein concentrate, soy protein isolate and moist extruded soy protein concentrate reduced the antigenic effects associated with conventional SBM (Li et al., 1991). Further processing such as micronization and fine grinding did not improve the nutritional value of SBM for piglets (Valencia et al., 2008).

Generally, animal protein sources appear to have superior feeding value to plant protein sources when fed to weaned pigs partly due to the fact that proteins of plant origin are less digestible than animal proteins (Yu et al., 2002). This could be attributed to the structural characteristics of protein and/or carbohydrate and the presence of anti-nutritional factors in plant protein sources (Makkink et al., 1994; Salgado et al., 2002). Feeding animal protein sources, whey protein concentrate and fish meal, to piglets

resulted in better growth performance, nutrient digestibility and gut morphology compared with plant protein sources, SBM, fermented soy protein, and rice protein concentrate (Yun et al., 2005).

Also, differences in nutritive value are not uncommon when different protein sources of animal origin are fed to piglets. Vente-Spreeuwenberg et al. (2004) reported that pigs fed a diet containing skim milk powder had better growth performance and higher VH than those fed a diet containing hydrolyzed feather meal probably due to lower nutrient digestibility in the latter. In another study, piglets fed a diet containing fish meal plus lactose after weaning had better growth performance compared with those fed a diet containing dried whey (Lopes et al., 2004). Compared with fish protein, spray-dried plasma improved growth performance, reduced intestinal expression of TNF-α and interleukin-8 and prevented jejunal ulceration and edema in piglets challenged with ETEC K88 (Bosi et al., 2004). The authors concluded that spray-dried plasma protected against *E. coli*-induced inflammatory status and this was suggested to be due to the presence of immunoglobulins and glycoproteins in spray-dried plasma. Indeed, Owusu-Asiedu et al. (2002) demonstrated that spray-dried animal plasma products contain antibodies against ETEC.

Although, the effects of dietary protein sources on growth and gut health of pigs have been studied extensively, there is still paucity of information on effect of dietary protein sources on intestinal microbial composition. Generation of the available data was limited to culture-based technique (Wellock et al., 2008a). The use of molecular techniques in evaluating the modulatory effects of dietary protein sources on gut microbiology will facilitate the study of important but not culturable bacteria.

Considering the critical role of microbes in maintaining enteric health, availability of data on gut microbial composition and diversity as influenced by dietary protein sources will help to better manage weaner pig nutrition, especially in the absence of in-feed antibiotics.

Level. One of the main factors that may influence the proliferation of gut microflora is the availability of the required substrates (Wellock et al., 2006b). Many pathogens such as ETEC preferentially ferment protein and thus, manipulation of dietary CP level has been suggested to be an important nutritional strategy for reducing scours in weaned pigs (Macfarlane, 1995; Stein and Kil, 2006). Typical diets for weaned pigs contain high level of CP, usually 21 to 25%, and sub-therapeutic levels of antibiotics (Pluske et al., 2002; Stein and Kil, 2006). Considering the fact that apparent ileal protein digestibility in piglets ranged from 75 to 85%, high dietary CP level will increase the amount of undigested CP entering the hindgut and may, thus, promote the growth of opportunistic pathogens in the absence of in-feed antibiotics.

It has been consistently demonstrated in the literature that low CP diets improved the indices of gut health compared with high CP diets. For example, Prohaszka and Baron (1980) reported an increase in the hemolytic *E. coli* count in the SI of weaned pigs fed a 21% CP diet compared with those fed a 13% CP diet. Feeding low CP, AA-supplemented diets to piglets reduced the incidence of diarrhea compared with high CP diets (Wellock et al., 2006a; Yue and Qiao, 2008; Heo et al., 2008). On the other hand, other studies reported no differences in fecal consistency score of pigs fed a high CP diet and those fed low CP diets and this was suggested to be due to cleanliness of the research facility where the studies were conducted (Le Bellego and Noblet, 2002; Nyachoti et al., 2006). Indeed,

low CP, AA-supplemented diets reduced the incidence of diarrhea in piglets challenged with ETEC (Wellock et al., 2008a).

Data on the effect of dietary CP level on growth performance are controversial. Le Bellego et al. (2002) and Htoo et al. (2007) reported that there were no differences in growth performance of pigs fed the low CP, AA-supplemented diets and those fed the high CP diets. Similarly, Heo et al. (2008) demonstrated that feeding a 17% CP, AA-supplemented diet to weaned piglets for a duration of 5, 7, 10 or 14 d did not impair their growth performance up to 106 d post-weaning compared with a 24% CP diet. Conversely, other studies showed that growth performance was impaired in piglets fed low CP, AA-supplemented diets compared with those fed high CP diets (Nyachoti et al., 2006; Wellock et al., 2006a).

Differences in weaning age, age and BW at which dietary treatments were allotted, pig genotype, adaptation period, and dietary CP content classified as low and high could account for discrepancies in growth performance and indices of gut health observed among studies. For instance, the CP content of the low CP and high CP diets were 20 and 24%, respectively, for the study of Htoo et al. (2007) and 13 and 23%, respectively, for the study of Wellock et al. (2006a).

Mechanisms by which dietary CP level modulate enteric health in weaned pigs are mediated primarily through changes in microbial population and activities (Figure 7). High dietary CP content is expected to increase the amount of substrate available for the proliferation of pathogenic bacteria (Ball and Aherne, 1987) and subsequently favor

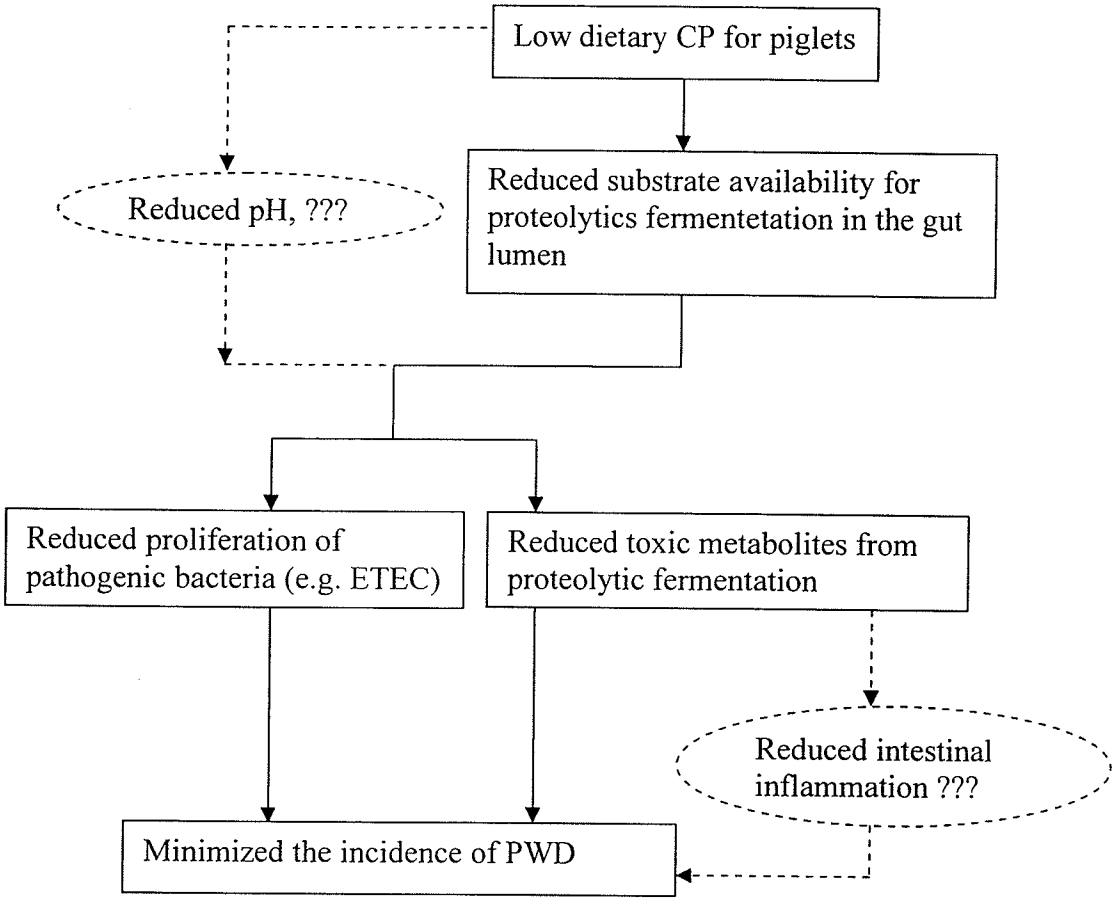

Figure 7. Schematic representation of the proposed mechanisms of action of low dietary CP content as a means of managing post-weaning diarrhea (PWD)

proteolytic fermentation and the associated production of toxic metabolites (Gaskins, 2001). High dietary CP content increased colon and fecal coliform population (Pierce et al., 2007), reduced fecal *Bifidobacteria* population (Wellock et al., 2006a) and increased fecal hemolytic *E. coli* level (Heo et al., 2008) in unchallenged, weaned piglets. Currently, our knowledge on the effect of dietary CP level on gut microflora is based on culture-based techniques. A large number of bacteria such as *Bacteriodes, Clostridium* and *Streptococcus* are capable of proteolytic fermentation (Gaskins, 2001). Utilization of culture-independent techniques in future research will increase our understanding of modulatory effects of dietary CP content on gut microbiology.

In terms of the proteolytic fermentation, low dietary CP level reduced the intestinal concentration of ammonia N (Bikker et al., 2006; Nyachoti et al., 2006), branched chain VFA, putrescine (Htoo et al., 2007) and cadaverine (Porter and Kenworth, 1969). These metabolites of proteolytic fermentation are known to be toxic to intestinal cells and may predispose piglets to PWD (Porter and Kenworth, 1969; Lin and Visek, 1991; Htoo et al., 2007). A reduction in the concentration of products of proteolytic fermentation in the SI and LI was probably achieved by a reduction in the ratio of protein to carbohydrate in the gut. This was demonstrated in the study of Pierce et al. (2007) who reported that high level of lactose reversed the elevated level of branched chain VFA in the feces of pigs fed a 21% CP diet. It has also been proposed that high dietary CP level support the growth of pathogenic bacteria such as ETEC by increasing the pH of the gut environment through its buffering capacity (Prohaszka and Baron, 1980). While this notion has been demonstrated by Wellock et al. (2008a) and Nyachoti et al. (2006), Htoo et al. (2007) reported no such effects.

Another potential mechanism of action of low dietary CP level is that of modulating the immune system. However, knowledge is still limited in this regard and requires further research. Houdijk et al. (2007) reported that plasma concentration of haptoglobin increased 3 d after ETEC infection in piglets fed a 23% CP diet but not in those fed a 13% CP diet. Since ETEC infection induces intestinal inflammation (Bosi et al., 2004), low dietary CP content may help to reduce inflammatory responses associated with ETEC infection by reducing substrate availability for ETEC proliferation. Further studies are also required for evaluating the effects of dietary protein level on diarrhea-associated responses at the intestinal level. This will provide insights on possible role of dietary protein level in modulating reponses associated with ETEC-induced diarrhea at the local site of infection.

SUMMARY AND PERSPECTIVES

Growth check and enteric diseases including PWD are the major concerns associated with the immediate post-weaning period in piglets. Post-weaning diarrhea represents a major source of revenue loss to the swine industry. Traditional practice of including sub-therapeutic levels of antibiotics in starter diets is very effective in mitigating the risk of PWD in piglets. However, growing concerns over a possible link between the emergence of antibiotic-resistant strains of bacteria and subtherapeutic usage of antibiotics in livestock diets led to a full ban of in-feed antibiotics in the EU and pressure to remove similar usage in other parts of the world. Antibiotic removal from swine diets increased the risk of infection, usage of therapeutic antibiotics, and production costs. To minimize production and economic consequences associated with

in-feed antibiotics removal from swine diets, a search for effective alternatives to antibiotics is imperative.

A number of nutritional strategies have been suggested as alternative means of enhancing post-weaning growth performance and controlling PWD in piglets. Manipulation of dietary protein content is one of such strategies. Indeed, low CP, AA-supplemented diet improves indicators of gut health in piglets. However, the effect of dietary CP level on growth performace is far from conclusive. Hence, there is a need to further investigate the effect of low CP, AA-supplemented diet on the performance and health of piglets especially those housed under conditions similar to those of the commercial production setting. The studies on the effect of dietary CP level on gut microbiology have been limited to culturable bacteria only. Utilization of culture independent techniques will expand our current understanding on how changes in dietary protein level affect gut ecology. Another area of research that deserves investigation is the effect of low protein, AA-supplemented diet on systemic and local (mucosal) immune system of piglets. A comprehensive understanding of how dietary CP level affect the physiology, immulogy, microbiology and environment of GIT is required to optimize its used as a dietary tool in mitigating enteric diseases in piglets especially in the absence of in-feed antibiotics .The outlined areas of further research will help to identify novel potential mechanisms of action of low CP, AA-supplemented diet in enhancing gut health in piglets.

CHAPTER 3

HYPOTHESIS AND OBJECTIVES

In this thesis, it was hypothesized that supplementation of low CP diets with crystalline AA, on the ideal protein basis, to meet the requirements of weaned pigs would improve their health status and minimize the impact of infection on their growth performance and gut health by:

1. reducing the amount of substrate available for the proliferation of pathogenic bacteria,
2. minimizing the severity of post-weaning diarrhea,
3. reducing ETEC-associated inflammatory responses, and
4. minimizing diarrhea-associated responses at the intestinal level.

The overall objective of this project was to evaluate the efficacy of low CP, AA-supplemented diets as a nutritional strategy of controlling PWD and enhancing GIT health in piglets.

Specific objectives of this project were:

1. to determine whether Ile supplementation, in addition to Lys, Met, Thr, and Trp, to a low CP diet is required to support growth performance of piglets after weaning. This objective was based on a previous study (Nyachoti et al., 2006) in

our laboratory suggesting that poor performance of pigs fed low CP diets supplemented with Lys, Met, Thr and Trp after weaning was probably due to deficiency and/or imbalance of other essential AA, especially Ile and Val.

2. to determine the effects of low CP, AA-supplemented diet on indices of GIT health in weaned pigs using incidence of diarrhea, microbial population and activities, and gut morphology as response criteria.
3. to assess the susceptibility of weaned pigs fed low CP, AA-supplemented diet, as opposed to a high CP diet, to ETEC K88 challenge using incidence and severity of scours, shift in GIT microbial population (culture and culture-independent techniques), immunological responses and growth performance as response criteria.
4. to determine the effects of dietary CP level on gut development as indicated by intestinal brush border enzymes.
5. to evaluate the effect of dietary CP level on diarrhea-associated responses in the SI of pigs induced experimentally with PWC.

CHAPTER 4

MANUSCRIPT 1

Effect of low-protein amino acid-supplemented diets on the growth performance, gut morphology, organ weights and digesta characteristics of weaned pigs.

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ABSTRACT

A 21-d study was conducted to determine whether Ile might limit the performance of piglets fed low CP, AA-supplemented diets and to investigate the potential benefits of low CP diets on gastrointestinal health in weaned pigs. Ninety-six piglets (initial BW = 6.44 ± 0.14 kg), housed 4 per pen, were randomly assigned to one of 4 diets resulting in 6 replicate pens per diet. Dietary treatments were: 1) 21% CP diet, 2) 19% CP diet deficient in Ile, 3) 19% CP diet supplemented with crystalline Ile up to the level in the 21% CP diet, and 4) 17% CP diet supplemented with Ile and Val on the ideal protein ratio basis (60 and 70% relative to Lys, respectively). Pigs were allowed to adapt to the new environment for 4 d before the experiment commenced. Overall, pigs fed the 21% CP diet had higher ($P < 0.05$) ADG and higher ($P < 0.05$) G:F ratio compared with those fed the other diets. Fecal consistency score of pigs fed the 21% CP diet was higher ($P < 0.05$) than those fed the other diets. Pigs fed the 17% CP diet had lower ($P = 0.02$) small intestine weight than those fed the 21% CP diet. Pigs fed the 21% CP diet had deeper ($P < 0.05$) crypt in the duodenum and ileum and higher ($P < 0.05$) ammonia N concentration in cecal digesta than those fed the other diets. There were no effects of diet on microbial population and VFA concentration in the cecal digesta except for propionic acid whose concentration was higher ($P < 0.05$) for pigs fed the 17% CP diet than those fed the 19% CP plus Ile and the 21% CP diets. The results indicate that the low CP, AA-supplemented diet reduced crypt hypertrophy, ammonia N concentration in the cecal digesta, small intestine weight and the performance of piglets. Also, the results of the current study were inconclusive with respect to whether Ile may limit the performance of pigs fed a low CP, AA supplemented diet.

Key words: amino acids, dietary protein, growth performance, intestinal morphology, weaned pig

INTRODUCTION

The period following weaning is usually characterized by low feed intake and poor weight gain (van Dijk et al., 2001) partly due to the sudden transition from a nutritious and readily digestible sow milk to dry feed. The poor performance associated with this period has been addressed by feeding diets containing high levels of CP supplemented with in-feed antibiotics as growth promoters. However, the recent ban of antibiotics usage in livestock diets in Europe as well as the ongoing interest to eliminate similar usage in North America and other parts of the world has led to exploration of other nutritional and management strategies to reduce the incidence and severity of digestive problems associated with weaning.

It is well documented that dietary CP levels play an important role in the health status of the weaned pigs (Gu and Li, 2004; Nyachoti et al., 2006; Wellock et al., 2006b). High levels of dietary CP could increase the amount of substrate available for proliferation of pathogenic bacteria that often colonize the pig's gut after weaning and, therefore, may be a predisposing factor to post-weaning diarrhea (**PWD**) (Ball and Aherne, 1987; Nollet et al., 1999). Thus, low CP, AA-supplemented diets may be used as part of the overall strategy for the nutritional management of the weaned pig. In fact, Le Bellego and Noblet (2002) reported that the performance of pigs (12 kg initial BW) fed

low CP, AA-supplemented diets was similar to that of pigs fed a high CP diet. However, Nyachoti et al. (2006) demonstrated that piglet (6.2 kg initial BW) performance may be impaired when dietary CP levels are reduced from 23% to 19% or 17% and suggested that essential AA, other than those normally added in pure form (Lys, Met, Thr, and Trp), especially Ile and Val, may limit the performance of pigs fed low CP diets. A similar observation was made by Kerr et al. (2004) who reported that Ile requirements of pigs fed a low protein diet was higher (0.61%) than the NRC (1998) value of 0.55%. Since deficiency of any essential AA may limit growth performance, we hypothesized that in addition to Lys, Met, Thr, and Trp, supplementation of Ile to a moderately reduced CP diet and Ile and Val to a low CP diet on an ideal protein basis will improve the performance of piglets, especially those weaned at an early age. Therefore, the objectives of this study were: 1) to further determine the growth performance of piglets fed low CP, AA-supplemented diets; 2) to determine whether Ile may limit the performance of piglets under a low CP, AA-supplemented feeding program; and 3) to further evaluate potential benefits of low CP diets on indicators of gut health.

MATERIALS AND METHODS

All experimental protocols were reviewed and approved by the University of Manitoba Animal Care Committee (Protocol # F05-024) and pigs were cared for according to the standard guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Pigs and Housing

A total of 96 crossbred Duroc × (Yorkshire × Landrace) piglets with an initial BW of 6.44 ± 0.14 kg and weaned at d 17 ± 2 were obtained from a commercial source. Pigs were weighed, balanced for BW and sex, and then assigned randomly to experimental treatments. Pigs were housed in groups of 4 (2 barrows and 2 gilts) in plastic covered, expanded metal floor pens (1.5 m × 1.16 m) equipped with a stainless steel feeder and a low-pressure nipple drinker and were allowed to adapt to the new environment for 4 d before the inception of the experiment. During the adaptation period, pigs received a non-medicated adaptation diet formulated to contain 22% CP and 10.7 MJ/kg NE and to meet or exceed requirements for other nutrients recommended by NRC (1998). Room temperature was maintained at 30°C during wk 1 of the experiment and then reduced by 1.5°C over the subsequent wks. Lights were on from 0700 to 2300. The experiment lasted 3 wks.

Experimental Diets and Performance Assessment

Four non-medicated diets based on corn, wheat, and soybean meal (**SBM**) were used in the current study (Table 5). Diets differed in CP content but were balanced for NE (Degussa feed composition data) and standardized ileal digestible Lys, Met + Cys, Thr, and Trp based on the ideal protein ratio (60, 62 and 20 for Met + Cys, Thr, and Trp, respectively) suggested by Rademacher et al. (2000). The dietary treatments were: 1) a high-protein, 21% CP control, 2) 19% CP, Ile deficient, 3) 19% CP supplemented with Ile up to the level in the 21% CP diet and 4) 17% CP supplemented with Ile and Val

Table 5. Composition of experimental diets, as fed basis

Item	Diet[1]			
	1	2	3	4
Ingredient, %				
Corn	31.82	39.64	39.90	60.98
Soybean meal, 48% CP	10.65	4.06	3.83	-
Wheat	30.00	30.00	30.00	13.55
Spray dried porcine plasma	4.00	4.00	4.00	4.00
Whey powder	10.00	10.00	10.00	10.00
Fish meal	6.00	6.00	6.00	6.00
Canola oil	4.22	2.46	2.31	0.52
Limestone	0.78	0.75	0.75	0.71
Biofos[2]	0.50	0.61	0.61	0.74
Vitamin and mineral premix[3]	1.00	1.00	1.00	1.00
Biolys®[4]	0.67	0.96	0.97	1.19
DL-Met	0.18	0.23	0.23	0.30
L-Thr	0.13	0.21	0.22	0.29
L-Trp	0.05	0.08	0.08	0.12
L-Ile	-	-	0.10	0.31
L-Val	-	-	-	0.29
Calculated nutrient content[5]				
NE, MJ/kg	10.70	10.70	10.70	10.70
CP, %	21.00	19.00	19.00	17.00
Lys, %	1.49	1.47	1.47	1.46
Met, %	0.51	0.53	0.53	0.57
Met + Cys, %	0.90	0.90	0.90	0.89
Thr, %	0.95	0.93	0.93	0.93
Trp, %	0.30	0.30	0.30	0.30
Ile, %	0.79	0.68	0.77	0.88
Leu, %	1.61	1.45	1.45	1.34

Val, %	0.99	0.88	0.86	1.04
His, %	0.52	0.46	0.46	0.41
Phe, %	0.72	0.60	0.60	0.49
Standardized ileal digestible AA, %				
Lys	1.35	1.35	1.35	1.35
Met + Cys	0.81	0.81	0.81	0.81
Thr	0.84	0.84	0.84	0.84
Trp	0.27	0.27	0.27	0.27
Ile	0.70	0.60	0.70	0.81
Val	0.86	0.77	0.77	0.95
Ca, %	0.85	0.85	0.85	0.85
Total P, %	0.66	0.67	0.67	0.67
Ca:P	1.28	1.28	1.28	1.28
Total NSP, g/kg of DM[6,7]	89.70	83.00	82.70	75.30
Starch, g/kg of DM[7]	417.70	469.90	471.60	509.00
Dietary fiber, g/kg of DM[7]	100.60	93.70	93.40	84.60

[1]1 = 21% CP diet; 2 = 19% CP diet; 3 = 19% CP diet plus crystalline Ile; 4 = 17% CP diet plus crystalline Ile and Val.

[2]Ca, 17%; P, 21% (Feed-Rite, Winnipeg, Manitoba, Canada).

[3]Supplied the following per kg of diet: 8,255 IU of vitamin A, 1,000 IU of vitamin D_3, 20 IU of vitamin E, 25 μg of vitamin B_{12}, 1.5 mg of vitamin K, 30 mg of niacin, 781 mg of choline chloride, 7.5 mg of riboflavin, 200 μg of biotin, 4.5 mg of pyridoxine, 1 mg of folic acid; 4 mg of thiamin, 40 mg of Mn (as MnO), 130 mg of Zn (as ZnO), 130 mg of Fe (as $FeSO_4.H_2O$), 10 mg of Cu (as CuO), 0.30 mg of Se (as Na_2SeO_3), 0.6 mg of I (as $Ca(IO_3)_2$).

[4]Contains 50.7% L-Lysine

[5]Based on analyzed AA content in feed ingredients and digestibility coefficients reported by Rademacher et al. (2000) and Degussa feed composition data for ME, Ca, and P.

[6]NSP = non-starch polysaccharides.

[7]Based on data reported by Bach Knudsen (1997).

according to the ideal protein ratio (60 and 70 for Ile and Val, respectively). Feed ingredients supplying CP and AA were analyzed for CP and AA content and the analyzed values used in diet formulation. The different dietary CP levels were achieved by using different combination of corn, wheat and soybean meal and by replacing soybean meal with crystalline AA supplied by Evonik Industries (Hanau-Wolfang, Germany). All other nutrients were supplied in amounts meeting or exceeding the NRC (1998) recommendations for a 6- to 10-kg pig.

Pigs had unlimited access to feed and water throughout the experimental period and diets were fed in a mash form. Body weight and feed disappearance were monitored weekly to determine ADG, ADFI, and G:F ratio.

Fecal Consistency Scoring

Severity of PWD was assessed using the fecal consistency (**FC**) scoring system recently used by Nyachoti et al. (2006). Fecal consistency scoring (0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea) was performed on d 4, 5 and 11 of the experiment by 2 trained individuals with no prior knowledge of treatment allocation. Feces were scored based on the visual observation of the pen and the area around the anus of the pigs. The FC by the 2 scorers was averaged to determine each day's value and the 3-d average was used to determine overall FC score.

Tissue Sampling and Histological Assessment

At the end of wk 3, 1 pig representing the average BW of each pen (6 pigs/treatment), was held under general anesthesia with Isoflurane. Once a plane of

surgical anesthesia was attained, two 10-cm adjacent segments were obtained from duodenum (5 cm away from pyloric junction), jejunum (10 cm distal of ligament of Treitz) and ileum (10 cm proximal of ileo-cecal junction) for histological measurement and adherent bacterial count. Pigs were euthanized by an intra-cardiac injection of sodium pentobarbital (55 mg/kg BW; Bimeda-MTC Animal Health Inc., ON, Canada). The first segment obtained from each section of the small intestine (**SI**) was fixed in 10% buffered formalin for histological measurements. The second segment was cut open longitudinally along the anti-mesenteric attachment plane; rinsed with sterile ice-cold physiological saline (0.9% saline) to remove any debris and then the mucosal tissue was scraped with a blunt sterile blade into a sterile tube, frozen immediately in liquid N and stored at -80˚C until cultured for adherent bacteria (14 d after sample collection). Following euthanasia, stomach, spleen, liver and the remaining parts of the SI were removed and weighed. All tissue samples were rinsed with ice-cold physiological saline (0.9% saline) and blotted dry with absorbent paper before the weights and length (SI only) of the organs were determined. The stomach and SI were emptied of any digesta before being weighed.

The formalin fixed segments were used for histological measurements as previously described (Owusu-Asiedu et al., 2002). Briefly, 6 cross-sections were obtained from each formalin-fixed sample and processed for histological examination using the standard Hematoxylin and Eosin method. Villus height (**VH**) and crypt depth (**CD**) were measured on 10 intact, well-oriented villi per specimen using a Nikon YS100 compound light microscope equipped with a Sony DSP 3CCD color video camera. Images were captured and processed using Northern Eclipse Image Processing Software version 6.0

(Empix Imaging, Inc, Mississauga, ON, Canada). The VH was measured from the tip of the villus to the crypt-villus junction and the depth of the crypt from the crypt-villus junction to the base.

Digesta Characteristics and Gut Microbial Population

Luminal content of cecum was collected into 2 separate sterile sample bags. One sample was snap frozen in liquid N and stored at -80˚C until analysed for ammonia N and VFA and the other was placed on ice and used immediately for bacterial count. Ammonia N concentration in digesta samples was determined as reported by Nyachoti et al. (2006) using the method described by Novozamsky et al. (1974). Volatile fatty acid concentration was determined using gas chromatography (Varian Chromatography Systems, Model Star 3400, Walnut Creek, CA, USA) according to the method described by Erwin et al. {Erwin, 1961 402 /id /d}.

For bacterial count, 1 g sample of cecum luminal content was diluted in sterile peptone water (0.1%, 9 mL) and then serially diluted using sterile PBS (pH 6.8). The diluted samples were analysed for aerobic and anaerobic sporeformers, *Enterobactericeae*, enterococci, *Escherichia coli*, and total coliforms as described by Nyachoti et al. (2006). Scraped mucosal samples (1 g each) were serially diluted as for the luminal samples and analysed for adherent total coliform and lactobacilli using Violet Red Bile (35°C, 34 h) and deMan Rogosa Sharpe agar (30°C, 24-48 h), respectively.

Other Chemical Analyses

Ingredients and diets were ground through a 1-mm screen (Cyclotec 1093, Tecator, Hogana, Sweden) and then analysed for AA composition by Degussa AG as described by Nyachoti et al. (2006).

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary NC). The pen was considered the experimental unit for performance and FC score data while the individual piglet was used as the experimental unit for other response criteria. The effects of gender and dietary treatment and the interaction between gender and dietary treatment were included in the statistical model as sources of variation. There were no effects of gender and interaction between gender and treatment in all the analyses, therefore, only the effect of dietary treatment was included in the model for the final analysis. The initial BW was used as a covariate in the analysis of the performance data. Treatment means were compared using Fisher's protected least significant difference procedure. Association between ammonia N and FC or CD was determined with Pearson's correlation coefficient. Statistical significance was accepted at $P < 0.05$ and $P \leq 0.10$ was accepted as a trend.

RESULTS

General

The analyzed CP and AA contents in the experimental diets (Table 6) were similar to the calculated values derived from the analyzed composition of individual feed ingredient except for CP and some AA in the 19% + Ile diet that were slightly lower than the calculated values.

Performance

Growth performance of the piglets during the 3-wk study period and FC score are shown in Table 7. Pigs fed the 21% CP diet had higher ($P < 0.05$) overall ADG and G:F compared with those fed the other diets. Overall ADFI of pigs fed the 17% CP diet was higher ($P < 0.05$) compared with other dietary treatments. Overall FC score of pigs fed the 21% CP diet was higher ($P < 0.05$) compared with other dietary treatments

Organ Weights and SI Morphology

There were no effects of dietary treatment on organ weights except for a tendency for a reduction ($P = 0.11$) in empty SI weight (expressed as a percent of final BW) as dietary CP level was reduced (Table 8). Pigs fed the 17% diet had lower ($P = 0.02$) SI weight than those fed the 21% CP diet. Piglets fed the 21% CP diet had deeper ($P < 0.05$) crypt in ileum compared with those fed the other diets (Table 8). The crypt tended to be deeper ($P < 0.10$) in duodenum and jejunum of pigs fed the 21% CP diet compared with those fed the other diets.

Table 6. Analyzed crude protein (CP) and amino acids (AA) composition of experimental diets, as fed basis

	Diet[1]			
Item, %	1	2	3	4
CP	21.3	19.1	18.7	17.0
Indispensable AA				
Arg	1.14	0.95	0.93	0.77
His	0.51	0.45	0.44	0.38
Ile	0.83	0.68	0.79	0.88
Leu	1.65	1.47	1.44	1.35
Lys	1.55	1.50	1.44	1.50
Met	0.53	0.53	0.52	0.57
Phe	0.94	0.80	0.80	0.67
Thr	0.96	0.93	0.87	0.92
Trp	0.32	0.31	0.30	0.30
Val	1.03	0.87	0.89	1.05
Dispensable AA				
Ala	1.02	0.93	0.90	0.87
Asp	1.74	1.43	1.38	1.20
Cys	0.39	0.36	0.35	0.31
Glu	3.79	3.33	3.35	2.58
Gly	0.92	0.81	0.79	0.70
Pro	1.35	1.25	1.23	1.04
Ser	0.93	0.84	0.77	0.69

[1]1 = 21% CP diet; 2 = 19% CP diet; 3 = 19% CP diet plus crystalline Ile; 4 = 17% CP diet plus crystalline Ile and Val.

Table 7. Performance of piglets fed different levels of dietary protein supplemented with amino acids[1]

Item	Diet[2]				SEM
	1	2	3	4	
Initial BW, kg	6.40	6.37	6.58	6.43	0.14
Final BW, kg	11.73^{a}	11.15^{b}	11.37^{ab}	11.23^{ab}	0.17
ADG, g/d					
d 1-7	119	131	102	134	9.98
d 7-14	339^{a}	288^{bc}	319^{ab}	254^{c}	12.81
d 14-21	354	306	315	351	15.08
Overall	266^{a}	239^{b}	237^{b}	240^{b}	6.38
ADFI, g/d					
d 1-7	201	202	209	229	10.80
d 7-14	434^{ab}	408^{a}	428	453	11.71
d 14-21	524	519	552	552	13.55
Overall	379^{a}	369^{a}	380^{a}	404^{b}	7.46
G:F					
d 1-7	0.59^{a}	0.65^{a}	0.49^{b}	0.58^{ab}	0.03
d 7-14	0.78^{a}	0.71^{a}	0.74^{a}	0.56^{b}	0.03
d 14-21	0.67^{a}	0.59^{b}	0.57^{b}	0.64^{ab}	0.02
Overall	0.70^{a}	0.65^{b}	0.62^{bc}	0.60^{c}	0.01
FC Score[3]					
d 4	1.92^{a}	1.08^{b}	1.00^{b}	0.50^{b}	0.28
d 5	1.50^{ab}	0.92^{bc}	1.75^{a}	0.58^{c}	0.23
d 11	0.92^{a}	0.50^{ab}	0.17^{bc}	0.00^{c}	0.16
Overall	1.45^{a}	0.78^{bc}	0.98^{b}	0.37^{c}	0.15

[1]Each value represents the mean of 6 pens.

[2]1 = 21% CP diet; 2 = 19% CP diet; 3 = 19% CP diet plus crystalline Ile; 4 = 17% CP diet plus crystalline Ile and Val.

[3]FC= fecal consisteny; 0 = normal; 1 = soft; 2 = mild diarrhea; 3 = severe diarrhea.

[a,b,c]Means within a row bearing different letters are different ($P < 0.05$).

Table 8. Organ weights and small intestine morphology of piglets fed different levels of dietary protein supplemented with amino acids[1]

Item	Diet[2]				SEM
	1	2	3	4	
Final BW, kg	11.7	11.2	11.4	11.2	0.17
Organ weights, % final BW					
Liver	3.48	3.16	3.17	3.19	0.14
Spleen	0.33	0.29	0.32	0.28	0.03
Stomach	0.97	0.97	0.89	1.00	0.07
Small intestine	5.34^{a}	4.95^{ab}	5.01^{ab}	4.37^{b}	0.27
Morphological measurements[3]					
Duodenum					
VH	437	424	486	442	19.14
CD	488	427	427	420	18.67
VH:CD	0.90	1.02	1.15	1.06	0.07
Jejunum					
VH	470	460	487	431	36.78
CD	632	540	566	534	24.94
VH:CD	0.76	0.85	0.88	0.82	0.08
Ileum					
VH	412	407	416	425	19.00
CD	377^{a}	313^{b}	300^{b}	313^{b}	16.42
VH:CD	1.10	1.31	1.41	1.40	0.10

[1]Each value represents the mean of 6 pens.

[2]1 = 21% CP diet; 2 = 19% CP diet; 3 = 19% CP diet plus crystalline Ile; 4 = 17% CP diet plus crystalline Ile and Val.

[3]VH = villus height; CD = crypt depth.

[a,b]Means within a row bearing different letters are different ($P < 0.05$).

Cecum Luminal Ammonia N and VFA Concentration and Gut Microbial Population

Effect of dietary treatment on cecum luminal ammonia N and VFA concentration is shown in Table 9. Pigs fed the 21% CP diet had higher ($P < 0.05$) cecum luminal ammonia N concentration compared with those fed the other diets. Ammonia N correlated positively to the overall FC ($R^2 = 0.47$, $P = 0.06$) and jejunum CD ($R^2 = 0.61$, $P = 0.01$). There was no effect of diet on VFA concentration except for pigs fed the 17% CP diet that had higher ($P < 0.05$) propionic acid concentration than those fed the 19% CP plus Ile and the 21% CP diet.

There was no effect of diet on adherent jejunal and ileal and cecal luminal microfloral count. Adherent ileal lactobacilli population averaged 4.6, 4.7, 5.1 and 4.3 $\log_{10}$ cfu/g (SEM = 0.58) for the 21% CP; 19% CP, Ile deficient; 19% CP; and 17% CP diets, respectively. Adherent jejunal and ileal total coliforms were not detected at 10^2 cfu/g. The total coliforms count in the cecal luminal content averaged 5.31, 5.97, 5.37 and 5.31 $\log_{10}$ cfu/g (SEM = 0.31) for the 21% CP; 19% CP, Ile deficient; 19% CP; and 17% CP diets, respectively.

DISCUSSION

It was hypothesized in the current study that low CP diets, supplemented with Lys, Met, Thr, Trp, Ile and Val will support performance of weaned pigs similar to that supported by a high CP diet. Contrary to the hypothesis, overall performance of pigs fed the low CP diets compared to those fed the high CP diet was reduced. This observation is in agreement with the study of Pierce et al. (2007) and Wellock et al. (2006a) who also

Table 9. Ammonia nitrogen and volatile fatty acid (VFA) concentration in the cecum of piglets fed different levels of dietary protein supplemented with amino acids

Item	Diet[1]				SEM
	1	2	3	4	
N	5	6	6	5	
Ammonia N, mg/L	125.7[a]	94.9[b]	80.4[bc]	64.2[c]	9.76
VFA, mmol/L					
Acetic	35.5	41.8	43.1	47.6	4.32
Propionic	17.6[a]	23.4[ab]	21.6[a]	31.5[b]	2.95
Butyric	12.7	8.7	10.7	9.9	2.36
Branched chain VFA[2]	0.4	0.3	0.5	0.6	0.12
Total VFA	68.7	76.5	78.9	93.2	7.65

[1]1 = 21% CP diet; 2 = 19% CP diet; 3 = 19% CP diet plus crystalline Ile; 4 = 17% CP diet plus crystalline Ile and Val.

[2]The branched chain VFA are valeric, isobutyric and isovaleric fatty acids.

[a,b,c]Means within a row bearing different letters are different ($P < 0.05$).

reported reduced ADG and G:F with a reduction in dietary CP level. The analyzed composition of experimental diets used in the current study showed that the 19% CP plus Ile diet was slightly lower in CP and some AA (Lys inclusive) than the formulated value. However, the 17% CP diet whose analyzed CP and AA profile were not different from the formulated values still performed poorly compared with the 21% CP control diet. Hence, it could be argued that the lower analyzed AA value relative to the calculated value in the 19% CP plus Ile diet was not solely responsible for the poor performance of pigs fed this diet. Compared with the other diets, the 21% CP diet contained a higher level of canola oil which is rich in omega 3 fatty acids. Omega 3 fatty acids have anti-inflammatory properties and could thus reduce the severity of intestinal inflammation associated with weaning stress (McCracken et al., 1999; Simopoulos, 2002; Pie et al., 2004).

Poor performance of pigs fed the low CP diets compared to those fed the high CP diet despite supplementation with Ile and Val suggests that other AA and /or other nutrients may have been deficient. Also, the lack of difference between the performance of pigs fed the 19% CP diet with and without Ile supplementation shows that other AA apart from Ile may be limiting in a low CP diet. However, it is possible that the lower levels of some AA determined in the 19% CP + Ile diet compared with the calculated values may have contributed to this observation. It has been suggested that supplementation of swine diets with some essential AA can make other essential AA limiting (Boisen et al., 2000) and that an attempt to reduce CP level in weaned pig diets could result in a deficiency of other indispensable AA apart from Lys, Met, Thr and Trp that are usually added to swine diets (Figueroa et al., 2002; Stein and Kil, 2006). This is

because swine diets are usually formulated using the ideal protein concept in which all indispensable AA requirements are expressed relative to the requirement of one individual AA (lysine) or protein (Boisen et al., 2000). Supplementation of diets with the first four limiting AA can alter the relative proportion of other indispensable AA to lysine and make them deficient. Since most indispensable AA are not commercially available, formulation of weaned pig diets to be moderately low in CP should be done carefully to ensure that all the essential AA requirements of the animals are met and contained at least the recommended ideal protein ratio. A moderate reduction in dietary CP with appropriate dietary levels of all indispensable AA should support similar performance of weaned pigs as high CP diet but further studies will be required to develop such diets.

The performance of pigs in the current study is contrary to the findings of Le Bellego and Noblet (2002) who reported similar performance for the pigs (weaned on d 28; adapted to the housing and experimental conditions for 12 d; 12 kg BW) fed low and high CP diets. This discrepancy could be explained by differences in the weaning age and the age and BW at which dietary treatment were allotted to the pigs used in the two experiments. Pigs weaned at 4 wks adapt to the environment and diet more quickly than those weaned at 2 wks of age (Leibbrandt et al., 1975). Likewise, Callesen et al. (2007) reported that pigs weaned at 33 d of age grew better than those weaned at 27 d of age. This observation suggests that weaning age plays an important role in maintaining growth performance of weaned pigs under a low CP, AA-supplemented feeding regimen. In addition, the essential AA requirements of the pigs used by Le Bellego and Noblet (2002) might have been lower, because they were heavier and older than those used in the

current study, such that a reduction in dietary CP did not result in a deficiency of any essential AA.

Although it has been suggested that low CP diets supplemented with crystalline AA could be used as part of overall strategy for reducing the incidence and severity of PWD in piglets, results from the literature have not consistently supported this opinion (Le Bellego and Noblet, 2002; Nyachoti et al., 2006). The FC score was significantly reduced with the 17% and 19% CP diets compared with the 21% CP control diet which is similar to the results of Wellock et al. (2006a). The discrepancies between studies on the effect of dietary CP on FC score may be due to differences in the pig genotypes, weaning age, age and BW of pigs during the experimental period, diet composition, adaptation period and level of infection. For example, pigs used in Nyachoti et al. (2006) study were adapted to a post-weaning diet for 7 d. Likewise, pigs used in Le Bellego and Noblet (2002) study were adapted to housing and experimental conditions for 12 d. These long adaptation periods compared with the 4 d used in the current experiment might have masked any dietary effects on FC scores.

It has been suggested that a severe reduction in dietary CP levels could result in poor gastrointestinal tract growth and development (Nunez et al., 1996) and that excess dietary protein might increase visceral organ weight (Le Bellego and Noblet, 2002). In the current study, the empty SI weight of pigs fed the 17% CP diet was about 21% lower than that of those fed the 21% CP diet. It does not appear that the low CP diets supplemented with AA in the current study impaired gastrointestinal tract development because gut morphology was not impaired compared with the high CP diet.

Pigs fed the 21% CP diet had or tended to have deeper crypts in all the sections of the SI evaluated compared with other dietary treatments. This finding is generally in agreement with Gu and Li (2004) who reported a linear increase in CD in the jejunum, but not in the duodenum and ileum, with increase in dietary CP level. Crypt hypertrophy may be an indication of insult to the gut which could be in the form of pathogen colonization, feed antigen or toxic microbial metabolic products (Li et al., 1991; Tang et al., 1999). High levels of SBM in weaned pigs' diet have been implicated with alterations in gut architecture (Dunsford et al., 1989; Li et al., 1990). Soybean meal composition in the 21% CP diet was 10%, an amount which is lower than over 30% inclusion level that have been associated with hypersensitivity reaction in weaned pigs. In a study by Jiang et al. (2000), there were no differences in the CD, VH and crypt cell proliferation in weaned pigs (14-d old) fed diets based on 15% SBM and those fed 10% porcine plasma. As a result, the 10% inclusion level of SBM in the 21% CP diet is not expected to have contributed significantly to the deeper crypts observed in pigs fed this diet

It was hypothesized in the current study that feeding weaned piglets a diet with a high CP level could provide substrate for proliferation of pathogenic bacteria and hence, promote production of toxic metabolic products. However, there was no effect of dietary treatment on measured cecal microbial population which is in agreement with the results of Nyachoti et al. (2006) but contrary to that of Pierce et al. (2007) and Wellock et al. (2006a) who reported lower fecal *bifidobacteria* and a higher fecal and colon coliform population, respectively, as the dietary CP level increased. It is also important to note that the different dietary CP levels in the current experiment were achieved by altering the ratios of corn, wheat and SBM in the diets and by replacing SBM with crystalline AA. As

a result, the levels of dietary fiber, starch and non-starch polysaccharides, which are known to affect microbial activities, in the experimental diets changed with differences in CP content (Table 5). Hence, the current results should be interpreted with caution. Furthermore, similar to the findings of Nyachoti et al. (2006), ammonia N concentration in cecal digesta was higher in pigs fed the high CP diet compared with those fed the low CP diets. Not only could a high ammonia N concentration cause inefficient use of dietary energy by increasing the amount of maintenance energy required for its detoxification and excretion, but could also cause insult to gut structure and, hence, impair gut function (Lin and Visek, 1991; Jensen, 1998). The results of the current experiment showed a positive correlation between ammonia N in the cecum digesta and FC or jejunal CD such that the pigs with the highest concentration of ammonia N in the cecum digesta also had the highest overall FC score and deepest crypt in the jejunum.

In conclusion, low protein diets supplemented with Lys, Met + Cys, Thr, Trp, Ile and Val in the current study reduced crypt hypertrophy, ammonia N concentration and the performance of pigs over the 3 wks period. Based on the results of the current experiment, the possible effect of Ile supplementation on the performance of weaned piglets is inconclusive and should be investigated further.

CHAPTER 5

MANUSCRIPT 2

Effect of dietary protein level on growth performance, indicators of enteric health and gastrointestinal microbial ecology of weaned pigs induced with post-weaning colibacillosis

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ABSTRACT

The effect of dietary CP level on performance, enteric health and gastrointestinal microbial ecology of weaned pigs challenged with enterotoxigenic *Escherichia coli* (**ETEC**) K88 was investigated in a 14-d study. Forty weaned pigs (BW = 5.32 ± 0.24 kg, mean ± SD), housed 4 per pen, were randomly assigned to 2 diets (5 pens/diet): 1) high, 22.5% CP or 2) low, 17.6% CP supplemented with AA. Diets contained the same amount of ME and standardized ileal digestible Lys, Met + Cys, Thr, Trp based on the ideal protein ratio. Isoleucine and Val were added to the 17.6% CP diet up to the level in the 22.5% CP diet. On d 8 post-weaning, pigs were challenged with 6 mL of ETEC suspension (10^{10} cfu/mL) by gavage. Feed disappearance and BW were measured on d 7, 9, 10, 12 and 14 for determination of ADG, ADFI and G:F. One pig from each pen was serially slaughtered on -1, 3 and 7 d post-challenge (10 pigs/d of slaughter) to evaluate gut morphology and gut microbial ecology and metabolites. Pigs fed the high CP diet had higher ($P < 0.05$) ADG and G:F than those fed the low CP diet prior to infection but performance was similar between the 2 diets after ETEC challenge and overall. On d 3 after challenge, ETEC was not detected in the ileal digesta of pigs fed the low CP diet but was detected in the ileal digesta of 80% of pigs fed the high CP diet (5.22 ± 1.07 cfu/g, mean ± SD). Pigs fed the low CP diet had a higher ($P < 0.01$) prevalence of order Clostridiales (73 vs. 50%), family Lachnospiraceae (43 vs. 18%), and genus Roseburia (13 vs. 3%) in the colon digesta 7 d after challenge compared with those fed the high CP diet. The richness and diversity of bacteria in the colon digesta were lower ($P < 0.05$) in pigs fed the low CP diet compared with those fed the high CP diet at -1, 3 and 7 d post-challenge. Pigs fed the high CP diet had higher ($P < 0.05$) ammonia N concentration in

the colon digesta on -1 and 7 d after challenge compared with those fed the low CP diet. Pigs fed the high CP diet had deeper ($P < 0.05$) crypts 1 d before challenge, shorter villi 3 d after challenge, and lower villus height:crypt depth 1 d before and 3 d after challenge compared with those fed the low CP diet. In conclusion, a reduction in the dietary CP level of weaned pigs from 22.5% to 17.6% with AA supplementation impaired growth performance before but not after the ETEC challenge and increased the relative composition of butyrate producing bacteria in the colon digesta after ETEC challenge.

Key words: gut ecology, performance, protein, weaned pigs.

INTRODUCTION

There is a growing need to develop alternative interventions to managing the poor growth performance and enteric disorders associated with weaning of piglets in view of the growing concerns regarding the use of antibiotics in animal feeds. Thus, it has been suggested that feeding low CP, AA supplemented diets to weaned pigs may serve as part of such interventions as long as performance is maintained (Nyachoti et al., 2006). Indeed, studies have shown improved gastrointestinal (**GIT**) health when low CP, AA supplemented diets are fed and that performance can be maintained (Manuscript 1; Heo et al., 2008).

However, to rigorously test the potential benefits of using low CP, AA supplemented diets as a nutritional strategy for managing weaned piglets, studies under conditions that are closer to commercial production conditions, where there is a high

pressure of enteric infection (Goransson et al., 1995), are critical. We have used the enterotoxigenic *Escherichia coli* (**ETEC**) challenge model to test the efficacy of various alternatives to antibiotics in weaned piglets (Owusu-Asiedu et al., 2003; Bhandari et al., 2008; Kiarie et al., 2008b). Recently, Wellock et al. (2007; 2008b) have shown that pigs fed a high CP diet have higher daily gain and better feed efficiency compared with those fed a low CP diet. However, in that study a very low level (13%) of dietary CP was used, which might have made some essential AA (**EAA**) to become limiting and thus, impairing growth performance. Furthermore, evaluation of the effects of dietary CP level on gut microbial composition of nursery pigs has been limited to culture-based techniques with no information on non-cultivable microorganisms. Therefore, the objective of this study was to evaluate the effect of dietary CP level on performance, indicators of enteric health, and gut microbial ecology in weaned piglets induced with post-weaning colibacillosis (**PWC**) using ETEC K88.

MATERIALS AND METHODS

The experimental protocol was approved by the Protocol Management and Review Committee of the University of Manitoba Animal Care Committee (Protocol # F05-024). Pigs were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Experimental Diets and Feeding Regimen

Two non-medicated diets based on corn, wheat and soybean meal were used in the current study. The diets (Table 10) were different in CP content but contained the same amount of ME and standardized ileal digestible Lys, Met plus Cys, Thr, and Trp on the ideal protein ratio basis suggested by Rademacher et al. (2000). Diet 1 contained 22.5%, CP (high CP) and diet 2 contained 17.6% CP (low CP) supplemented with crystalline AA. Isoleucine and Val were supplemented to the low CP diet up to the level in the high CP diet. All other nutrients were supplied in amounts meeting or exceeding NRC (1998) nutrient standards for a 6- to 10-kg pig. Prior to diet mixing, feed ingredients contributing AA were analyzed for AA composition as described by Nyachoti et al. (2006) and the analyzed values used in diet formulation. Representative samples of the experimental diets were also analyzed for AA composition. Diets were offered to pigs as pellets.

Animals and Housing

A total of 40 crossbred Duroc × (Yorkshire × Landrace) piglets, males and females, weaned at 17 ± 1 d of age (BW = 5.32 ± 0.24 kg, mean ± SD) were obtained from 7 litters in the University of Manitoba Glenlea Swine Research Farm and used in this 14-d experiment. On arrival, piglets were weighed and randomly allotted to dietary treatment according to litter and gender. Pigs were housed in groups of 4 resulting in 5 replicate pens per experimental diet. Each pen had a plastic-covered, expanded metal floor (1.5 m × 1.2 m), a single stainless steel self-feeder and a single low-pressure

Table 10. Composition of experimental diets, as fed basis

Item	High CP[1]	Low CP[1]
Ingredient, %		
Corn	53.21	67.63
Wheat	14.01	5.00
Soybean meal	4.00	4.00
Fish meal	6.00	6.00
Whey powder	7.00	7.00
Spray-dried blood plasma	2.00	2.00
Casein	7.90	-
Canola oil	3.64	3.34
Limestone	0.63	0.55
Biofos[2]	0.51	0.83
Vitamin and mineral premix[3]	1.00	1.00
Biolys®[4]	-	1.13
DL-Met	0.06	0.29
L-Thr	0.03	0.32
L-Trp	0.01	0.10
L-Ile	-	0.37
L-Val	-	0.44
Calculated nutrient composition[5]		
ME, MJ/kg	14.51	14.51

CP, %	22.50	17.64
Lys, %	1.47	1.47
Met, %	0.58	0.61
Ile, %	0.98	0.97
Thr, %	0.95	0.97
Trp, %	0.28	0.28
Val, %	1.23	1.21
Standardized ileal digestible AA, %		
Lys	1.35	1.35
Met + Cys	0.81	0.81
Thr	0.88	0.88
Trp	0.25	0.25
Ile	0.90	0.90
Val	1.12	1.12
Ca, %	0.80	0.80
Total P, %	0.65	0.65
Raffinose, g/kg of DM[6]	2.02	1.95
Starchyose, g/kg of DM[6]	2.69	2.66
Total NSP, g/kg of DM[6]	76.97	80.23

[1] The analyzed composition of the high CP and low CP diets, respectively, were: CP, 22.69 and 17.27; Arg, 0.97 and 0.80; His, 0.56 and 0.41; Ile, 1.01 and 0.96; Leu, 2.14 and 1.47; Lys, 1.43 and 1.42; Met, 0.56 and 0.61; Phe, 1.03 and 0.75; Thr, 0.92 and 0.95; Trp, 0.27 and 0.26; Val, 1.27 and 1.20; Ala, 1.02 and 0.94;

Asp, 1.69 and 1.31; Cys, 0.29 and 0.27; Glu, 4.30 and 2.63; Gly, 0.77 and 0.71; Pro, 1.71 and 1.03; and Ser, 1.01 and 0.71.

[2] Ca, 17%; P, 21% (Feed-Rite, Winnipeg, Manitoba, Canada).

[3]Supplied the following per kg of diet: 8,255 IU of vitamin A, 1,000 IU of vitamin D_3, 20 IU of vitamin E, 25 μg of vitamin B_{12}, 1.5 mg of vitamin K, 30 mg of niacin, 781 mg of choline chloride, 7.5 mg of riboflavin, 200 μg of biotin, 4.5 mg of pyridoxine, 1 mg of folic acid; 4 mg of thiamin, 40 mg of Mn (as MnO), 130 mg of Zn (as ZnO), 130 mg of Fe (as $FeSO_4.H_2O$), 10 mg of Cu (as CuO), 0.30 mg of Se (as Na_2SeO_3), 0.6 mg of I (as $Ca(IO_3)_2$).

[4]Contains 50.7% L-Lysine

[5]Based on analyzed AA content in feed ingredients and digestibility coefficients reported by Rademacher et al. (2000) and Degussa feed composition data for ME, Ca, and P.

[6]Based on data reported by Bach Knudsen (1997).

drinking nipple adjusted to the right height for pigs at this stage. Pigs had unlimited access to feed and water throughout the experimental period. Body weight and feed disappearance were monitored and the two were used to calculate feed efficiency. Room temperature was maintained at 31°C during wk 1 and then reduced to 29°C during wk 2. A 16 h lighting system was maintained in the room.

Bacterial Preparation, Oral Challenge, and Diarrhea Assessment

In order to evaluate the proliferation of ETEC and to differentiate the inoculum from the indigenous strains, a pure strain of ETEC expressing $K88^+$ fimbriae was made resistant to ciprofloxacin by exposing it to increasing doses of ciprofloxacin in Mueller-Hinton broth (Becton, Dickinson and Company, MN). The bacterial susceptibility testing to ciprofloxacin was performed using broth microdilution and disk diffusion test according to the methods of the National Committee for Clinical Laboratory Standards (NCCLS, 2002). The pure strain of ETEC K88 had minimum inhibitory concentration (**MIC**) and zone of inhibition (**ZI**) of 0.002 μg/mL and 34 mm, respectively, which were within the range of ≤ 0.015 μg/mL (MIC) and ≥ 30 mm (ZI) considered sensitive to ciprofloxacin by NCCLS (2002). The ciprofloxacin resistant ETEC K88 had MIC and ZI value of 0.625 μg/mL and 9 mm, respectively. Prior to being used to induce PWC, the ciprofloxacin resistant ETEC was confirmed to be positive for K88 fimbrial antigen, heat labile enterotoxin and heat stable enterotoxin (STb) genes by PCR genotyping using published primers (Kotlowski et al., 2007; Setia et al., 2009). On the morning of d 8, each pig was orally challenged with 6 mL of ETEC (5×10^{10} cfu/mL) suspended in PBS by gavage.

Occurrence and severity of post-weaning diarrhea was monitored and assessed using a fecal consistency (**FC**) scoring system (0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea) at 24 h, 30 h, 48 h, 72 h and 5 d post-challenge by 2 trained personnel with no prior knowledge of dietary treatment allotment (Marquardt et al., 1999).

Tissue Collection and Histological Assessment

Pigs (by random selection) were slaughtered on d -1, 3, and 7 d post ETEC challenge. On each day of slaughter, 10 pigs (1 pig/pen; 5pigs/diet) were anesthesized by an IM injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge, ON) and euthanized by an IV injection of sodium pentobarbital (50 mg/kg BW; Bimeda-MTC Animal Health Inc., ON, Canada). The abdominal cavity was exposed by midline laparotomy. A 10-cm section of ileum (starting 10 cm proximal of ileo-cecal junction) was collected, rinsed with cold physiological saline (0.9% saline), and stored in 10% buffered formalin to fix the villi and the crypts. Six cross-sections from each segment were processed for histological examination using the standard hematoxylin and eosin method as described by Owusu-Asiedu et al. (2002). Villus height (**VH**) and crypt depth (**CD**) were measured on 10 intact, well-oriented villi per specimen using a compound light microscope equipped with a video camera. Images were processed using the U. S. National Institute of Health Image J Software (version 1.37v). The VH was measured from the tip of the villous to the crypt-villous junction and the depth of the crypt from the crypt-villous junction to the base.

Digesta Collection and Assessment of Digesta Characteristics.

Digesta content from the ileum, distal colon and rectum were emptied into 3 separate sterile sample bags. Colon digesta was aseptically divided into 3 sterile sample bags. Digesta samples from the ileum and rectum, and a sample of colon digesta were placed on ice and transferred to the laboratory immediately for microbial count. Two samples of colon digesta were snap-frozen in liquid N and stored either at -80°C until used for DNA analysis or at -20°C until assayed for ammonia N and VFA.

Prior to ammonia N and VFA analysis, colon digesta was suspended in 0.1 *N* HCl (1:5, wt/vol) in a 125-mL conical flask and the mixture shaken for 5 h in an incubator shaker (New Brunswick Scientific Inc., Edison, NJ) at room temperature. The VFA concentration in the resulting digesta fluid was determined by gas chromatography (Varian Chromatography Systems, Model Star 3400, Walnut Creek, CA, USA) using the method described by Erwin et al. {Erwin, 1961 402 /id /d}. Briefly, 1 mL of 25% meta-phosphoric acid was mixed with 5 mL of digesta fluid in a centrifuge tube and the mixture was frozen overnight. Samples were then thawed, mixed with 0.4 mL of 25% NaOH and vortexed, followed by the addition of 0.64 mL of 0.3 *M* oxalic acid. The samples were centrifuged for 20 min at 3,000 x *g* at 4°C and 2 mL supernatant was transferred into a gas chromatography vial.

Digesta ammonia N concentration was determined using the method described by Novozamsky et al. (1974). Briefly, 1.5 mL of a reagent containing 200 mL of 0.05% sodium nitroprusside and 10 mL of 4% EDTA was added to 50 µL of digesta fluid in a 10-mL test tube and the mixture was vortexed. A solution containing 10% NaOCl (2.5

mL) was then added to the mixture and vortexed. Test tubes containing the resulting mixture were placed in complete darkness for 30 min followed by the reading of absorbance of the mixture at 630 nm (Milton Roy Spectronic 1001 Plus UV Visible Spectrophotometer, Milton Roy Co., Rochester, NY). Ammonia N concentrations were determined by calculating the concentrations from a regression equation of the standard curve (range: 2.5 to 20 mg/L). All samples were diluted to concentrations within the range used for the standard curve.

Gut Microbial Analysis

Culture-Based Analysis. One gram digesta from ileum or colon or rectum was added to 9 mL of sterile 0.1% peptone water, vortexed for 60 s, and then serially diluted 10-fold in sterile peptone water. Lactobacilli, total coliform and ETEC K88 in the serially diluted samples were quantified using deMan Rogosa Sharpe agar, and Eosin Methyl Blue (Becton Dickinson and Company, Sparks, MD) agar without and with ciprofloxacin (0.5 μg/mL), respectively. The plates were incubated aerobically at 37°C for 24 to 48 h. Fecal swab was collected from all pigs on arrival and plated on eosin methyl blue medium with ciprofloxacin (0.5 μg/mL) to screen them for the presence of ciprofloxacin resistant ETEC K88.

Molecular-Based Analysis. Deoxy-ribonucleic acid was extracted from the colon digesta using the ZR fecal DNA Kit™ (Zymo Research, Orange, CA, USA) following the manufacturer's protocol and the DNA quality was checked on a 1% agarose gel. Terminal restriction fragment length polymorphism (**T-RFLP**) analysis based on the PCR amplification of a highly variable section of the 16S rDNA fragments using 27

forward (labeled; 5'-GAAGAGTTTGATCATGGCTCAG-3') and 1100 reverse (5' CTGCTGCCTCCCGTAG 3') primers was used to assess the changes in microbial composition in the gut (Bhandari et al., 2009). The PCR reactions were performed using the following conditions: 5 min of the incubation mixture pre-denaturation at 94°C ; 35 cycles of repeated denaturation (94°C for 1 min), primer annealing (61°C for 1 min), and chain elongation (72°C for 2 min); and 5 min of post-cycling final extension at 72°C. Terminal restriction fragments (**T-RF**) were produced through *Hha*I digestion of 27 to1100 amplicons. Briefly, a mixture of 10 μL of PCR product, 10 units of *Hha*I, 1x *Hha*I buffer, 20 μg of bovine serum (Promega, Madison, WI) was incubated at 37°C for 8 h. The digested PCR product was cleaned using a method described by Paddison et al., (2004). Briefly,0.25 μL of glycogen (2mg/mL), 1.5 μL 3 *M* NaOAc (sodium acetate, pH 5.2) solution and 37.5 μL 95% (v/v) ethanol were added to 15 μL of PCR product and the mixture was centrifuged at 13,500 × *g*, 4°C for 15 min. The supernatant was decanted, rinsed twice with 200 μL of 70% (v/v) ethanol for 5 min at 13,500 × *g* at 4°C and the resulting pellet was allowed to dry for 30 min. The pellet was then re-suspended in 15 μL of sample loading solution. The precise lengths of T-RF were determined on a CEQ 8800 Genetic analysis system (Beckman Coulter Inc., Brea, CA). Briefly, 6 μL of fluorescently labeled fragments, 25.75 μL of sample loading solution, and 0.75 μL of a DNA size standard (1000 bp for T-RFLP, Beckman Coulter Inc., Brea, CA) were mixed together before application to the capillaries. An electropherogram with peaks of different sizes was obtained for each sample, and each peak represented an operational taxonomic unit. The bioinformatics analysis of T-RFLP data was performed as described by Bhandari et al. (2008).

Estimates 7.5 software (Colwell, 2005) was used to calculates richness indicators (Chao2, incidence-based coverage estimator and Michaelis-Menten function mean) and diversity indices (Shannon and Simpson) as described by Bhandari et al. (2008).

Statistical Analysis

All data were analyzed as repeated measures using Proc Mixed procedure of SAS (SAS Inst. Inc., Cary NC) except for the phylogenetic lineage data. The pen was considered the experimental unit. Effect of diet, period and their interaction were included in the model. Treatment means were compared using t-test. Statistical significance was accepted at $P < 0.05$ except for the phylogenetic lineage determined with molecular analysis where statistical significance was accepted at $P < 0.01$ according to the recommendation of the ribosomal database project (RDPII, Cole et al., 2005). The treatment means of the phylogenetic lineage data were compared with Fiser's Exact Test.

RESULTS

The analyzed CP and AA contents in the experimental diets were similar to the calculated values (Table 10).

Performance and Diarrhea Assessment

During the first 7 d after weaning, pigs fed the low CP diet had lower ($P < 0.05$) ADG and G:F compared with those fed the high CP diet (Table 11). No effect of diet was further observed on performance after the ETEC challenge (d 9-10, d 12-14) and overall

Table 11. Effect of dietary crude protein (CP) level on growth performance and fecal consistency score of weaned pigs challenged with *Escherichia coli* K88[1,2]

Item	High CP	Low CP	SEM
Initial BW, kg	5.34	5.30	0.24
Final BW, kg	8.51	8.07	0.30
ADG, g/d			
d 1-7	201[a]	147[b]	8.50
d 9-10	333	304	21.81
d 12- 14	322	343	29.39
Overall	286	265	13.01
ADFI, g/d			
d 1-7	200	179	8.67
d 9-10	302	260	20.62
d 12- 14	302	317	16.90
Overall	268	252	11.35
G:F			
d 1-7	1.00[a]	0.82[b]	0.02
d 9-10	1.20	1.11	0.10
d 12- 14	1.08	1.06	0.07
Overall	1.06	1.04	0.05
Fecal consistency score[3]			
24 h after challenge	0.68	0.32	0.19
30 h after challenge	1.00	0.60	0.30
48 h after challenge	1.40[a]	0.50[b]	0.29
72 h after challenge	1.40[a]	0.60[b]	0.17
5 d after challenge	1.10[a]	0.30[b]	0.23
Overall	1.12[a]	0.46[b]	0.17

[1]Each value represents the mean of 5 pens.

[2] Pigs were challenged with 6 mL of ETEC (10^{10} cfu/mL) on the morning of d 8.

[3]0 = normal; 1 = soft; 2 = mild diarrhea; 3 = severe diarrhea.

[a,b]Values within a row bearing different letters are different ($P < 0.05$).

(d 0-14). Pigs fed the high CP diet had higher ($P < 0.05$) FC scores compared with those fed the low CP diet starting from 48 h after ETEC challenge up to 5 d after challenge.

Microbial Analysis

Culture-Based. None of the pigs tested positive for ciprofloxacin resistant ETEC upon arrival at the research facility. There were no effects of diet on total coliforms and lactic acid producing bacteria population in the ileum, colon and rectum digesta before and after ETEC challenge.On d 7 after ETEC challenge, the bacteria population in the high and low CP diets, respectively, were (mean ± SD): coliform, 7.57 ± 1.84 and 8.48 ± 0.93; and lactic acid producing bacteria, 4.80 ± 2.27and 4.00 ± 1.03. A similar pattern of result was observed in the ileum and rectum for the high and low CP diets.

Before ETEC challenge, ciprofloxacin-resistant ETEC was not detected in the ileum, colon or rectum digesta of pigs fed both diets. After ETEC challenge, a similar pattern of ciprofloxacin-resistant ETEC population was observed in the digesta content from the ileum, colon and rectum, hence only ileal results are presented (Figure 8). On d 3 after challenge, ciprofloxacin-resistant ETEC was detected in 4 out of 5 pigs fed the high CP diet (5.22 ± 1.07 $\log_{10}$ cfu/mL, mean ± SD) while none was detected in the pigs fed the low CP diet. On d 7 after ETEC challenge, the ciprofloxacin-resistant ETEC was detected in 2 out of 5 pigs fed the high CP diet (4.24 ± 0.65 $\log_{10}$ cfu/mL, mean ± SD) and in 1 out of 5 pigs fed the low CP diet (4.30 $\log_{10}$ cfu/mL).

Molecular-Based. The T-RFs generated from colon digesta were not different between the two diets 1 d before and 3 d after ETEC challenge (data not shown). However, colon digesta of pigs fed the high CP diet tended to have a higher prevalence of

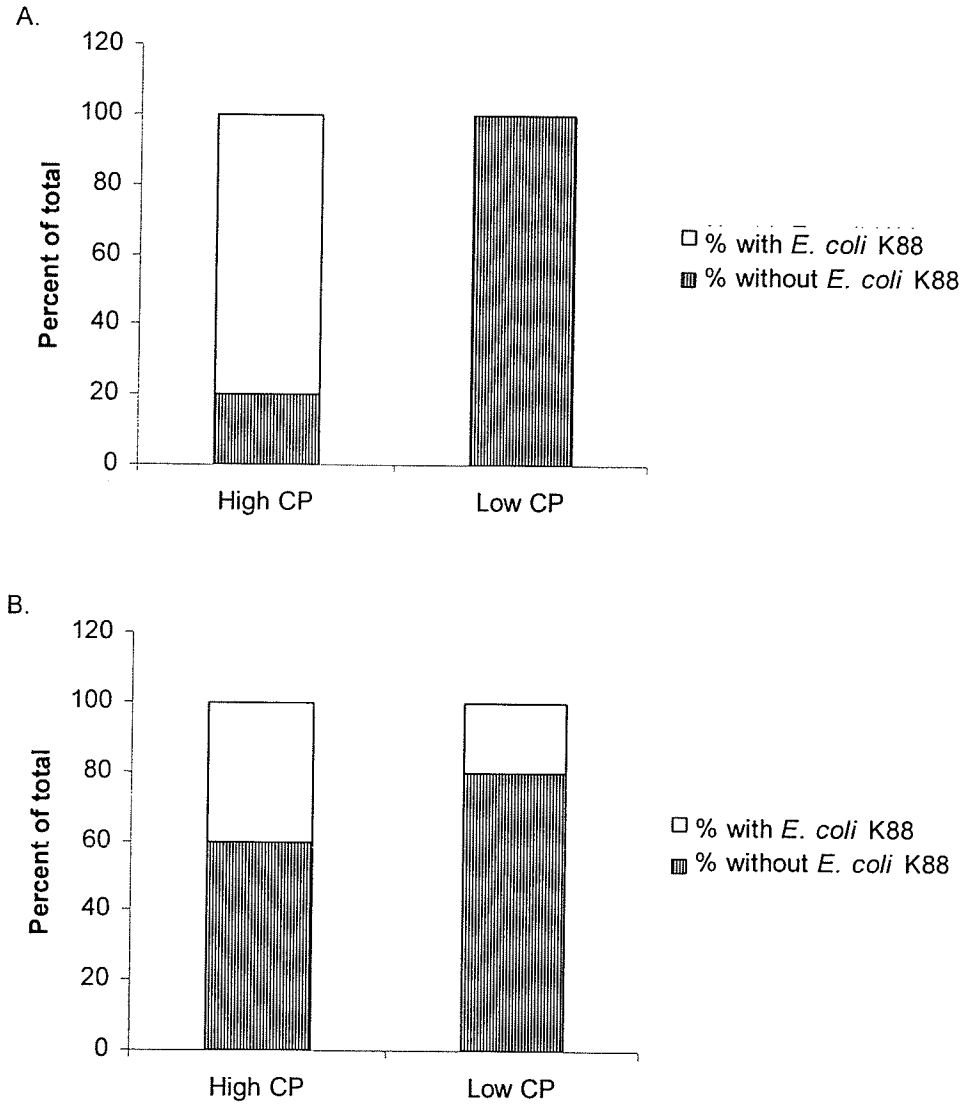

Figure 8. Effect of dietary crude protein (CP) level on *Escherichia coli* K88 population in the ileal digesta of weaned pigs challenged with *Escherichia coli* K88 3 d (A) and 7 d (B) after challenge.

family Clostridiaceae 1 d before (6.4% vs. 2.7%, $P = 0.17$) and 3 d after challenge (11.1% vs. 4.8%, $P = 0.07$), and genus Clostridium (2.7% vs. 0%, $P = 0.15$) 1 d before challenge compared with that of pigs fed low CP diet. On d 7 after ETEC challenge, the prevalence of order Unclassified clostridia, order Clostridiales, family Lachnospiraceae and genus Roseburia were higher ($P < 0.01$, 95% CI) in colon digesta of pigs fed the low CP diet compared with those fed the high CP diet (Table 12). Microbial richness and diversity in the colon digesta follow similar pattern on -1, 3 and 7 d after challenge, hence, only d 7 data were presented here. Pigs fed the high CP diet had higher ($P < 0.05$) microbial richness and diversity in their colon digesta compared with those fed the low CP diet 7 d after ETEC challenge (Table 13).

Digesta Characteristics and Gut Morphology

The ammonia N and VFA concentration are shown in Table 14. Ammonia N concentration was lower ($P < 0.05$) in the colon digesta of piglets fed the low CP diet 1 d before and 7 d after ETEC challenge compared with those fed the high CP diet. Diet had no effect on the concentration of acetic acid, propionic acid, butyric acid, branched chain VFA (data not shown) and total VFA (Table 14) in the colon digesta. Pigs fed the high CP diet had shorter ($P < 0.05$) villi on d 3 after challenge, deeper ($P < 0.05$) crypt and lower ($P < 0.05$) VH:CD 1 d before challenge compared with those fed the low CP diet. This was also the case for VH:CD on d 3 after challenge (Table 15).

Table 12. Terminal restriction fragment length polymorphism analysis of colon digesta of weaned pigs fed the high and low crude protein (CP) diet 7 d after *Escherichia coli* K88 challenge[1]

Taxonomic level, %[2]	High CP	Low CP	SEM
Phylum Bacteroidetes	1.5	2.5	0.50
Class unclassified Bacteroidetes	1.5	2.5	0.50
Phylum Firmicutes	92.4	88.6	1.90
Class Bacilli	11.6	13.9	1.15
Order Lactobacillales	11.1	13.9	1.40
Order Bacillales	0.5	0.0	0.25
Class Clostridia	77.3	74.7	1.30
Order Clostridiales	50.0[a]	73.4[b]	11.7
Family Lachnospiraceae	18.2[a]	43.0[b]	12.4
Genus Roseburia	2.5[a]	12.7[b]	5.10
Order unclassified Clostridia	27.3[a]	1.3[b]	13.0
Class unclassified Firmicutes	3.5	0.0	1.75
Phylum Lentisphaerae	0.5	1.3	0.40
Class Lentisphaerae	0.5	1.3	0.40
Order Victivallales	0.5	1.3	0.40
Phylum Proteobacteria	5.6	7.6	1.00
Class Epsilonproteobacteria	0.0	2.5	1.25
Order Campylobacterales	0.0	2.5	1.25
Class Deltaproteobacteria	0.5	2.5	1.00
Order Desulfovibrionales	0.0	1.3	0.65
Order unclassified Deltaproteobacteria	0.5	1.3	0.40
Class Gammaproteobacteria	2.5	0.0	1.25
Order Pasteurellales	0.5	0.0	0.25
Order Enterobacteriales	2.0	0.0	1.00
Class Betaproteobacteria	2.0	1.3	0.35
Order Burkholderiales	1.5	0.0	0.75
Order unclassified Betaproteobacteria	0.5	1.3	0.40
Class unclassified Proteobacteria	0.5	1.3	0.40
Phylum unclassified Bacteria	0.5	0.0	0.25

[1]Each value represents the mean of 5 pens.

[2]Prevalence data.

[a,b]Values within a row bearing different letters are different ($P < 0.01$).

Table 13. Richness and diversity indices of microbiota in the colon digesta of weaned pigs fed the high and low crude protein (CP) diet 7 d after *Escherichia coli* K88 challenge[1]

Item	High CP	Low CP	SEM
Richness[2]			
Chao2	71.00	59.69	6.37
ICE	71.00	54.13	5.44
MM Mean	71.00[a]	44.42[b]	0.20
Diversity[3]			
Shannon	4.26[a]	3.45[b]	0.02
Simpson	96.28[a]	46.71[b]	4.09

[1]Each value represents the mean of 5 pens.

[2] Species richness is an indicator of the number of distinct species present in a sample. Chao2, the incidence-based coverage estimator (ICE) and the Michaelis-Menten mean (MM mean) are estimators of richness and are different based on the sampling distribution that is assumed.

[3] Species diversity is a weighting of the abundance of distinct species. The Shannon and Simpson indices are estimators of diversity and are different based on the mathematical approach of estimating diversity. Simpson is a simple index based on species' richness and abundance and Shannon index involves the use of natural logarithm transformation.

[a,b]Values within a row bearing different letters are different ($P < 0.05$).

Table 14. Effect of dietary crude protein (CP) level on ammonia N and volatile fatty acids (VFA) concentration in the colon of weaned pigs challenged with *Escherichia coli* K88[1,2]

Item	High CP	Low CP	SEM
Ammonia N, mg/L			
1 d before challenge	237.85[a]	168.97[b]	20.68
3 d after challenge	293.99	211.77	71.28
7 d after challenge	414.97[a]	182.65[b]	73.89
Total VFA, mmol/L			
1 d before challenge	17.24	21.85	2.45
3 d after challenge	16.75	15.32	1.47
7 d after challenge	17.85	18.65	2.48

[1]Each value represents the mean of 5 pens.

[2] The individual VFA composition of high CP and low CP diets, respectively, on d 7 after challenge were: acetic, 8.20 and 9.28 (SE = 1.68); propionic, 6.23 and 5.40 (SE =1.02); butyric, 2.69 and 3.22 (SE = 0.56); branched chain AA, 0.73 and 0.76 (SE = 0.22)

[a,b]Values within a row bearing different letters are different ($P < 0.05$).

Table 15. Effect of dietary crude protein (CP) level on ileal morphology of weaned pigs challenged with *Escherichia coli* K88[1]

Item	High CP	Low CP	SEM
Villus height, μm			
1 d before challenge	402	406	16.15
3 d after challenge	330[a]	459[b]	26.38
7 d after challenge	413	410	9.13
Crypt depth, μm			
1 d before challenge	352[a]	243[b]	21.12
3 d after challenge	325	312	19.28
7 d after challenge	358	299	24.75
Villus height:Crypt depth			
1 d before challenge	1.18[a]	1.67[b]	0.10
3 d after challenge	1.03[a]	1.47[b]	0.06
7 d after challenge	1.20	1.39	0.11

[1]Each value represents the mean of 5 pens.

[a,b]Values within a row bearing different letters are different ($P < 0.05$).

DISCUSSION

Several pathogens preferentially ferment protein and high levels of dietary CP have been identified as one of the predisposing factors to PWC, a disease which is associated with the proliferation of ETEC in the GIT (Prohaszka and Baron, 1980; Macfarlane, 1995). Previous studies conducted in clean research facilities have shown consistently that low CP diets help to maintain indicators of a healthy gut in weaned pigs (Nyachoti et al., 2006; Wellock et al., 2006a). However, growth performance is often impaired. Therefore, we set out to investigate the effects of feeding low CP diets to weaned pigs under commercial conditions (simulated with ETEC challenge model) where there is a high pressure of infection. Since a high dietary CP level could increase the risk of ETEC infection in piglets, we hypothesized that feeding a low CP diet supplemented with AA to weaned pigs challenged with ETEC would minimize ETEC proliferation and infection, and hence, would improve performance compared with pigs fed a high CP diet.

The performance of pigs fed the low CP, AA supplemented diet was lower prior to the ETEC challenge (1 wk after weaning) compared with those fed the high CP diet and this is in agreement with literature (Wellock et al., 2006a; Pierce et al., 2007). The analyzed AA content of the dietary treatments shows that the EAA composition of the 2 diets met the NRC (1998) recommended levels for pigs at this stage. Hence, similar performance between the piglets fed the low and high CP diets was expected. This observation suggests that some non-essential AA (**NEAA**) such as Glu which is a major fuel for enterocytes might be deficient in the low CP, AA supplemented diet (Wu, 1995; Yue and Qiao, 2008). In the current study, analyzed Glu content in the low CP diet was

39% lower than that of high CP diet. Also, of importance in N and AA utilization is the ratio between N from essential AA and N from NEAA. The ratios between N from EAA and N from NEAA in the current study were 49:51 and 54:46, for the 22.5 and low CP diets, respectively. The ratio between N from EAA and N from NEAA in the low CP diet was different from the optimal ratio of 50:50 reported by Lenis et al. (1999) for growing pigs (30-60 kg BW) fed low protein diets (11.8 % and 14.3% CP).

Irrespective of the dietary CP level, growth performance was similar after ETEC challenge. The results of the current experiment suggest that pigs fed the high CP diet had higher enteric challenge after ETEC infection compared with those fed the low CP diet and hence were expected to have poorer performance. None of the pigs in the current study had severe diarrhea suggesting sub-clinical rather than clinical induction of PWC. This probably explains the similar performance observed in pigs fed both diets after ETEC challenge. Nevertheless, infection appeared to have a larger impact on the performance of pigs fed the high CP diet compared with those fed the low CP diet. There was a numeric 13% increase and 3% reduction in ADG from 3 to 7 d post-challenge, for pigs fed low and high CP diet, respectively. Likewise, there was a numeric 22% increase and no difference in ADFI from 3 to 7 d post-weaning challenge, for pigs fed low and high CP diet, respectively. The lack of dietary effect in the post-challenge performance observed in the current study does not agree with that of Wellock et al. (2008b) who reported that piglets fed a high CP diet (23%) have higher ADG and better G:F compared with those fed a low CP (13%) diet after ETEC challenge. The difference in the CP level of the low CP diet used in the two studies might explain the discrepancy in growth performance. However, in agreement with Wellock et al. (2007; 2008a), pigs fed the low

CP diet had a lower fecal consistency score and a lower number of ETEC in the distal small intestine and large intestine digesta compared with those fed the high CP.

Although previous studies (Wellock et al., 2007; 2008a) have investigated the effects of dietary protein level on gut microbial population in ETEC-challenged nursery pigs, these studies only utilized culture-based methods and non-cultivable bacteria were not determined. The T-RF data demonstrated that pigs fed the low CP diet had a higher prevalence of bacteria in the order Clostridiales particularly family Lachnospiraceae and genus Roseburia and tended to have a lower prevalence of family Clostridiaceae and genus Clostridium in their colon digesta compared with those fed the high CP diet. Members of genus Clostridium preferentially ferment protein and some of them, for example *C. perfringens*, are responsible for some clinical diseases in pigs and other animals (Gibert et al., 1998). Roseburia/*Eubacterium rectale* group are butyrate (preferred energy source for colonocytes) producing bacteria and they represent about 7% of the total bacteria in pig gut (Bergman, 1990; Leser et al., 2002). The results of this experiment demonstrate the potential for a low CP diet to increase the prevalence of butyrate-producing bacteria in the hindgut.

Roseburia/*Eubacterium rectale* group preferentially ferments carbohydrates (Louis et al., 2007). A lower amount of undigested CP is expected to reach the hind gut of pigs fed the low CP diet, hence, the ratio of protein to carbohydrate in the colon was expected to be lower compared with pigs fed the high CP diet. Furthermore, the growth of Roseburia/*Eubacterium rectale* group is favored at a slightly acidic pH (Walker et al., 2005). Although the colon pH was not measured in the current experiment, a higher level of dietary CP could increase the gut pH due to the buffering effects of protein (Prohaszka

and Baron, 1980; Wellock et al., 2008a). Therefore, we proposed that a lower buffering effect and a lower protein: carbohydrate ratio in the hind gut might be responsible for the higher proportion of Roseburia in the colon of pigs fed the low CP diet compared with those fed the high CP diet.

Furthermore, the results of the current experiment indicate that the microbial richness and diversity in the colon digesta of pigs fed the low CP diet was lower than those fed the high CP diet after ETEC challenge. This observation is probably a direct effect of reduced substrate availability for protein fermenters, which are often pathogens, in pigs fed the low CP diet (Macfarlane, 1995). Increase in microbial diversity has been associated with increased ecosystem stability and resistance to pathogen invasion (Konstantinov et al., 2004). However, in the context of the current data, the reduced diversity may not necessarily increase the susceptibility of piglets fed the low CP diet to enteric infection. By providing lesser amounts of substrate to protein fermenters, low dietary CP level may help to reduce the richness and abundance of potential pathogens in the gut of piglets. However, this hypothesis needs to be tested further.

There were no effects of dietary CP level on VFA concentration in the colon digesta. The lack of dietary effects on VFA concentration is in agreement with observations of Bikker et al. (2006) in non-challenged weaned pigs (8.7 kg BW). This observation is probably due to similar concentration of dietary factors such as non-starch polysaccharides that influence microbial concentration and organic acid production in both diets. For example, overall calculated total non-starch polysaccharides intake was similar for pigs fed both diets and averaged 202 and 206 g/kg DM for the low and high CP diet, respectively. Conversely, ileal and cecal VFA concentration decreased with a

reduction in dietary CP level in other studies (Nyachoti et al., 2006; Htoo et al., 2007). In addition, more proteolytic fermentation, as indicated by higher concentration of ammonia N in the colon digesta, was observed in pigs fed the high CP diet before and after ETEC challenge compared with those fed the low CP diet. This observation is in agreement with our previous studies and those of others in non-challenged weaned pig (Manuscript 1; Htoo et al., 2007).

Deeper crypts were observed in the ileum of pigs fed the high CP diet compared with those fed the low CP diet before challenge and this observation is similar to our previous study in non-challenged pigs (Manuscript 1). Likewise, a notable villi atrophy was also observed in pigs fed the high CP diet 3 d after ETEC challenge and this observation corresponded with the detection of ETEC K88 in 80% of pigs fed the high CP compared with 0% in those fed the low CP diet. The effect of high dietary CP content on the small intestine morphology was transient as no differences were detected in pigs fed both diets at 7 d after ETEC challenge. Contrary to our results, Wellock et al. (2008a) reported that dietary CP level had no effect on ileal morphology 3 d after ETEC challenge.

In conclusion, the piglets fed the low CP, AA-supplemented diet had similar performance as those fed the high CP diet after but not before ETEC K88 challenge. The low CP diet reduced proteolytic fermentation as indicated by ammonia N concentration in the colon digesta before and after ETEC K88 challenge; reduced the number of ETEC K88 in the small and large intestine digesta; increased villus:crypt depth ratio before and after ETEC challenge; and increased the prevalence of butyrate producing bacteria in the hindgut of weaned pig after ETEC K88 challenge.

CHAPTER 6

MANUSCRIPT 3

Imflammation-associated responses in piglets induced with post-weaning colibacillosis are influenced by dietary protein level

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ABSTRACT

Forty piglets (average BW = 5.32 kg) were used to investigate the effect of dietary CP content on immunological responses following a challenge with enterotoxigenic *Escherichia coli* (**ETEC**) K88. Pigs, housed 4 per pen, were randomly allotted to 2 diets: 1) a high, 22.5% CP diet or 2) a low, 17.6% CP diet supplemented with crystalline AA. Pigs were orally challenged with 6 mL of an ETEC K88 suspension containing 10^{10} cfu/mL on d 8 after weaning. Blood samples were collected from 10 pigs (1 pig/pen) on d 7 (at weaning), -24 h, 8 h, 72 h and 7d after challenge for determination of plasma urea N (**PUN**) and serum concentrations of tumor necrosis factor alpha (**TNF-α**), interleukin 1 beta (**IL-1β**) and haptoglobin (**Hp**). Tumor necrosis factor alpha, IL-1β and Hp were measured as indicators of inflammatory responses. The concentrations of serum TNF-α at 8 h, 72 h and 7 d after challenge were similar to the level observed at 24 h before challenge but higher ($P < 0.05$) than the weaning level. Pigs fed the low CP diet had lower ($P = 0.032$) concentrations of IL-1β (72 vs. 116 pg/mL) at 8 h post-challenge compared with those fed the high CP diet. Likewise, pigs fed the low CP diet tended to have lower ($P = 0.088$) concentration of Hp (9 vs. 25 mg/dL) compared with those fed the high CP diet at 8 h post-challenge. Compared with the weaning concentration, PUN concentration at 72 h after ETEC challenge was higher ($P < 0.05$) in pigs fed the high CP diet. The results indicate that the low CP diet supplemented with crystalline AA reduced inflammatory responses, as indicated by serum IL-1β, in piglets infected with ETEC K88.

Keywords: dietary protein, enterotoxigenic *Escherichia coli*, haptoglobin, piglets, post-weaning colibacillosis, pro-inflammatory cytokines.

INTRODUCTION

Post-weaning colibacillosis (**PWC**), a disease associated with proliferation of enterotoxigenic *Escherichia coli* (**ETEC**), is a major source of revenue loss to the swine industry due to losses resulting from mortality, growth stasis and treatment-associated cost (Amezcua et al., 2002; Fairbrother et al., 2005; Cutler et al., 2007). Previous studies showed improvements in indicators of animal well-being when nursery pigs were fed low CP, AA supplemented diets such as a reduction in ETEC population and the amount of toxic nitrogenous metabolites in the gut lumen (Nyachoti et al., 2006; Htoo et al., 2007; Wellock et al., 2008a). Utilization of low protein diets as a means of managing post-weaning diarrhea requires that such diets do not compromise piglets' performance. It was recently demonstrated that a low CP (17%) diet supplemented with Lys, Met, Thr, Trp, Ile and Val according to the ideal protein ratio supported similar piglets performance as the high CP diet (23%) up to 106 d of age (Heo et al., 2008).

Enterotoxigenic *E. coli* K88 infection has been associated with inflammatory responses in piglets and porcine intestinal cell lines (Bosi et al., 2004; Roselli et al., 2007). Stimulation of the immune system, including inflammatory responses, is an important defense mechanism in animals. However, it has been associated with production penalties such as partitioning of dietary nutrients away from growth towards synthesis of proteins and cells of the immune system (Klasing and Korver, 1997). Fimbrial colonization factor antigen and heat labile enterotoxin of *E. coli* K88 are potent stimulators of the immune system (Nataro and Kaper, 1998; Nagy and Fekete, 2005) suggesting that it is intestinal colonization by ETEC K88 that triggers immune responses (Verdonck et al., 2002). Thus, diets with low CP level may help to

reduce ETEC-associated inflammatory reactions in piglets by reducing ETEC proliferation and subsequent colonization of the gut.

Pro-inflammatory cytokines, interleukin1 beta (**IL-1β**), interleukin 6 (**IL-6**) and tumor necrosis factor-alpha (**TNF-α**), are important mediators of inflammatory responses including production of acute phase proteins (**APP**) such as haptoglobin (**Hp**) (Dube et al., 2001; Burger and Dayer, 2002; Chen et al., 2003). Previously, it was demonstrated in an ETEC challenge model that plasma Hp concentration was reduced in piglets fed a low CP diet compared with those fed a high CP diet on d 3 after challenge and the authors suggested that this might be due to protein scarcity in pigs fed the low CP diet (Houdijk et al., 2007). It is unknown whether a low CP diet supplemented with AA based on the ideal protein ratio will produce similar result. Likewise, there is paucity of information on the effect of dietary CP content on circulating pro-inflammatory cytokines in pigs.

This study investigated the effect of dietary CP content on inflammatory responses in weaned pigs challenged with ETEC K88 using serum pro-inflammatory cytokines (IL-1β and TNF-α) and Hp as response criteria. Additional data on the effect of dietary protein content on growth performance, enteric health and gut microbiology were reported elsewhere (Manuscript 2).

MATERIALS AND METHODS

Animals and Housing

The experimental protocol was approved by the Protocol Management and

Review Committee of the University of Manitoba Animal Care Committee. Pigs were cared for in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Forty crossbred [Duroc × (Yorkshire × Landrace)] piglets, male and female, weaned at 17 ± 1 d of age (average initial BW = 5.32 ± 0.24 kg, mean ± SD) and obtained from 7 litters were used in a 14-d experiment. On arrival, piglets were weighed and randomly allotted to dietary treatment according to litter and gender. Pigs were housed in groups of 4. Each pen (1.5 m × 1.2 m) had a plastic-covered, expanded metal floor, a single stainless steel self-feeder and a single low-pressure drinking nipple adjusted to the right height for pigs at this age. The room temperature was maintained at 31°C during wk 1 and then reduced to 29.5°C during wk 2. Throughout the experimental period, light was constantly on for 16 h (0700 to 2300). Five replicate pens were assigned to each of the 2 experimental diets in a completely randomized design.

Experimental Diets and Feeding Regimen

At weaning, 5 replicate pens were randomly allotted to one of the 2 non-medicated diets: a high, 22.5% CP diet and a low, 17.6% CP diet. The diets were based on corn, wheat and soybean meal. The feed ingredients contributing protein were analyzed for AA composition and the analyzed values were used in diet formulation. The diets contained similar amount of ME and standardized ileal digestible AA content based on the ideal protein ratio (Rademacher et al., 2009). In addition to Lys, Met, Thr, and Trp, the low CP was supplemented with Ile and Val to meet the level in the high CP diet. Both diets were formulated to meet or exceed NRC (1998) requirement for all other

nutrients. For detailed description of the experimental diets, refer to Manuscript 2. Pigs had *ad libitum* access to feed and water throughout the experimental period. Diets were offered to pigs as pellets.

Bacterial Challenge

On the morning of d 8, piglets were orally challenged with ciprofloxacin-resistant ETEC to differentiate the inoculum from the indigenous strains. The ETEC strain was confirmed to be positive for $K88^+$ fimbrae, heat labile and heat stable enterotoxin genes by PCR genotyping using published primers (Kotlowski et al., 2007; Setia et al., 2009). The ETEC culture was grown in Luria Bertani broth (Becton, Dickinson and company, MN) containing 0.5 μg/mL ciprofloxacin (Sigma) at 37°C. After 16 h of incubation, the culture was centrifuged at 4,000 × *g* for 10 min to harvest the bacteria cells, and then washed twice and re-suspended in PBS. Each pig was orally challenged with 6 mL of ETEC (10^{10} cfu/mL) by gavage. A sub-sample of the inoculum was serially diluted and plated on Eosin Methylene Blue agar (Becton, Dickinson and company, Sparks, MD) containing 0.5 μg/mL ciprofloxacin to confirm the concentration of ETEC in the inoculum.

Blood Sampling

Blood samples were collected by jugular vein puncture from 10 randomly selected pigs (1 pig/pen) into vacutainer tubes with and without heparin (Becton Dickinson, Rutherford, NJ) on 7 d (at weaning) and 24 h before challenge, and 8 h, 72 h and 7 d after challenge. The blood samples were placed on ice during collection and

were centrifuged immediately after collection at 3000 x *g* for 10 min at 4°C to recover plasma and serum. Aliquots of plasma and serum were stored immediately at -20°C until required for analysis.

Pro-inflammatory Cytokines, Haptoglobin and Plasma Urea N Assays

Serum samples were used for TNF-α, IL-1β and Hp determination and urea nitrogen (**PUN**) was determined in plasma samples. Haptoglobin concentration was determined using a commercially available solid phase sandwiched ELISA kit specific for porcine Hp (American Laboratory Product Company, Windham, NH). Intra- and inter-assay coefficient of variation was 5.3% and 9.1%, respectively.

The TNF-α and IL-1β concentrations were determined using a commercially available solid phase ELISA kit specific for porcine TNF-α and IL-1β (Quantikine R and D Systems Inc., Minneapolis, MN). Assay kit for each cytokine included a standard of known concentration and a control specimen. The controls for TNF-α and IL-1β assay kits are recombinant porcine TNF-α and recombinant porcine IL-1β, respectively. The intra- and inter-assay coefficient of variation for TNF-α was 4.9% and 8.9%, respectively, and the sensitivity (minimum detectable dose) of the assay was <3.7 pg/mL. For IL-1β, the intra- and inter-assay coefficient of variation was 4.7% and 8.7%, respectively, and the sensitivity of the assay was <10 pg/mL.

Plasma urea nitrogen was determined using a Nova Stat profile M blood and electrolyte analyzer (Nova Biomedical Corporation, Waltham, MA).

Statistical Analysis

Data were analyzed using the Mixed procedure of SAS (SAS Inst. Inc., Cary NC). The pig was considered the experimental unit. Effect of treatment, time, and interaction between treatment and time were included in the model. Association between pro-inflammatory cytokines and Hp was determined with Pearson's correlation coefficient. Treatment means were compared with t-test and statistical significance was accepted at $P < 0.05$ and $P \leq 0.10$ was presented as a trend.

RESULTS

There was an effect of time ($P = 0.007$) on serum TNF-α concentration (Figure 9). The TNF-α concentration was higher ($P < 0.05$) at 8 h, 72 h and 7d after challenge compared with the weaning level but similar to the concentration at 24 h before challenge. In pigs fed the high CP diet, serum TNF-α concentration increased ($P = 0.046$) at 24 h before challenge compared with the weaning concentration and remained elevated till 7 d after challenge. However, pigs fed the low CP diet had similar serum TNF-α concentration before and after ETEC challenge.

There were no effects of time or interaction between diet and time on serum IL-1β concentration. Diet had an effect ($P = 0.028$) on serum IL-1β concentration (Fig. 10). Pigs fed the the low CP diet had a lower ($P = 0.032$) serum IL-1β concentration at 8 h after challenge compared with those fed the high CP diet.

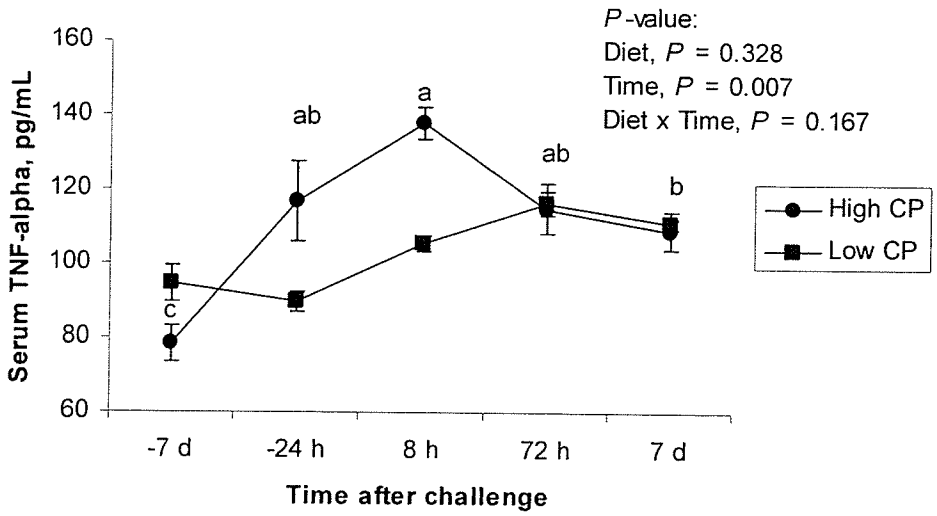

Figure 9. Effect of dietary crude protein (CP) level on serum tumor necrosis factor alpha (TNF-α) concentration of weaned pigs challenged with *Escherichia coli* K88.

Values are means ± SEM, n = 5. Pigs were challenged with 6 mL of ETEC (10^{10} cfu/mL) by gavage on d 8 after weaning. Labeled means within the high CP diet without a common letter differ, $P < 0.05$. Serum TNF-α concentration at weaning is represented by the -7 d value. *P*-value represents the effects of diet, time after infection and their interactions (Diet × Time).

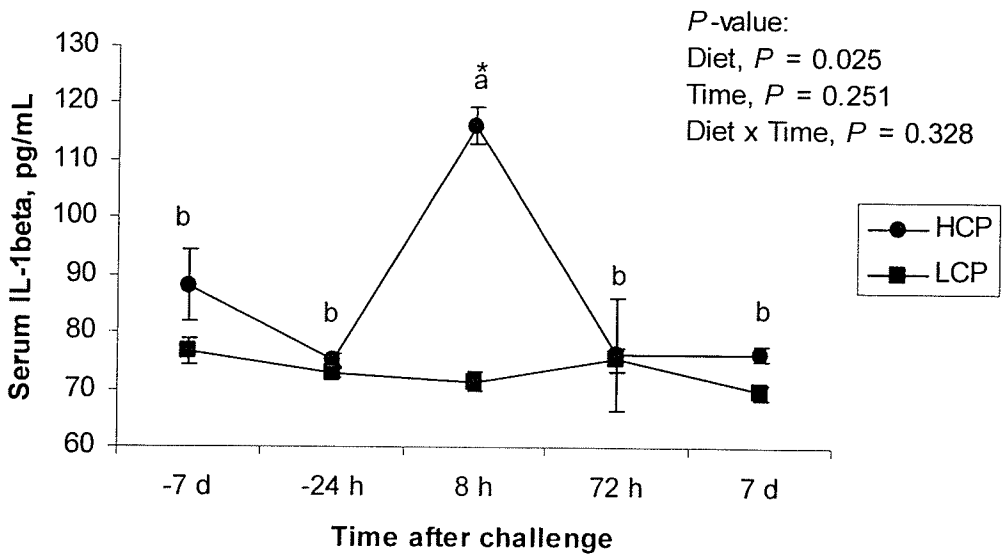

Figure 10. Effect of dietary crude protein (CP) level on serum interleukin 1 beta (IL-1β) concentration of weaned pigs challenged with *Escherichia coli* K88.

Values are means ± SEM, n = 5. Pigs were challenged with 6 mL of ETEC (10^{10} cfu/mL) by gavage on d 8 after weaning. Labeled means within the high CP diet without a common letter differ, $P < 0.05$. Asterisks indicate differences between dietary means at a given time period, $P < 0.05$. Serum IL-1β concentration at weaning is represented by the -7 d value. *P*-value represents the effects of diet, time after infection and their interactions (Diet × Time).

Time ($P = 0.082$) tended to have an effect on serum Hp concentration (Figure 11). Likewise, interaction between diet and time tended to be significant ($P = 0.088$) such that pigs fed the low CP diet tended to have a lower serum Hp concentration at 8 h after challenge compared with those fed the high CP diet. There was a positive correlation between serum Hp and TNF-α concentration ($R^2 = 0.43$; $P = 0.003$).

Diet ($P = 0.0002$), time ($P = 0.002$) and interaction between diet and time ($P = 0.002$) had effects on PUN concentration (Figure 12). Pigs fed the low CP diet had lower ($P < 0.05$) PUN concentration compared with those fed the high CP diet before and after ETEC challenge. Compared with the weaning concentration, PUN concentration was higher ($P < 0.05$) at 72 h after challenge in pigs fed the high CP diet. In pigs fed the low CP diet, PUN concentration decreased ($P < 0.05$) at 24 h before ETEC challenge up to the end of the experiment.

DISCUSSION

Manipulation of dietary protein content has been suggested as one of the nutritional means of managing PWC (Ball and Aherne, 1987; Nyachoti et al., 2006). Enterotoxigenic *E. coli* K88 infection induces intestinal inflammation (Bosi et al., 2004) and high levels of dietary CP promote proliferation of ETEC (Wellock et al., 2008a) in piglets. Thus, it was hypothesized in the current study that a reduction in dietary CP level would reduce the amount of substrate available for ETEC proliferation and colonization of intestinal epithelium of piglets, and would subsequently reduce inflammatory responses associated with ETEC infection including production of pro-inflammatory

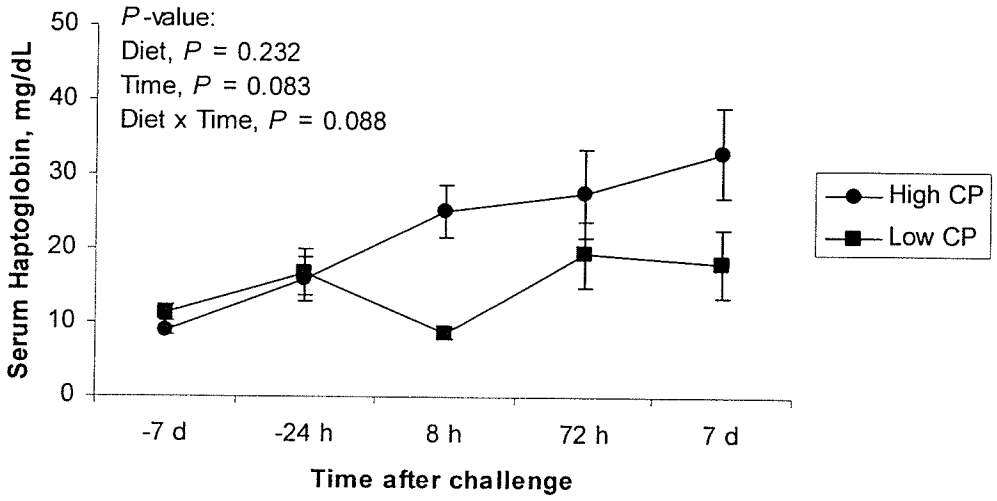

Figure 11. Effect of dietary crude protein (CP) level on serum haptoglobin concentration of weaned pigs challenged with *Escherichia coli* K88.

Values are means ± SEM, n = 5. Pigs were challenged with 6 mL of ETEC (10^{10} cfu/mL) by gavage on d 8 after weaning. Serum haptoglobin concentration at weaning is represented by the -7 d value. *P*-value represents the effects of diet, time after infection and their interactions (Diet × Time).

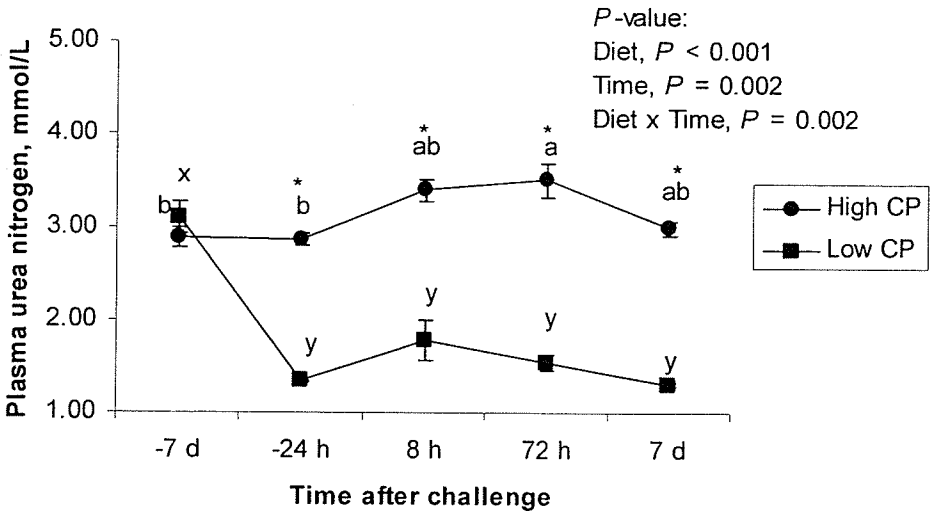

Figure 12. Effect of dietary crude protein (CP) level on plasma urea N concentration of weaned pigs challenged with *Escherichia coli* K88.

Values are means ± SEM, n = 5. Pigs were challenged with 6 mL of ETEC (10^{10} cfu/mL) by gavage on d 8 after weaning. Labeled means within a dietary treatment without a common letter differ, $P < 0.05$; a, b high CP diet and x, y low CP diet. Asterisks indicate differences between dietary means at a given time period, $P < 0.05$. Plasma urea N concentration at weaning is represented by the -7 d value. *P*-value represents the effects of diet, time after infection and their interactions (Diet × Time).

cytokines and APP. Although secretion of pro-inflammatory cytokines such as IL-1β and TNF-α is beneficial to the host for recruitment of immune cells to fight infection and eliminate pathogens, it may impair optimization of dietary nutrients for growth (Klasing and Korver, 1997).

All pens were observed to have diarrhea after the infection although the severity was higher in pigs fed the high CP diet compared with those fed the low CP diet. However, no pigs used in the current experiment had severe diarrhea suggesting an induction of subclinical rather than clinical PWC (Manuscript 2). Infection had a greater impact on health of pigs fed the high CP diet compared with those fed the low CP diet. Compared with those fed the high CP diet, pigs fed the low CP diet had lower number of ETEC K88 in their ileum and large intestine (Manuscript 2) and this is in agreement with our hypothesis and the reports of Wellock et al. (2007; 2008a). Although we did not measure acute feed intake (feed intake between 0-24 h after ETEC challenge) in the current experiment, a period of stasis and a numeric 22% increase in feed intake was observed from d 3 to 7 after challenge in pigs fed the high CP and low CP diet, respectively.

In accordance with our hypothesis, feeding a high CP diet to piglets caused a transient elevation in serum concentration of IL-1β after ETEC K88 challenge suggesting an induction of a mild inflammation. Following a bacterial infection, an immunocompetent animal will mount strong immunological responses such as recruitment of neutrophils and macrophages to the site of infection, a procees which is mediated by pro-inflammatory cytokines (Dube et al., 2001; Burger and Dayer, 2002). Hence, the concentration of pro-inflammatory cytokine is expected to increase following

ETEC challenge. In contrast to the pattern observed in pigs fed the high CP diet, circulating concentration of IL-1β remained unaltered in pigs fed the low CP diet after the challenge. Immune function is sensitive to nutrient availability and it has been repeatedly demonstrated that immune function is compromised during a period of malnutrition (Akira et al., 1990; Reid et al., 2002). In the current experiment, the low CP diet was formulated to be similar to the high CP diet in AA and other nutrient composition except for CP. Hence, low CP diet was not expected to depress the immune response. This observation, however, might be due to a reduction in infection intensity associated with low CP diets (Prohaszka and Baron, 1980; Wellock et al., 2007; 2008a). Data from the current study but reported separately showed that ETEC was not detected ($<10^2$ cfu/mL) in ileal digesta of pigs fed the low CP diet on d 3 after challenge but was detected in 80% of pigs fed the high CP diet (Manuscript 2).

Out of the 2 pro-inflammatory cytokines measured in the present study, only IL-1β responded specifically to ETEC challenge. Tumor necrosis factor-α can be produced in response to specific and non-specific stimuli and this probably explains the observed elevated concentration of serum TNF-α in pigs fed the high CP prior to the ETEC challenge. This observation demonstrated that other factors, apart from ETEC, present in the gut environment of pigs fed the high CP diet are capable of initiating an acute phase response. For example, pigs fed the high CP diet had higher concentration of ammonia N in their colon digesta compared with those fed the low CP diet before and after ETEC challenge (Manuscript 2).

Serum concentration of TNF-α was not affected by ETEC challenge in pigs fed the low CP diet. To our knowledge, this is the first study to report the effect of dietary CP

content on serum pro-inflammatory cytokines in piglets challenged with ETEC. Hence, we could not compare our results to the literature.

As a component of the host defence mechanisms, APP are synthesized in the liver and then released into circulation in response to various immunological challenges (Kushner, 1993). Tumor necrosis factor-α and IL-1β, which are primarily secreted by activated phagocytic cells, are important mediators of acute phase response (Akira et al., 1990; Murtaugh, 1994). These cytokines mediate APP synthesis indirectly by acting as stimulants for the production of IL-6 which is the primary mediator of APP synthesis (Webel et al., 1997; Johnson, 1997). Following the same pattern observed for serum concentrations of TNF-α and IL-1β, pigs fed the high CP diet had higher serum Hp concentration compared with those fed the low CP diet at 8 h after ETEC challenge. The observation is in agreement with the results of Houdjik et al. (2007) who reported that plasma concentration of Hp increased 3 d after ETEC infection in piglets fed a 23% CP diet but not in those fed a 13% CP diet. Compared with the weaning level, there was a numeric decline in serum concentration of Hp in pigs fed the low CP diet at 8 h after challenge despite a numeric increase in serum TNF-α concentration. It might be due to the fact that TNF-α is not the only stimulant required for APP synthesis. Nevertheless, there was a positive correlation between serum TNF-α and Hp concentration and this was probably due to the effects of the high CP diet since the serum concentration of TNF-α and Hp remained relatively constant throughout the experimental period.

Urea is the primary end product of protein and AA catabolism in mammals (Brown and Cline, 1974). Throughout the experimental period, pigs fed the low CP diet had lower concentration of PUN compared with those fed the high CP and this might be

due to ingestion of more protein in this group of pigs. Indeed, high dietary CP level has been associated with increased PUN concentration in pigs especially during the immediate postprandial period (Zervas and Zijlstra, 2002). In addition, the observed higher ammonia N concentration in the cecum of pigs fed the high CP diet compared with the low CP diet (Manuscript 2) might be partly responsible for the elevated PUN concentration. Ammonia N can be absorbed from the intestine and used for urea synthesis in the liver (Deguchi et al., 1978; van der Meulen and Jansman, 1997).

Plasma urea N concentration was 22% more than the weaning concentration at 72 h after ETEC challenge in pigs fed the high CP diet. The elevated PUN concentration could be attributed to proteolysis of muscle protein (Webel et al., 1997). This is because PUN concentration only peaked after there was a peak in serum concentration of IL-1β and TNF-α. Similar observation was reported by Webel et al. (1997) in an *E. coli* LPS challenge model although with a more profound increase in PUN concentration. In the current experiment, diet had no effect on ADG despite the higher concentration of IL-1β at 8 h after challenge. This might be due to the fact that the effect of dietary treatment on serum IL-1β was only transient and may not have a long lasting effect on muscle proteolysis in manner that will reflect on weight gain.

During acute phase response, IL-1β and TNF-α stimulate catabolism of skeletal muscle protein into free AA for APP synthesis (Johnson, 1997). The muscle protein degradation occurs in response to high demands for aromatic AA because APP are rich in aromatic AA, especially Phe (Le Floc'h et al., 2004). Since skeletal muscle contains a lower proportion of aromatic AA compared with APP, muscle protein must be broken down in a quantity exceeding that of the synthesized APP (Reeds et al., 1994; Le Floc'h

et al., 2004). For example, based on the assumption that skeletal muscle protein is the major source of AA for APP synthesis and that all Phe is completely utilized for APP synthesis, approximately 2 g of muscle protein must be catabolized to support the synthesis 1 g of APP (Reeds et al., 1994). The excessive non-limiting AA for APP synthesis would be oxidized and would thus increase ureagenesis (Brown and Cline, 1974).

A number of mechanisms could be responsible for the modulatory effects of dietary CP content on circulating pro-inflammatory cytokines concentration in ETEC-challenged pigs. First, dietary CP content affects ETEC proliferation and infection. Previous studies have shown that high dietary CP content increased the amount of undigested protein in the gut lumen and promoted ETEC proliferation (Prohaszka and Baron, 1980; Wellock et al., 2008a). Bacterial infection induces a cascade of immune responses including activation of macrophages to produce pro-inflammatory cytokines (Glasser et al., 2001). Second, low CP diets have been shown to reduce alteration to gut architecture compared with a high CP diet in weaned pigs as evidenced by deeper crypts (Manuscript 1). Damage to intestinal mucosa surface could compromise its barrier function resulting in intestinal malabsorption and translocation of bacteria and toxins across the gut wall (Owusu-Asiedu et al., 2003). In a separate report (Manuscript 2), gut morphology component of the current study showed that intestinal damage was higher in pigs fed the high CP diet compared with those fed the low CP diet after ETEC infection.

Third, due to the buffering effects of protein, a low CP diet may help to reduce digesta pH (Prohaszka and Baron, 1980). Low digesta pH is detrimental to the growth of pathogenic bacteria and could stimulate the growth of beneficial bacteria (Gibson, 1999;

Mikkelsen et al., 2007; Hansen et al., 2007). Wellock et al. (2008a) reported a higher lactobacilli count and a lower pH in the proximal colon digesta of piglets fed a 13% CP compared with those fed a 23% CP diet. In the current study, pH was not measured and diet had no effects on lactobacilli and coliform count (Manuscript 2). However, compared with those fed the high CP diet, pigs fed the low CP diet had higher prevalence of genus Roseburia which are butyrate-producing bacteria (Manuscript 2). Dietary treatment had no effect on butyric acid concentration due to high variability between animals. Fourth, as in the current study, evidence in the literature show that proteolytic fermentation and resulting toxic metabolites such as ammonia N, phenolic compounds and amines are reduced with low dietary CP content (Geypens et al., 1997; Le Leu et al., 2007; Htoo et al., 2007). These metabolites can cause a significant damage to colonic epithelium (Lin and Visek, 1991; Blaut and Clavel, 2007) and hence, could indirectly initiate inflammatory reactions.

In conclusion, feeding a low CP, AA-supplemented diet to piglets reduces inflammatory-associated responses induced by ETEC challenge as indicated by serum IL-1β concentration. The possible role of low CP, AA-supplemented diets in reducing intestinal inflammation in weaned pigs induced with PWC should be investigated further.

CHAPTER 7

MANUSCRIPT 4

Effect of dietary crude protein level on jejunal brush border enzyme activities in weaned pigs.

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ABSTRACT

Forty weaned pigs (average BW = 7.0 ± 0.5 kg, mean ± SD) were used to determine the effects of feeding a low CP, AA supplemented diet to piglets on the activities of jejunal brush border enzymes. Pigs were randomly allotted to 2 diets: a high, 22.2% CP diet and a low, 17.3% CP AA-supplemented diet. Pigs fed the high CP diet had higher small intestine weight compared with those fed the low CP diet on d 7 after weaning. Diet had no effects on the specific activities of jejunal sucrase, lactase, leucine aminopeptidase, aminopeptidase A, aminopeptidase N and dipeptidyl peptidase IV. The activities of sucrase and lactase decreased ($P < 0.05$) from d 3 after weaning to d 7 but the activities of leucine aminopeptidase and aminopeptidase N increased. The results showed that feeding a low CP diet supplemented with AA according to the ideal protein ratio to piglets had no negative effect on the development of jejunal brush border enzymes.

Keywords: amino acids; brush border enzymes; piglets; protein

INTRODUCTION

Piglets are highly susceptible to enteric disorders and infections during the immediate period after weaning (Fairbrother et al., 2005; Lalles et al., 2007a). Feeding a low CP diet supplemented with AA to weaned pigs has been suggested as one of the nutritional means of enhancing their gastrointestinal health especially in the absence of in-feed antimicrobial growth promoters (Nyachoti et al., 2006; Wellock et al., 2008a).

The major concern with the routine feeding of a low CP diet to weaned pigs is the potential growth and gastrointestinal tract (**GIT**) development disadvantage (Gu and Li, 2004; Wellock et al., 2007). It was recently demonstrated by Heo et al. (2008) that growth performance can be maintained up to 15 wk after weaning when pigs are fed a low CP (17%) diet supplemented with limiting AA according to the ideal protein pattern during the first 5, 7, 10, or 14 d after weaning.

Using the activities of intestinal enzymes as an indicator of gut maturity, few studies have evaluated the effects of dietary CP content on GIT development in weaned pigs (Gu and Li, 2004; Bikker et al., 2006; Yue and Qiao, 2008). However, the results from these studies were not consistent. Compared with a 22% CP diet, Bikker et al. (2006) reported that a 15% CP, AA-supplemented diet had no adverse effect on jejunal activities of maltase, sucrase and aminopeptidase N (**APN**) in piglets weaned at 26 d of age. In contrast, Yue and Qiao (2008) reported a reduction in the jejunal activities of sucrase, lactase and maltase in pigs weaned at 18 d of age as the dietary CP content was reduced from 23% to 17% . Hence, the effect of dietary CP level on GIT maturity is inconclusive.

In our previous study (Manuscript 1), we demonstrated that pigs fed a 17% CP, AA-supplemented diet had a lower empty small intestine (**SI**) weight compared with those fed a 21% CP diet. This observation could be an indication of poor GIT development in pigs fed the 17% CP diet (Nunez et al., 1996; Dudley et al., 1997; Guay et al., 2006) or overgrowth of visceral organs in pigs fed the 21% CP diet (Schoknecht and Pond, 1993; Le Bellego and Noblet, 2002). The objective of the current experiment therefore, was to determine the effect of a low CP, AA-supplemented diet on intestinal

development during the first wk after weaning using the activities of jejunal brush border enzymes as response criteria. The activities of jejunal enzymes were evaluated from d 3 to 7 after weaning to explore potential time-course effects of dietary CP level on enzyme activities. It was hypothesized that the low CP, AA-supplemented diet will not impair the jejunal brush border enzyme activities.

MATERIALS AND METHODS

Experimental Diets

Two, non-medicated diets were formulated to be different in dietary CP content: a high, 22.3% CP diet and a low, 17.3% CP diet (Table 16). Diets contained the same amount of standardized ileal digestible Lys, Met plus Cys, Thr, and Trp based on the ideal protein ratio (60, 62 and 20 for Met plus Cys, Thr and Trp, respectively) suggested by Rademacher et al. (2009). Isoleucine and Val were supplemented to the 17.3% CP diet up to the level in the 22.3% CP diet. All other nutrients were supplied in amounts meeting or exceeding NRC (1998) nutrient standards. Prior to diet mixing, feed ingredients contributing AA were analyzed for AA composition as described by (Nyachoti et al., 2006) and the analyzed values were used in diet formulation. Representative samples of the experimental diets were also analyzed for AA composition to ensure that formulation targets were met (Table 17). Diets were offered to pigs as mash. Pigs had unlimited access to feed and water throughout the experimental period. Feed intake was determined on d 3, 5 and 7 after weaning.

Table 16. Composition of experimental diets, as fed basis.

Item	High CP	Low CP
Ingredient, %		
Corn	60.417	66.281
Wheat	5.000	5.000
Soybean meal	4.000	4.000
Fish meal	3.000	3.000
Whey powder	7.000	7.000
Spray-dried blood plasma	3.000	3.000
Casein	10.800	2.500
Canola oil	3.900	3.680
Limestone	0.970	0.950
Biofos[1]	0.730	1.050
Vitamin and mineral premix[2]	1.000	1.000
Biolys®[3]	0.080	1.111
DL-Met	0.093	0.300
L-Thr	0.001	0.283
L-Trp	-	0.092
L-Ile	-	0.337
L-Val	-	0.416
Calculated nutrient composition[4]		
ME, MJ/kg	14.50	14.50
CP, %	22.22	17.31
Fiber, %	1.79	1.92
Lys, %	1.45	1.45
Met, %	0.59	0.54
Ile, %	0.97	0.95
Leu, %	2.09	1.49
Thr, %	0.94	0.93

Trp, %	0.28	0.28
Val, %	1.24	1.22
Standardized ileal digestible AA, %		
Lys	1.35	1.35
Met + Cys	0.81	0.81
Thr	0.85	0.85
Trp	0.25	0.25
Ile	0.89	0.89
Val	1.14	1.14
Ca, %	0.80	0.80
Total P, %	0.65	0.65

[1] Ca, 17%; P, 21% (Feed-Rite, Winnipeg, Manitoba, Canada).

[2]Supplied the following per kg of diet: 8,255 IU of vitamin A, 1,000 IU of vitamin D_3, 20 IU of vitamin E, 25 µg of vitamin B_{12}, 1.5 mg of vitamin K, 30 mg of niacin, 781 mg of choline chloride, 7.5 mg of riboflavin, 200 µg of biotin, 4.5 mg of pyridoxine, 1 mg of folic acid; 4 mg of thiamin, 40 mg of Mn (as MnO), 130 mg of Zn (as ZnO), 130 mg of Fe (as $FeSO_4.H_2O$), 10 mg of Cu (as CuO), 0.30 mg of Se (as Na_2SeO_3), 0.6 mg of I (as $Ca(IO_3)_2$).

[3]Contains 50.7% L-Lysine

[4]Based on analyzed AA content in feed ingredients and digestibility coefficients reported by Rademacher et al. (2009) and Degussa feed composition data for ME, fiber, Ca, and P.

Table 17. Analyzed crude protein (CP) and amino acids (AA) composition of the experimental diets, as is basis.

Item, %	High CP	Low CP
CP	22.5	17.8
Indispensable AA		
Arg	1.02	0.83
His	0.63	0.45
Ile	1.01	0.96
Leu	2.13	1.51
Lys	1.55	1.55
Met	0.61	0.63
Phe	1.09	0.76
Thr	1.01	0.96
Trp	0.28	0.28
Val	1.29	1.27
Dispensable AA		
Ala	1.05	0.88
Asp	1.78	1.34
Cys	0.30	0.27
Glu	4.25	2.82
Gly	0.74	0.64
Ser	1.15	0.79

Animals and Housing

Forty crossbred Duroc × (Yorkshire × Landrace) piglets (20 barrows and 20 gilts) with an average initial BW of 6.96 ± 0.45 kg (mean ± SD) were obtained from the University of Manitoba Glenlea Swine Research Unit. Pigs were weaned at 19 ± 1 day of age and were from 7 litters. The pigs were randomly allotted to dietary treatments such that pigs from the same litter with similar body weights and sex were allotted to 1 of the 2 diets. Pigs were housed in groups of 4 per pen resulting in 5 replicate pens per diet. Each pen had plastic-covered expanded metal floor (1.5 m x 1.16 m) and was equipped with a stainless steel self-feeder and a nipple-type drinker. Room temperature was maintained at 30°C with a 16-h lighting system. The experimental protocol was approved by the Protocol Management and Review Committee of the University of Manitoba Animal Care Committee. Pigs were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Tissue Collection

On d 3, 5 and 7 after weaning, 1 pig was randomly selected from each pen for euthanasia. Pigs were anesthesized by an IM injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Ontario, Canada) and euthanized by an IV injection of sodium pentobarbital (50 mg/kg BW; Bimeda-MTC Animal Health Inc., ON, Canada). The abdominal cavity was exposed by midline laparotomy. A 25-cm section of jejunum (10 cm distal of ligament of Treitz) was collected, flushed with ice-cold physiological saline (0.9% NaCl), cut open lengthwise, blotted dry, snap-frozen in liquid N and stored

at -80°C until used for enzyme analyses. Following euthanasia, the remaining part of small intestine was removed, emptied of digesta, rinsed with physiological saline, blotted dry and weighed.

Mucosal Protein and Brush Border Enzyme Analysis

Jejunal samples were allowed to thaw on ice and the mucosa was scraped off from the underlying muscular layer using a glass slide. Mucosal weight was determined only on the jejunal samples of pigs slaughtered on d 7 after weaning. Mucosal homogenate was prepared as described by Dahlqvist (1984) by homogenizing the mucosa in ice-cold 0.9% NaCl (5 mL/g of mucosa) using PowerGen 125 homogenizer (Fisher Scientific, Edmonton, AB). The homogenate was then centrifuged at 20,000 × *g* for 1 h at 4°C. The supernatant fraction was then used for the protein and enzyme analysis.

Total protein concentration was determined according to Lowry et al. (1951) procedure using BSA as the standard (Biorad, Hercules, CA). Sucrase (EC 3.2.1.48) and lactase (EC 3.2.1.23) activities were determined by the method of Dahlqvist (1984) and the produced glucose was measured by spectrophotometry (Ultrospec 3100pro, Biochrom Ltd, Cambridge, UK) using a glucose kit (Sigma, St. Louis, MO).

Leucine aminopeptidase (**LAP**, EC 3.4.11.1), aminopeptidase A (**APA**, EC 3.4.11.7), APN (EC 3.4.11.2) and dipeptidyl peptidase IV (**DPP**, EC 3.4.14.5) activities were determined using L-leucine *p*-nitroanilide hydrochloride, L-glutamic acid 1-(4-nitroanilide) , L-alanine *p*-nitroanilide hydrochloride and glycyl L-proline *p*-nitroanilide hydrochloride (Sigma, St. Louis, MO, USA), respectively, as substrates (Nagatsu et al., 1976; Sjostrom et al., 1978). All peptidases activities were determined

spectrophotometrically at 405 nm after the reaction was stopped by 30% acetic acid (Erlanger et al., 1961).

The enzyme activities were expressed as units per mg of protein. One unit was defined as the amount of enzyme that hydrolysed 1 μmol of substrate per minute.

Statistical Analysis

All data, except for the mucosal weight, were analyzed as repeated measures using the Mixed procedure of SAS (SAS Inst. Inc., Cary, NC). The pen was considered the experimental unit. Effect of gender, diet, time (d after weaning) and their interaction were included in the model. Since there were no effects of gender ($p > 0.05$) on all the parameters, only the effect of diet, time and their interaction were included in the model. Average daily feed intake was initially used as a covariate for protein and enzyme analysis to eliminate the possible influence of ADFI on enzyme development in piglets. However, ADFI was removed from the model since its effect was not significant. Jejunum mucosal weight was analyzed using the GLM procedures of SAS. Only the effect of diet was included in the model since no gender effect was detected. For all the analysis, treatment means were compared using Students t- test. Statistical significance was accepted at $P < 0.05$ and $P \leq 0.10$ was considered a trend.

RESULTS

There was no effect ($P > 0.10$) of diet and interaction between diet and time on feed intake (Table 18). Feed intake increased ($P < 0.05$) from d 3 to 7 after weaning. Diet

Table 18. Effect of dietary crude protein (CP) level on average daily feed intake of weaned pigs during the first wk after weaning. [1]

Item, g	High CP	Low CP	SEM	*P*-value
Day 1 to 3	53	59	20.57	0.782
Day 4 to 5	229	209	34.99	0.576
Day 6 to 7	321	291	45.34	0.518

[1]Each value represents the mean of 5 pens.

had no effect on SI weight (Figure 13). Small intestine weight increased ($P < 0.0001$) from d 3 to 7 after weaning. Interaction between diet and time had an effect on the weight of SI such that pigs fed the low CP diet had lower ($P = 0.043$) SI weight compared with those fed the high CP diet only at d 7 after weaning. There was no effect of diet on jejunal mucosal weight expressed per unit of jejunum length on d 7 after weaning and this averaged 170 and 176 mg/cm for the high CP and low CP diets, respectively. However, mucosal weight expressed per unit of jejunum weight was higher ($P = 0.025$) in pigs fed the low CP diet (425 mg/g) compared with those fed the high CP diet (342 mg/g).

Diet and interaction between diet and time had no effect ($P > 0.10$) on mucosal protein content (Figure 14). The concentration of mucosal protein tended ($P = 0.095$) to increase from d 3 to 7 after weaning. The activity of lactase declined ($P = 0.0005$) and that of sucrase tended to decline ($P = 0.096$) from d 3 to 7 after weaning (Figure 15). There were no effects of diet and interaction between diet and time on lactase and sucrase activities ($P > 0.10$).

There was no effect of diet ($P > 0.10$) on all the analyzed peptidases (Figure 16). Contrary to what was observed for lactase, the activities of LAP ($P = 0.008$) and APN ($P = 0.031$) increase from d 3 to 7 after weaning. The activity of APA tended to decline ($P = 0.056$) from d 3 to 7 after weaning. There was no interaction between diet and time ($P > 0.10$) on all the analyzed peptidases.

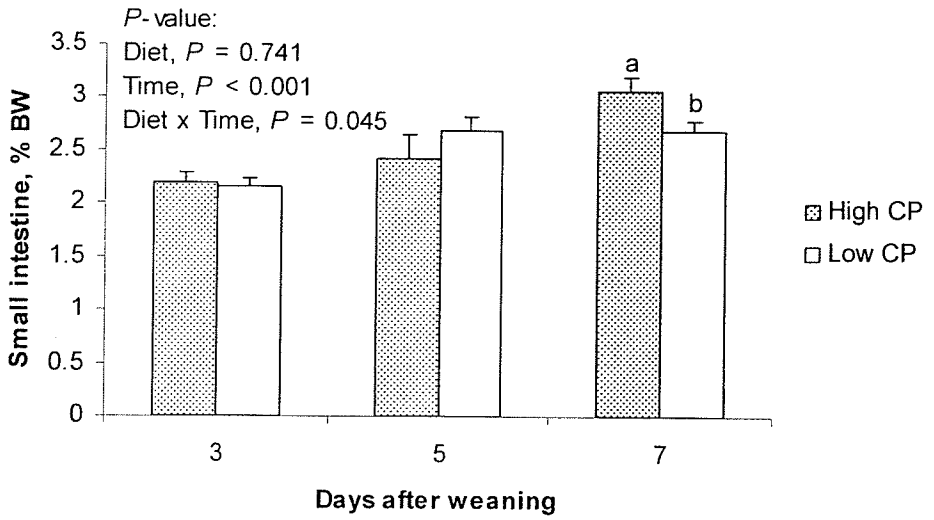

Figure 13. Effect of dietary crude protein (CP) level on empty small intestinal weight of weaned pigs during the first wk after weaning.

Values are means ± SEM, n = 5. *P*-value represents the effects of diet (Diet), day after weaning (Time) and their interactions (Diet × Time). Labeled means within a day without a common letter differ, $P < 0.05$.

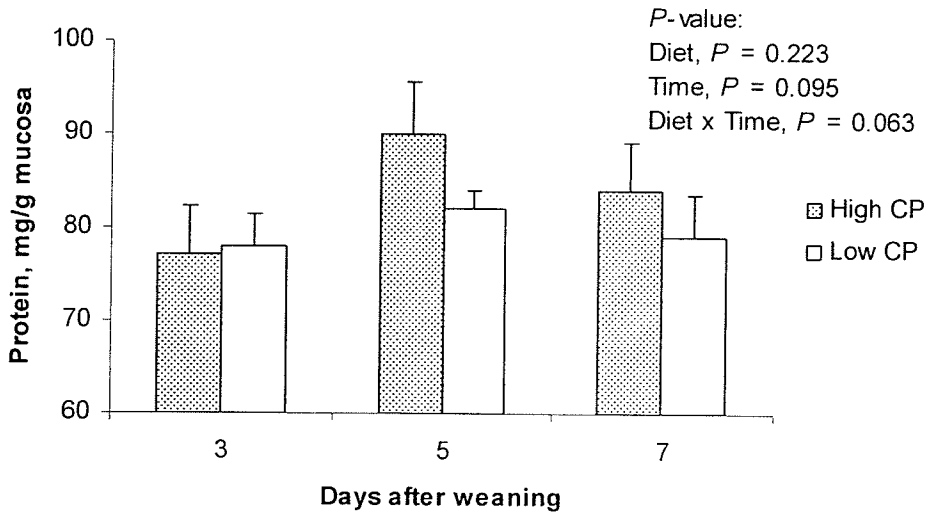

Figure 14. Effect of dietary crude protein (CP) level on the concentration of jejunal mucosal protein in piglets during the first wk after weaning.

Values are means ± SEM, n = 5. *P*-value represents the effects of diet (Diet), day after weaning (Time) and their interactions (Diet × Time).

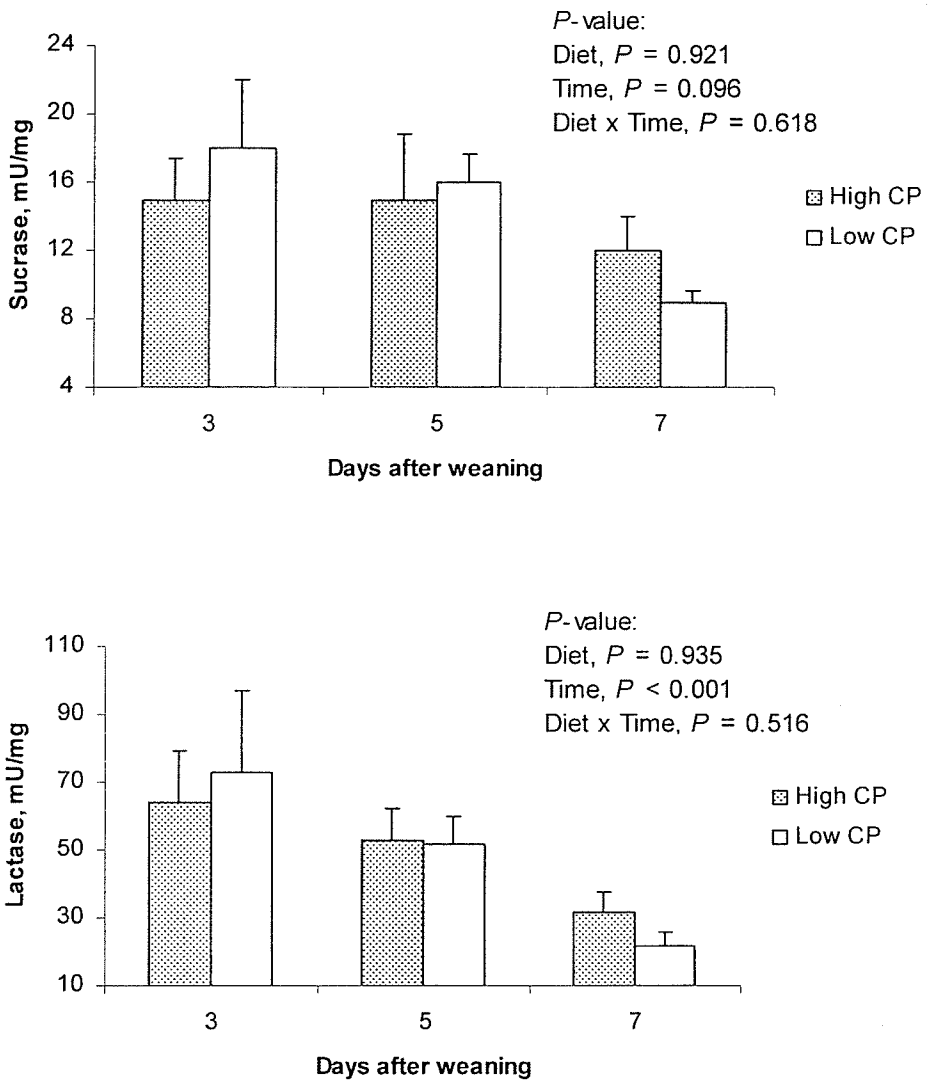

Figure 15. Effect of dietary crude protein (CP) level on the activities of jejunal disaccharidases in piglets during the first wk after weaning.

Values are means ± SEM, n = 5. *P*-value represents the effects of diet (Diet), day after weaning (Time) and their interactions (Diet × Time).

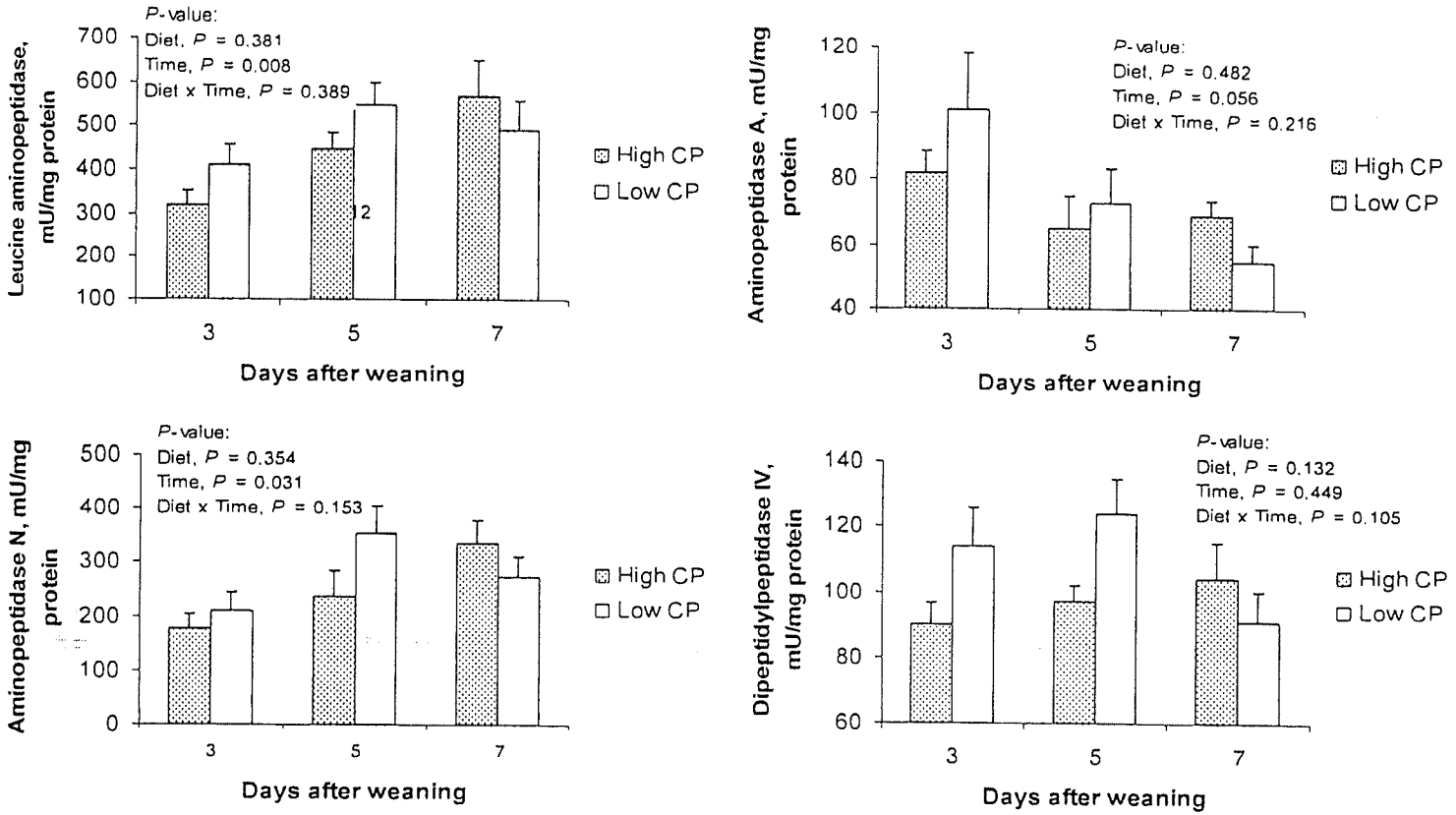

Figure 16. Effect of dietary CP level on the activities of jejunal peptidases in piglets during the first wk after weaning.

Values are means ± SEM, n = 5. P-value represents the effects of diet (Diet), day after weaning (Time) and their interactions (Diet × Time)

DISCUSSION

In the absence of in-feed antibiotics, a low CP, AA-supplemented diet has been suggested as one of the nutritional means of managing enteric disorders associated with weaning in pigs (Nyachoti et al., 2006; Wellock et al., 2007). To be an effective tool in managing post-weaning diarrhea, low CP, AA-supplemented diet must be able to support optimal organ and tissue growth and development of the piglets. It has been demonstrated that a low CP, AA-supplemented diet had no detrimental effects on the immediate and subsequent growth performance of weaned pigs (Heo et al., 2008). However, whether a low CP, AA-supplemented diet could sustain the growth of the GIT has not been fully elucidated. Hence, we set out to investigate the effects of feeding a low CP, AA-supplemented diet to piglets after weaning on jejunal digestive function.

The SI weight was 14% lower in pigs fed the low CP diet compared with those fed the high CP diet on d 7 after weaning. This was in agreement with our previous study where we reported a smaller empty SI weight in pigs fed the 17% CP diet compared with those fed the 21% CP diet (Manuscript 1). Increase in visceral organ mass has also been reported in weanling pigs fed a high CP diet compared with those fed a low CP diet (Schoknecht and Pond, 1993; Le Bellego and Noblet, 2002). In agreement with Hedemann et al. (2003), the SI weight increased from d 3 to 7 after weaning in the current study. During post-weaning period, the SI of pig undergoes a progressive increase in size as part of an adaptation process for digestion and absorption of solid feed (Pluske et al., 2003; Adeola and King, 2006). Indeed, feed intake increased from d 3 to 7 after weaning.

Despite the differences in the intestinal weight of pigs fed the high CP and low CP diet, protein concentration in the jejunal mucosa was unaffected by dietary treatment. In agreement with our study, Schoknecht and Pond (1993) reported that the protein concentration in the jejunal tissue of pigs fed a high CP diet was similar to that of pigs fed a low CP diet. Unlike the current study, Yue and Qiao (2008) reported a reduction in jejunal mucosal protein concentration in piglets (weaned at 18 d of age) as the dietary CP content was reduced from 23% to 17% . The analyzed AA composition of the high CP and low CP diets were fairly similar between the two studies. The discrepancy between our result and that of Yue and Qiao (2008) might be due to differences in the experimental design, the time after weaning at which the samples were taken, and the BW of piglets at the time of weaning. Pluske et al. (2003) reported that the pigs weaned at a lighter weight at 2 or 4 weeks of age had a less developed GIT compared with their heavier counterparts.

The activities of sucrase and lactase were not affected by dietary treatment indicating that feeding a moderately low CP diet with adequate AA supplementation had no negative effects on the ability of weaned pigs to digest lactose and starch. As in the present study, Bikker et al. (2006) reported that the activity of sucrase in the jejunum of pigs (weaned at 26 d of age) fed a 15% CP diet and a 22% CP diet was similar. In contrast, Yue and Qiao (2008) reported a linear decrease in sucrase and lactase activities as dietary CP decreased from 23% to 17%. In agreement with literature, lactase activity declined with time after weaning and this was probably due to ontogenic decline in the brush border lactase activity (Hampson and Kidder, 1986; Cranwell, 1995; Motohashi et al., 1997). The activity of sucrase also tended to increase from d 3 to 7 after weaning.

Other studies where pigs were weaned at 3 wk of age have also reported transient decline in intestinal sucrase during the first week after weaning (Hampson and Kidder, 1986; Bruininx et al., 2002).

Diet had no effect on the activities of LAP, APA, APN and DDP. Limited information is available on the effect of dietary CP content on intestinal peptidases in piglet. Thus far, only Bikker et al. (2006) has reported the effect of dietary CP content on APN activity. In that study, jejunal activity of APN was similar between pigs fed the 15% CP diet and those fed the 22% CP diet which is in agreement with the results of the current study. The effect of weaning on jejunal activities of peptidases was not consistent and this is in agreement with literature (Hedemann et al., 2003; Marion et al., 2005). The activities of LAP and APN increased from d 3 to 7 after weaning. The activity of APA tended to decrease from d 3 to 7 after weaning and the activity of DPP remained unchanged. In early weaned pigs (weaned at 7 d of age), Marion et al. (2005) reported that jejunal activity of APN increased and that of APA did not change during the first 14 d after weaning compared with the unweaned pigs. Likewise, Hedemann et al. (2003) reported that the proximal jejunal activity of APN in pigs weaned at 28 d of age decreased from the d 0 to 3 but increased up to the pre-weaning level from d 3 to 10. The authors also reported that weaning had no effects on γ-glutamyl activity but it decreased the activity of DDP. Information on developmental change in LAP activity during the first week after weaning is scarce.

The lower SI weight observed in pigs fed the low CP diet on d 7 had no effect on the specific activities of analyzed disaccharidases and peptidases in the jejunum. It has been suggested, however, that expression of intestinal enzymatic activity on a segmental

basis is a better approach as this usually reflects the actual capacity of the organ to perform a particular biochemical reaction (Kelly et al., 1991c; Nunez et al., 1996). Mucosal weight expressed per unit length of jejunum was not affected by dietary treatment. However, feeding the low CP diet to piglets led to an increase in the mucosal weight expressed as milligram per g tissue. This observation suggests that the increased small intestine weight in pigs fed the high CP diet compared with those fed the low CP diet was due to an increase in muscular serosal tissue mass and may not necessarily indicate a higher total digestive capacity. This is because an increase in the digestive and absorptive capacity of the small intestine is more associated with mucosal weight rather than serosal weight (Ewtushik et al., 2000). In fact enzyme activities expressed as unit per gram mucosa/tissue were not affected by dietary treatment (data not shown).

Results of the current experiment collectively indicate that diet had no effect on jejunal enzyme activity and therefore suggest that feeding a low CP, AA-supplemented diet to weaned pigs does not impair digestive capacity of the SI. This probably explains the results of Heo et al. (2008) who reported that feeding a 17% CP diet to pigs for 5, 7, 10 or 14 d after weaning had no negative effect on total tract apparent digestibility of DM, energy, and CP compared with pigs fed a 24% CP diets. Similar observation was made by Pierce et al. (2007). We have also previously demonstrated (Manuscript 1) that pigs fed a 21% CP diet and those fed a 17% CP diet had similar villus height which is often used an indicator of digestive and absorptive function of the SI (Hampson and Kidder, 1986; Ewtushik et al., 2000; Zhang and Xu, 2003). Adequate nutrition, including protein, is required to support intestinal development and function (Livshin et al., 1987; Nunez et al., 1996; Dudley et al., 1997). Since the analyzed AA composition of the high

CP and low CP diets (Table 17) showed that both diets met or exceed the AA requirement of 5 to 10 kg pigs (NRC, 1998), the low CP diet is not expected to have any negative effect on intestinal brush border enzymes as the case was in the current study.

CONCLUSIONS

The current experiment demonstrated that feeding a low CP diet, supplemented with Lys, Met, Thr, Trp, Ile and Val to piglets after weaning does not impair intestinal digestive enzyme development. Reduction of dietary CP from 22.3% to 17.8% with AA supplementation reduced SI weight without affecting intestinal mucosal weight, jejunal protein concentration and specific activities of jejunal enzymes. The heavier SI observed in pigs fed the high CP diet might be due to muscular serosal tissue accretion. The results confirm the fact that intestinal enzymes are differently regulated and that they respond differently to weaning.

CHAPTER 8

MANUSCRIPT 5

Intestinal response of weaned pigs to *Escherichia coli* K88 challenge when fed a low or a high crude protein diet

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ABSTRACT

Effects of dietary CP content on electrolyte concentration in digesta, mucin histochemistry, and intestinal indicators of infection and diarrhea in pigs challenged with enterotoxigenic *Escherichia coli* (**ETEC**) K88 were investigated in this study. Forty piglets, housed 4 per pen, were randomly allotted to 2 diets: a high, 22.2% CP diet and a low, 17.3% CP, AA-supplemented diet. Three piglets per pen were serially slaughtered from d 3 to 7 after weaning for evaluation of intestinal hydrolases (Manuscript 4). On d 8, the remaining pigs were inoculated with ETEC suspension (10^9 cfu/mL) and slaughtered at 20 h after challenge. Mucosa-associated ETEC K88 was detected in jejunum of 60% and 20% of pigs fed the high CP and low CP diet, respectively. Similar observation was made in jejunal digesta. Pigs fed the high CP diet tended to have a lower ($P = 0.09$) number of goblet cells with sialic mucins in the jejunal villi. Expression of jejunal sodium-coupled glucose transporter 1 was higher ($P = 0.04$) in pigs fed the high CP diet compared with those fed the low CP diet and this was accompanied by a numeric increase in Na^+ and Cl^- concentration. Expression of toll-like receptor 4 and 5 was not affected by dietary treatments. The results show that low dietary CP decreased colonization of jejunal mucosa by ETEC and reduced jejunal expression of sodium-coupled glucose transporter 1 in piglets challenged with ETEC K88.

Key words: dietary protein, electrolytes, *Escherichia coli*, sodium-coupled glucose transporter 1, toll-like receptors, weaned pigs

INTRODUCTION

Weaned pigs often suffer from post weaning diarrhea (**PWD**) which is mostly caused by enterotoxigenic *Escherichia coli* (**ETEC**) (Pluske et al., 2002; Fairbrother et al., 2005). This disease is usually controlled by supplementing starter diets with subtherapeutic levels of antibiotics (Verstegen and Williams, 2002). Currently, there are growing world-wide concerns over a possible link between the emergence of antibiotic-resistant bacteria and subtherapeutic usage of antibiotics (van den Bogaard and Stobberingh, 2000; Lusk et al., 2006). Hence, the use of nutritional interventions such as low CP, AA-supplemented diets have been suggested as possible alternative means of managing PWD in piglets (Prohaszka and Baron, 1980; Nyachoti et al., 2006).

The effect of dietary CP content on gut health in piglets challenged with ETEC has only been investigated in the gut lumen (Wellock et al., 2007; Opapeju et al., 2008; Heo et al., 2008). Since an interaction between intestinal epithelium and ETEC is required prior to initiation of PWD in piglets (Nataro and Kaper, 1998; Fairbrother and Gyles, 2006; Moeser and Blikslager, 2007), it would be logical to also evaluate possible benefits of low dietary CP content on gut health at the intestinal level. Sodium-coupled glucose cotransporter 1 (**SGLT1**) which mediates glucose transport across the intestinal brush border membrane also serves as a passive transporter of salt and water (Loo et al., 1999; Wright et al., 2007). Hence, we postulated that at the onset of diarrhea, when fluid and electrolyte flux into the intestinal lumen is initiated, intestinal expression of SGLT1 may be increased as part of host adaptive response to facilitate fluid and electrolyte absorption. Indeed, up-regulation of SGLT1 has been identified as the basis for the

WHO's oral rehydration therapy for treating acute diarrhea (Turk et al., 1993; Wright, 1993).

Toll-like receptors (**TLR**) are members of pattern-recognition receptors which are important components of innate immunity (Sandor and Buc, 2005; Kaisho and Akira, 2006; Uenishi and Shinkai, 2009). Toll-like receptor 4 recognizes cell wall lipopolysaccharide (**LPS**) from gram negative bacteria and TLR5 is involved in recognition of flagellin (Smith et al., 2003; Hawlisch and Kohl, 2006). Enterotoxigenic *E. coli* is a gram negative, flagellated bacterium (Fairbrother and Gyles, 2006). Hence, TLR4 and TLR5 could play a role in ETEC infection. Moue et al. (2008) reported that the expression of TLR4 and TLR5 was increased in porcine intestinal epithelial cells treated with LPS.

Abundance of acidic mucins in the mucus layer covering the gut epithelium protects against bacterial infection (Strous and Dekker, 1992). Deplancke and Gaskins (2001) suggested that *E. coli* may alter intestinal mucin chemistry to their own advantage by stimulating the synthesis of neutral mucins. The objective of the current experiment was to determine the effects of dietary CP content on intestinal indices of ETEC infection and diarrhea in piglets challenged with ETEC K88. The hypothesis tested in the current study was that high dietary CP protein would support ETEC proliferation and colonization of the small intestine, increase the small intestine expression of TLR4 and TLR5 and up-regulate the expression of SGLT1 at the onset of diarrhea.

MATERIALS AND METHODS

The experimental protocol was approved by the Protocol Management and Review Committee of the University of Manitoba Animal Care Committee. Pigs were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

Animals and Housing

Forty piglets (Duroc × (Yorkshire × Landrace); average initial BW = 6.96 ± 0.45 kg), weaned at 19 ± 1 d of age, were obtained from the University of Manitoba Glenlea Swine Research Unit. Pigs were of mixed gender (20 barrows and 20 gilts) and were from 7 litters. The pigs were randomly allotted to dietary treatments such that pigs from the same litter with similar BW and sex were allotted to 1 of the 2 diets. Pigs were housed in groups of 4 per pen resulting in 5 replicate pens per diet. Each pen had plastic-covered expanded metal floor (1.5 m x 1.16 m) and was equipped with a stainless steel self-feeder and a nipple-type drinker. Room temperature was maintained at 30°C with a 16-h lighting system. On d 3, 5 and 7 after weaning, one pig form each pen was euthanized for evaluation of intestinal hydrolases (Manuscript 4) leaving only 1 pig per pen.

Experimental Diets

Two experimental diets were formulated to be different in CP content: a high, 22.2% CP diet and a low, 17.3% CP diet (Manuscript 4). Diets were supplemented with

Lys, Met plus Cys, Thr, and Trp to achieve the ideal protein ratio suggested by Rademacher et al. (2009). In addition, the low CP diet was supplemented with Ile and Val up to the level in the high CP diet. The amount of sodium, chlorine and potassium were similar between the 2 diets and averaged 0.20, 0.21, and 0.48%, respectively. All other nutrients were supplied in amounts meeting or exceeding NRC (1998) nutrient standards. Feed ingredients contributing AA were analyzed for AA composition as described by Nyachoti et al. (2006) prior to diet formulation. Representative samples of the experimental diets were also analyzed for AA composition to ensure that formulation targets were met. Diets were offered to pigs as mash. Pigs had unlimited access to feed and water throughout the experimental period.

Bacterial Preparation and Oral Challenge

At 1300 h on d 8, piglets were challenged with ciprofloxacin-resistant ETEC K88 in order to differentiate the inoculum from the indigenous strains. Each pig received 6 mL of ETEC K88 (10^9 cfu/ml) suspension by gavage. The ETEC K88 strain was confirmed by PCR genotyping as possessing K88 fimbrial antigen, heat labile enterotoxin and heat stable enterotoxin genes using published primers (Kotlowski et al., 2007; Setia et al., 2009). The ETEC culture was grown in Luria Bertani broth (Becton, Dickinson and company, MN) containing 0.5 μg/ml ciprofloxacin (Sigma) at 37°C for 16 h. The culture was then centrifuged at 4,000 × *g* for 10 min to harvest the bacteria cells. The cell pellet was washed twice and re-suspended in sterile PBS. The concentration of the inoculum was confirmed by serial dilution and plating of the inoculum on Eosin Methylene Blue agar (Becton, Dickinson and company, Sparks, MD) containing 0.5 μg/ml ciprofloxacin.

Tissue and Digesta Collection

Pigs were anesthesized by an intramuscular injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Ontario, Canada) at 20 h after inoculation and were euthanized by an IV injection of sodium pentobarbital (50 mg/kg BW; Bimeda-MTC Animal Health Inc., ON, Canada). Immediately after midline laparotomy, two 10-cm, a 20-cm and a 60-cm sections of jejunum (10 cm distal of ligament of Treitz) were clamped and freed of mesentery. The first 10-cm segment was flushed with ice-cold physiological saline (0.9% NaCl) to remove digesta particles, snap-frozen in liquid N and stored at -80°C until used for mRNA analysis. The second 10-cm segment was fixed in 10% buffered formalin for histological measurements. The 20-cm segment was placed in a sterile sample bag (Fisher Scientific) for adherent ETEC count. Digesta from the 60-cm segment were divided into two sterile sample bags for ETEC count and electrolytes analysis. All the samples for bacterial count were placed on ice immediately after collection and transferred within 25 min to the laboratory. The digesta sample for electrolyte analysis was quickly frozen in liquid N and stored at -80°C until used for analysis.

Bacteria Enumeration

The jejunal segment was cut open longitudinally along the anti-mesenteric attachment plane; rinsed with sterile ice-cold physiological saline (0.9%) to remove any debris and then the mucosal tissue was scraped with a blunt sterile blade into a sterile tube. One gram each of jejunal mucosa and digesta was added to 9 ml of sterile peptone

water (0.1%), vortexed for 60 sec, and then serially diluted 10-folds (10^{-2} to 10^{-9}) in sterile peptone water. The ETEC K88 in the serially diluted samples was quantified using Eosin Methyl Blue (Becton Dickinson and Company, Sparks, MD) agar containing 0.5 μg/ml ciprofloxacin. The plates were incubated aerobically at 37°C for 24 to 36 h. Representative colonies were picked by random selection and were confirmed by PCR to possess heat labile enterotoxin, heat stable enterotoxin and K88 antigen. Fecal swab was collected from all pigs on arrival and plated on eosin methyl blue medium with ciprofloxacin (0.5 μg/ml) to screen them for the presence of ciprofloxacin resistant ETEC K88.

Electrolyte Determination

Digesta sample was centrifuged at 20,000 × *g* for 30 min at 4°C to separate the solids from the fluid (Hamilton and Roe, 1977). The supernatant was removed and filtered through a 0.45 μm syringe filter (Fisher Scientific). The filtrate was then analyzed for Na^+, K^+ and Cl^- using an ion chromatography (ICS 1000 Ion Chromatograph; Dionex Canada).

Histological Assessment

Staining of samples for histological measurements was done at the Veterinary Services Laboratory of Manitoba Agriculture and Rural Initiatives (Winnipeg, MB). Staining for carbohydrate histochemistry was done using either the periodic acid-Schiff reaction or the alcian blue reaction at either pH 2.5 or pH 1.0. The periodic acid-Schiff, alcian blue - pH 2.5 and alcian blue- pH 1.0 reactions stain for neutral mucins, non-

sulfated types of acidic mucin (sialomucins) and sulfated types of acidic mucins (sulfomucins), respectively (Brown et al., 2006). Images of the histological slides were captured on computer using a compound light microscope equipped with a video camera. Positively stained goblet cells in the villus and secretory cells in the crypts were counted within 10 well-oriented villi and crypts.

Tissue Preparation and RNA Extraction

Total RNA was extracted using the TRIzol reagent (Invitrogen™, Carisbad, CA) according to the manufacturer's instructions. Briefly, about 80 mg frozen jejunal sample was homogenized in 1 mL TRIzol using Ultra-Turrax T25 homogenizer (IKA®-Labortechnik, Staufen, Germany) and then mixed with 0.2 ml of chloroform, shaken vigorously and centrifuged at 12,000 × *g* for 15 min at 4°C. The upper aqueous phase was transferred to a clean 1.5 ml centrifuge tube and 0.5 ml of isopropanol was added to precipitate the RNA. The RNA pellet was harvested by centrifugation, rinsed twice with 75% ethanol and then re-suspended in 80 µl of diethyl pyrocarbonate treated water (Invitrogen™, Carisbad, CA). Total RNA was quantified using Du® 800 spectrophotometer (Beckman Coulter, Fullerton, CA) at a wave length of 260 nm. The mRNA purity was estimated using the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) and the value ranged from 2.0 to 2.1.

Reverse-transcription and Primer Design

Reverse transcription was performed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). One microgram of total RNA was used in a reaction volume of 20 µl. Random primers were used in cDNA synthesis. The program used was: 10 min at 25°C; 120 min at 37°C and 5 min at 85°C. Primers for quantitative real time PCR amplification of TLR4, TLR5 and SGLT1 were designed using Oligo™ Perfect Designer (Invitrogen) based on the nucleotide sequences obtained from GenBank. Beta-actin was used as a housekeeping gene. The primers were then synthesized by Core DNA Services (University of Calgary, Calgary, AB). Beta-actin primer was synthesized based on a published sequence (Duvigneau et al., 2005). The primers are described in detail in Table 19.

Real-time PCR

Real-time PCR reaction was carried out with a Step-One real time PCR system (Applied Biosystems, Foster City, CA). The cDNA was diluted to 5 ng/µl. The 20 µl reaction mixture contained 2 µl cDNA, 1 µl each of specific forward and reverse primers (500 nM) and 10 µl of Fast SYBR® Green master mix (Applied Biosystems, Foster City, CA). Amplification was done in triplicates. Prior to mRNA quantification, primer concentrations were optimized with respect to formation of primer dimer, and primer melting temperature. The PCR cycling conditions were as follows: a 20 sec enzyme activation at 95°C, followed by 40 cycles of 3 sec denaturation at 95°C and 30 sec

Table 19. Nucelotide sequences of PCR oligonucleotide primers used in this study

Gene	Primer sequence (5'- 3')		Product size (bp)	Source/GenBank accession number
	Forward	Reverse		
TLR4	TCCCTCAGGATTCTGGATTG	CGTTCCTGAAACTCAGCACA	244	NM001113039
TLR5	CTGGAAGCCTTCAGTTACGC	TGTTGAGAAACCAGCTGACG	187	NM001123202
SGLT1	GGCTGGACGAAGTATGGTGT	ACAACCACCCAAATCAGAGC	153	M34044
β-actin	CTCGATCATGAAGTGCGACGT	GTGATCTCCTTCTGCATCCTGTC	114	Duvigneau et al., 2005

annealing at 60°C. A melting curve analysis was performed after the completion of the last cycle to verify specific amplification. Water was used as the negative control and no PCR product was detected in it. Results were normalized to the housekeeping gene β-actin using the comparative cycle threshold method.

Statistical Analysis

The GLM procedure of SAS (version 9.1, SAS Inst., Inc., Cary, NC) was used to analyze all data. The model included the effects of gender, litter of origin and treatment. The effects of gender and litter were not significant and were eliminated from the model. Means were compared using Students t- test and differences were considered significant at $P < 0.05$. Tendencies were accepted at $0.05 < P < 0.10$.

RESULTS

Fecal swabs collected before the challenge showed that none of the pigs tested positive for ciprofloxacin-resistant ETEC. Enterotoxigenic *Escherichia coli* K88 was detected in jejunal digesta of all pigs fed the high CP diet but was detected only in 3 out of 5 pigs fed the low CP diet (Figure 17). The ETEC count averaged 3.65 ± 1.44 and 2.49 ± 0.85 $\log_{10}$ cfu/g digesta for pigs fed the high CP and low CP diets, respectively. In the jejunal mucosa, ETEC K88 colonies were detected in 4 (3.17 ± 0.63 $\log_{10}$ cfu/g) out of 5 pigs fed the high CP diet and in 1 (2.00 $\log_{10}$ cfu/g) out of 5 pigs fed the low CP diet (Figure 17).

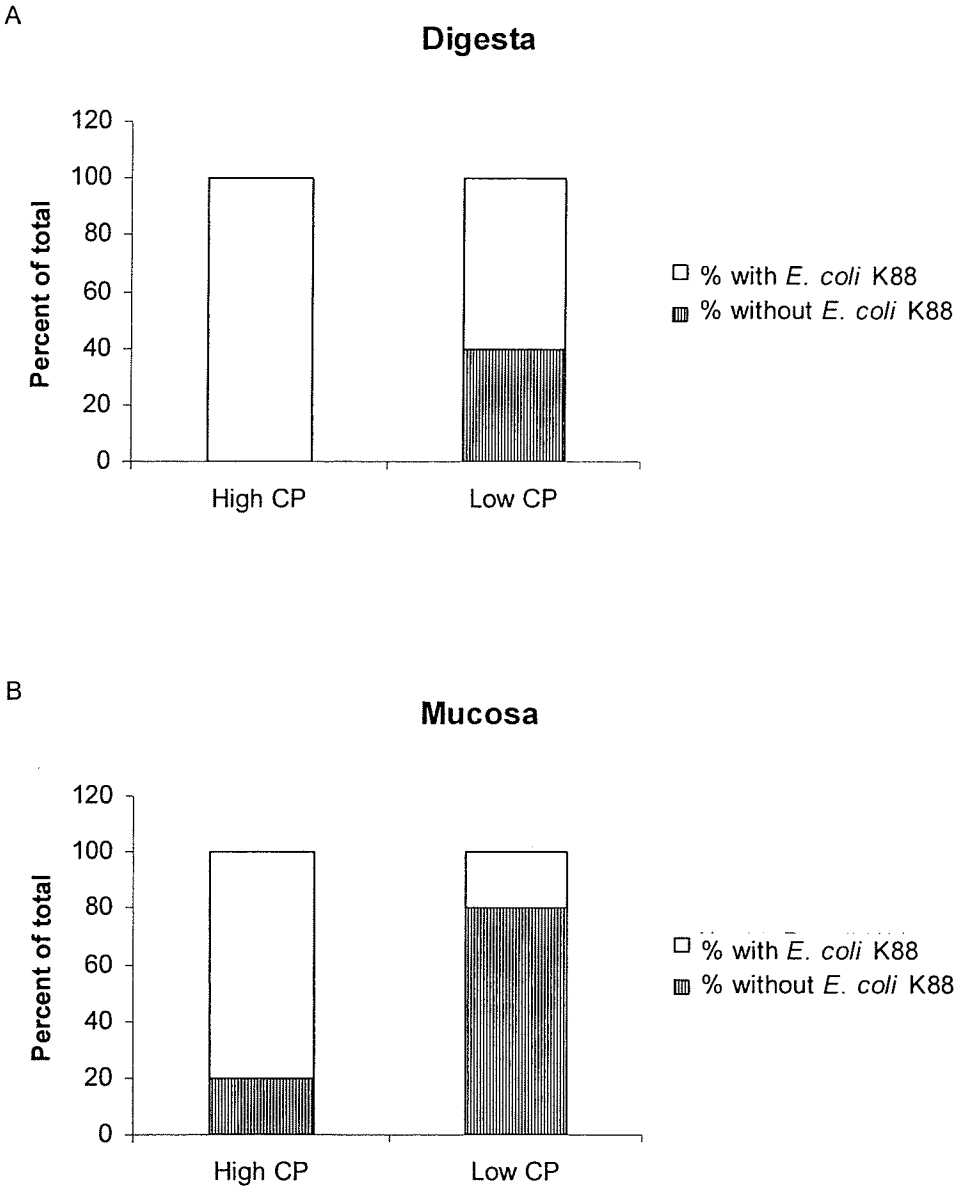

Figure 17. Effect of dietary crude protein (CP) level on *Escherichia coli* K88 population in the ileal digesta (A) and ileal mucosa (B) of weaned pigs challenged with *Escherichia coli* K88.

In jejunal villus, diet had no effect on number of goblet cell with sulfo- and neutral mucins. However, pigs fed the high CP diet tended to have lower ($P = 0.09$) number of goblet cells with sialomucins in their villi compared with those fed the low CP diet (Table 20). There were no effects of diet on goblet cells with sulfo-, sialo- and neutral mucins in jejunal crypts.

There was no effect of diet on the expression of TLR4 and TLR5 (Figure 18). The expression of SGLT1 was higher ($P = 0.04$) in jejunum of pigs fed the high CP diet compared with those fed the low CP diet (Figure 19). Diet had no effect on the electrolyte concentration in the jejunal digesta (Table 21).

DISCUSSION

Post weaning diarrhea is a major source of economic loss to the swine industry (Cutler et al., 2007) and it is mostly caused by ETEC (Fairbrother et al., 2005; Nagy and Fekete, 2005; Fairbrother and Gyles, 2006). The pathogenesis of ETEC K88 diarrhea involves the colonization of small intestinal mucosal surface by ETEC followed by secretion of their enterotoxins which then stimulate electrolyte and fluid secretion into the intestinal lumen (Nataro and Kaper, 1998; Nagy and Fekete, 1999). Previous studies have shown that low CP, AA-supplemented diets improved indicators of enteric health and reduced the incidence of diarrhea in weaned pigs compared with high CP diets (Manuscript 1; Heo et al., 2008; Wellock et al., 2008a). Apart from the architectural evaluation of the small intestine (**SI**) (Manuscript 2; van den Bogaard and Stobberingh, 2000; Wellock et al., 2008a), effect of dietary protein content on intestinal response to ETEC challenge has not been investigated. Thus, the current experiment determined the

Table 20. Effect of dietary crude protein (CP) level on goblet cell count in the jejunum of weaned pigs challenged with *Escherichia coli* K88[1]

Item	High CP	Low CP	SEM	*P*-value
Sulfomucins				
Villus	2.38	3.16	0.375	0.180
Crypt	8.10	9.02	0.385	0.130
Acidic mucins				
Villus	1.64	2.80	0.430	0.093
Crypt	7.94	9.12	0.684	0.257
Neutral mucins				
Villus	1.66	2.60	0.393	0.130
Crypt	7.36	7.78	0.369	0.444

[1]Each value represents the mean of 5 pigs. Pigs were slaughtered 20 h after the challenge.

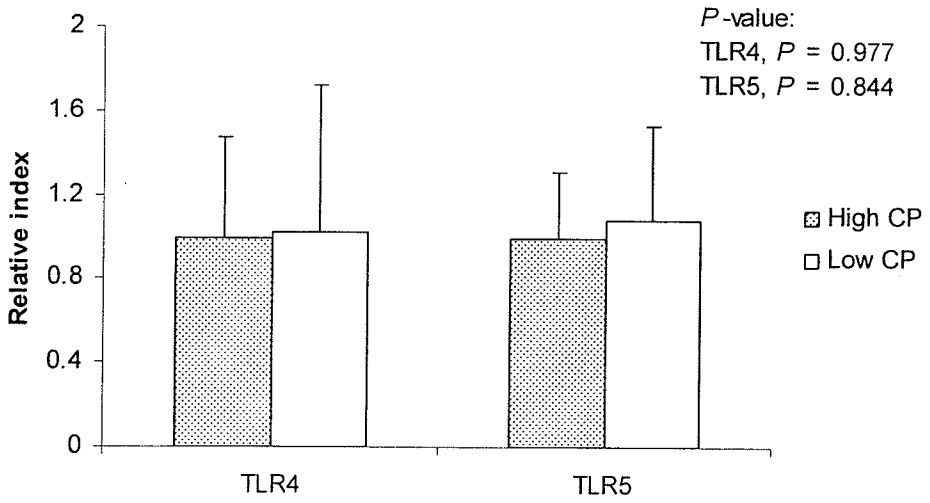

Figure 18. Effect of dietary crude protein (CP) level on the expression of toll-like receptor 4 (TLR4) and toll-like receptor 5 (TLR5) in jejunum of weaned pigs challenged with *Escherichia coli* K88.

Values are means ± SEM, n = 5. *P*-value represents the effects of diet.

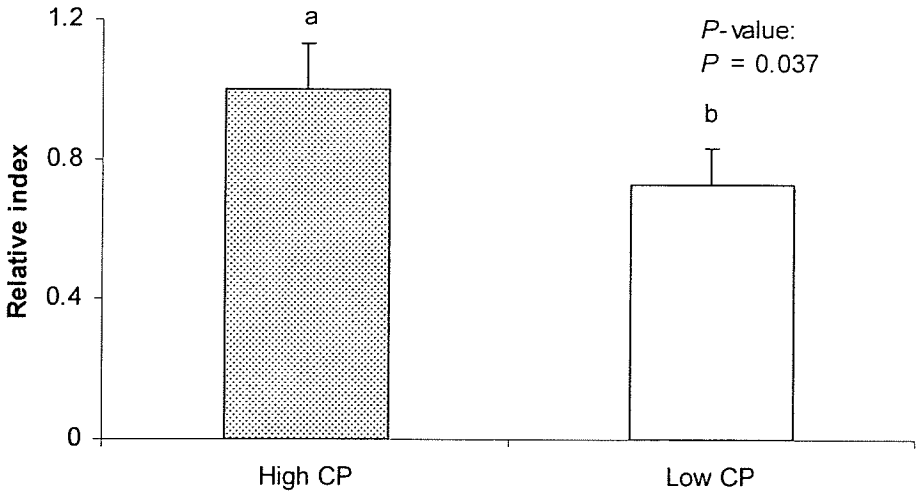

Figure 19. Effect of dietary crude protein (CP) level on sodium-coupled glucose transporter 1 expression in jejunum of weaned pigs challenged with *Escherichia coli* K88.

Values are means ± SEM, n = 5. Means without a common letter differ, $P < 0.05$. *P*-value represents the effects of diet.

Table 21. Effect of dietary crude protein (CP) level on the concentration of electrolytes in jejunual digesta of weaned pigs challenged with *Escherichia coli* K88[1]

Electrolytes, mmol/L	High CP	Low CP	SEM	*P*-value
Na^+	98.9	83.8	7.14	0.173
K^+	8.9	8.6	0.64	0.749
Cl^-	80.6	67.3	5.91	0.151

[1]Each value represents the mean of 5 pigs. Pigs were slaughtered 20 h after the challenge

effects of dietary CP level on the intestinal indices of infection and diarrhea in weaned pigs challenged with ETEC.

Enterotoxigenic *Escherichia coli* K88 was detected in the jejunal digesta of all pigs fed the high CP diet compared with 60% of those fed the low CP diet. In addition, ETEC K88 count in pigs fed the high CP diet was one log higher than those fed the low CP diet. These observations further confirm the notion that high dietary CP content could increase the amount of substrate available for ETEC growth in the gut lumen. For example, it has been demonstrated previously that ETEC proliferation is favoured by high dietary CP content (Prohaszka and Baron, 1980; Wellock et al., 2008a). The number of ETEC expressing K88 fimbriae in jejunal mucosa was evaluated in order to check whether the ETEC actually adhered to the SI and that responses reported in the current study were due to ETEC infection. Mucosa-associated ETEC count was also evaluated to measure how dietary protein level would influence the degree intestinal colonization by ETEC. We have used mucosa-associated ETEC count as an indicator of adherent ETEC in our previous studies (Kiarie et al., 2008b; Bhandari et al., 2009). Mucosa-associated ETEC K88 was detected in 80% of the pigs fed the high CP diet compared with only 20% of the pigs fed the low CP diet. This, to the best of our knowledge, is the first study to report the effect of dietary CP content on adherent ETEC and it indicates that ETEC colonization of the SI was reduced in pigs fed the low CP diet compared with those fed the high CP diet. This is probably due to reduced proliferation of ETEC in the gut lumen of pigs fed the low CP diet as indicated by the luminal ETEC count data. As observed for the jejunal digesta, ETEC count in jejunal mucosa of pigs fed the high CP diet was one log higher than that of pigs fed the low CP diet.

Mucins, secreted by goblets cell, are the predominant component of the mucus layer covering the epithelial surface of the gastrointestinal tract (Montagne et al., 2004). They protect the epithelial surface from acidic luminal content, endogenous and bacteria digestive enzymes and colonization by pathogenic bacteria (Deplancke and Gaskins, 2001; Montagne et al., 2004). In the current experiment, there was a tendency for lower numbers of goblet cells with sialic mucins in the jejunal villi of pigs fed the high CP diet compared with those fed the low CP diet, which suggest that the low CP diet could offer more protection against ETEC colonization of the mucosal surface. Sialo- and sulfo-mucins are acidic, more mature and more beneficial in maintaining gut health compared with the neutral mucins (Roberton and Wright, 1997; Nieuw et al., 1998; Deplancke et al., 2000). Lack of dietary CP content effect on the crypt mucin histochemistry suggest that the observed trend for a lower number of goblet cells with sialomucins in jejunal villi of pigs fed the high CP diet was due to mucin loss and not mucin synthesis.

There is scarcity of data on effect of dietary CP content on intestinal mucin histochemistry in pigs. However, dietary CP content has been shown to influence endogenous N losses and possibly mucin flow in the SI of pigs (Mariscal-Landin et al., 1995; Eklund et al., 2008). Mariscal-Landin et al.(1995) suggested an increase in mucin excretion, based on the ileal hexosamine concentration, as dietary CP content increased from 5.5% to 16.5%. Since dietary protein source could also influence mucin endogenous flow (Nyachoti et al., 1997), protein sources were kept constant between the 2 diets used in the current study.

Toll-like receptors play a crucial role in the initiation of the innate immune response (Kaisho and Akira, 2006). Innate immunity serves as the first line of defence

against infection and helps to shape the subsequent adaptive response (Medzhitov and Janeway, 1997; Fleer and Krediet, 2007). Toll-like receptor 4 and TLR5 recognize LPS and flagellin, respectively (Hayashi et al., 2001; Smith et al., 2003; Hawlisch and Kohl, 2006). Hence, it was hypothesized in the current study that high dietary CP content will provide a suitable condition for colonization of intestinal epithelial surface by ETEC K88 and will, therefore, increase the expression of intestinal TLR4 and TLR5 in pigs challenged with ETEC. Contrary to our hypothesis, expression of TLR4 and TLR5 was not affected by dietary treatment.

It is unclear why there was no dietary effect on jejunal expression of TLR4 and TLR5 despite the fact that mucosa-associated ETEC K88 was detected in most pigs fed the high CP diet. However, four possible explanations are proposed here. First, the presence of other bacteria with LPS and flagellin in the gut probably masked the response of TLR4 and TLR5 to ETEC infection. For instance, lipid A, a component of LPS that is responsible for its immunological activities is conserved in both pathogenic and non-pathogenic strains of *E. coli* (Harris et al., 2006). Second, LPS and flagellin might not be the major contributing factors in the pathogenesis of ETEC. Harris et al. (2006) suggested that *E. coli* LPS may have minimal interaction with TLR4 due to selective relocalization of TLR4 from outer apical membrane into the cytoplasm by commensal bacteria LPS as demonstrated by Cario et al. (2002). Although enteroaggressive *E. coli* derived flagellin has been shown to mediate immunological responses in human cell lines (Steiner et al., 2000; Donnelly and Steiner, 2002) and in mice (Roy et al., 2009), the role of ETEC flagellin in activating immune response in pigs is unknown. Third, other receptors apart from TLR4 and TLR5 may be involved in initiating immune response to ETEC infection

in pigs. For example, ETEC challenge had no effect or depressed the expression of TLR4 in porcine intestinal epithelial cells but it up-regulated the expression of TLR2 and Th1 cytokines (Moue et al., 2008). Fourth, the observation that effect of diet on serum IL-1β concentration (Manuscript 3) was relatively short term probably influenced the TLR4 and TLR5 results such that dietary effects were not observed at 20 h after challenge.

Sodium-coupled glucose transporter 1 is the basis for the WHO oral rehydration therapy for treating diarrhea (Turk et al., 1993; Wright, 1993). With the transport of 1 molecule of glucose, SGLT1 transports 2 molecules of Na and 200-260 molecules of water (Loo et al., 1999; Wright et al., 2007). Also, SGLT1 serves as a passive Na^+ and water transporter in the absence of sugar (Loo et al., 1999). In the current experiment, SGLT1 expression in the jejunum was evaluated as an indicator of intestinal fluid and ion transport. The expression of SGLT1 was higher in pigs fed the high CP diet compared with those fed the low CP diet. Since high dietary CP has been associated with higher incidence of diarrhea in pigs (Nyachoti et al., 2006; Heo et al., 2008; Wellock et al., 2008a), this observation was probably a reflection of host adaptive defence mechanism to the initiation of diarrhea considering the fact that intestinal samples were obtained at 20 h after challenge.

Unlike rotavirus infection (Halaihel et al., 2000; Boshuizen et al., 2003), ETEC infection does not impair nutrient/water absorption capability of the intestine except in cases of severe fluid loss and dehydration (Field, 2003; Moeser and Blikslager, 2007). Under commercial conditions, PWD usually last 4 to 14 d and in severe cases death may occur (Fairbrother and Gyles, 2006). Post-weaning diarrhea is a multi-factorial disease and using experimental challenge models to reproduce diarrhea disease similar to that

often encountered in the field is difficult (Madec et al., 2000). Although we did not measure the incidence of diarrhea in the current study, our previous studies (Manuscript 2; Bhandari et al., 2009) and those of others (Wellock et al., 2008b) showed that the experimental ETEC challenge model hardly results in severe diarrhea or mortality in piglets weaned at 17 d of age or more. Piglets suffering from mild diarrhea usually recover without any medication. The results of the current experiment thus suggest that increase in the absorption of fluid and electrolyte from the gut lumen via SGLT1 is one of the mechanisms underlying the recovery.

Fluid and electrolyte secretion into the gut lumen were expected to be higher in pigs fed the high CP diet compared with those fed the low CP diet. It is possible that the higher expression of SGLT1 in the jejunum of pigs fed the high CP diet might have facilitated the absorption of electrolytes from the gut lumen and thereby masking dietary effects on digesta electrolyte concentrations. We did not quantify the impact of infection on secretion of electrolyte in the current study, however, Na^+ concentration observed in the jejunal digesta of pigs fed the low CP diet was similar to the value reported for the unchallenged pigs (Hamilton and Roe, 1977). Sodium, K^+ and Cl^- concentrations were kept constant between the diets in order to rule out confounding effects of dietary electrolyte concentration on the results.

CONCLUSION

The results of the current experiment showed that high dietary CP content resulted in a greater colonization of the jejunal mucosal by ETEC K88 at 20 h after challenge and

this was accompanied by an increase in the expression of SGLT1. Increase in jejunal SGLT1 expression in pigs fed the high CP diet might be a result of initial host adaptive response to ETEC infection.

CHAPTER 9

GENERAL DISCUSSION

Diarrhea caused by ETEC is a common disease of weaned pigs and it often has serious production and financial consequences (Nataro and Kaper, 1998; Hayes et al., 2002; Amezcua et al., 2002). This disease has been managed in the past by adding pharmaceutical levels of trace minerals such as ZnO and $CuSO_4$ and sub-therapeutic levels of antibiotics to the piglet diets. However, due to increasing emergence of antibiotic resistant strains of bacteria and its possible link to in-feed antibiotics, the EU has banned the use of subtherapeutic levels of antibiotics in livestock diets. Likewise, there is an ongoing interest to completely eliminate similar usage of antibiotics from livestock diets in North American and around the globe. There are also environmental concerns over supplementation of pharmacological levels of inorganic trace minerals to piglets due to the excretion of excessive metal ions in feces. Hence, the swine industry is currently under pressure to find effective alternative(s) to in-feed antibiotics. Nutritional and management strategies have been suggested as possible means of minimizing the incidence and severity of PWD.

The use of diets low in CP content and supplemented with crystalline AA has been suggested as one of the nutritional strategies for managing enteric disorders associated with weaning (Nyachoti et al., 2006; Stein and Kil, 2006; Wellock et al., 2006b). However, in comparison to high CP diets, the growth performance of weaned

pigs fed low CP diets supplemented with Lys, Met, Thr and Trp is often impaired and this was suggested to be due to imbalance of some other essential AA such as Ile and Val. In addition, there is paucity of information on the effects of low CP, AA-supplemented diets on performance and health of weaned pigs under conditions of greater enteric infection with ETEC. Although previous studies showed improvement in indices of gut health when piglets were fed low CP, AA-supplemented diets, most of these studies were conducted in research facilities under hygienic conditions which are not representative of commercial production conditions. Thus, the general objective of this project was to evaluate the role of low CP, AA-supplemented diets as a dietary tool in managing PWD diarrhea and enhancing gut health in weaned pigs.

With the primary goal of optimizing production, low CP, AA-supplemented diets will only become an acceptable routine feeding system in the swine industry if it does not compromise performance. Thus, the first experiment in this thesis (Manuscript 1) was conducted to determine whether Ile supplementation, in addition to Lys, Met, Thr and Trp, to a low CP diet is required in order to support the similar growth performance of piglets as high CP diets and to determine the effect of dietary CP content on indicators of gut health. The AA supplementation was done according to the ideal protein ratio suggested by Rademacher et al. (2000). The overall results of this feeding study showed that pigs fed the high CP (21%) diet had higher overall ADG and G:F ratio compared with those fed the 19% CP diets with and without Ile supplementation and 17% with Ile and Val supplementation. This observation is in agreement with our previous study (Nyachoti et al., 2006) but contrary to the report of Le Bellego and Noblet (Le Bellego and Noblet, 2002) and to our hypothesis that Ile supplementation to a low CP diet would

yield similar performance as the high CP diet. The results should be interpreted with caution since the low CP diet supplemented with Ile in addition to Lys, Met, Thr and Trp had a slightly lower analyzed AA composition compared with the calculated values which may have confounded the effects of dietary treatment on pig performance. Generally, indicators of enteric health were improved in pigs fed the low CP diets. Incidence of diarrhea as indicated by FC score was higher in pigs fed the high CP diet compared with those fed low CP diets. In agreement with the results of Nyachoti et al. (2006), low dietary CP content reduced the concentration of ammonia N in cecal digesta indicating a reduced proteolytic fermentation. Crypts were deeper in the duodenum and ileum of pigs fed the high CP diets compared with other treatments, which is in agreement with the report of Gu and Li (2004). Crypt hypertrophy observed in pigs fed the high CP diet may be in part due to increased ammonia N concentration because crypt depth and ammonia N were positively correlated. High concentration of intestinal ammonia is toxic and could increase the turnover rate of the intestinal epithelial cells (Visek, 1978). It was also observed that pigs fed the 21% CP diet had higher empty SI weight compared with those fed the 17% CP diet. This observation might be a sign of impaired GIT development in pigs fed the 17% CP diet (Nunez et al., 1996) or an indicator of excessive viscera growth in those fed the 21% CP diet (Le Bellego and Noblet, 2002).

To further understand the role of low dietary CP content in mitigating the effects of PWD in piglets, Experiment 2 (Manuscript 2 and 3) was conducted to determine the responses of weaned piglets to ETEC K88 challenge when fed a high (22.5%) CP diet or a low (17.6%) CP diet supplemented with Lys, Met, Thr, Trp, Ile and Val according to

the ideal protein ratio. This approach was used to allow induction of PWD and evaluation of the impact of dietary CP content on performance and gut health of piglets under conditions similar to those in commercial situations where there is a high pressure of enteric infection. In Manuscript 2, effect of dietary CP level on growth performance, gut health and gut ecology in piglets challenged with ETEC K88 was evaluated. The results showed that pigs fed the high CP diet outperformed those fed the low CP diet prior to infection (d 1 to 7 post-weaning) but similar performance was observed between the 2 treatments after infection and overall. The impact of infection seemed to be greater in pigs fed the high CP diet such that ADG from d 3 to 7 post-challenge increased by 13% in pigs fed the low CP diet and reduced by 3% in pigs fed the high CP diet. Wellock et al. (2008b) on the other hand reported that piglets fed a high CP diet (23%) had higher ADG and better G:F compared with those fed a low CP (13%) diet after ETEC challenge. Discrepancy between the 2 studies is probably a reflection of differences in the CP content of the low CP diets.

Severe diarrhea was not observed in any of the piglets after ETEC challenge suggesting sub-clinical induction of PWC. However, pigs fed the low CP, AA-supplemented diet had a lower FC score and a lower ETEC count in the ileal, colon and rectum digesta compared with those fed the high CP diet. Similar results have also been reported by other authors (Wellock et al., 2007; Heo et al., 2008; Wellock et al., 2008a). At d 3 after challenge, ETEC was detected in 80% and 0% of pigs fed the high and low CP diets, respectively. The result is in support of the hypothesis that high dietary CP level will increase the amount of substrate available for ETEC proliferation in the gut. Diets high in CP content may buffer stomach contents (Prohaszka and Baron, 1980) and, hence,

could cause an increase in gastric pH. A rise in gastric pH provides suitable environment for ETEC proliferation (Smith and Jones, 1963). Previous evaluation of the effect of dietary CP content on gut ecology was determined using the culture-dependent approach (Nyachoti et al., 2006; Wellock et al., 2008a). Using culture-independent technique, it was demonstrated for the first time that low dietary CP content increased the prevalence of genus Roseburia in the colon digesta. Roseburia species are butyrate-producing bacteria and they preferentially ferment carbohydrates. Thus, it was proposed that low CP diets supported the prevalence of such bacteria by reducing the protein:carbohydrate ratio in the hind gut.

Indices of enteric health were improved with low dietary CP level before and after ETEC challenge. The high CP diet increased ammonia N concentration in colon digesta before and after ETEC challenge but had no effect on VFA concentration. Deeper crypts and shorter villi were observed in pigs fed the high CP diet before and after ETEC challenge. This observation further confirms greater intensity of infection in pigs fed the high CP diet. Pathogenesis of PWD involves colonization of SI surface by ETEC (Nagy and Fekete, 2005) and this could result in villus atrophy.

Manuscript 3 evaluated the effect of dietary CP level on inflammatory-associated responses in piglets challenged with ETEC K88. Only IL-1β responded specifically to ETEC K88 challenge while TNF-α concentration increased non-specifically to ETEC at 24 h before challenge. High dietary CP content resulted in a higher serum IL-1β (72 vs. 116 pg/mL) at 8 h after challenge compare with the low CP diet. Likewise, serum concentration of Hp tended to be higher in pigs fed the high CP diet compared with those fed the low CP diet at 8 h after challenge (9 vs. 25 mg/dL). Higher count of ETEC in the

gut of pigs fed the high CP diet (Manuscript 2) could partly explain the higher concentration of IL-1β in the serum of those pigs. During inflammation, pro-inflammatory cytokines mediate synthesis of APP in the liver which are then released into circulation in response to various immunological challenges (Kushner, 1993). During acute phase response, IL-1β and TNF-α stimulate catabolism of skeletal muscle protein into free AA for APP synthesis (Johnson, 1997). The excessive non-limiting AA for APP synthesis would be oxidized and would thus increase ureagenesis (Brown and Cline, 1974). Indeed, plasma urea N concentration was elevated at 72 h post-infection in pigs fed the high CP diet. However, dietary treatment had no effect on weight gain during the post-infection period. This is probably due to the fact that the observed spike in serum IL-1β after ETEC challenge in pigs fed the high CP diet was transient, only at 8 h after challenge, and thus skeletal muscle proteolysis for acute phase protein synthesis may not have had any long lasting effect on weight gain (Manuscript 2).

The effect of dietary CP content on intestinal development was determined in Manuscript 4. This experiment was conducted to determine whether a lower SI weight observed in pigs fed the 17% CP diet compared with those fed the 21% CP diet in Manuscript 1 has a negative impact on brush border enzyme development. As observed in Manuscript 1, pigs fed the low CP diet had smaller empty SI weight compared with those fed the high CP diet. However, diet had no effect on intestinal mucosal weight suggesting that the increased SI weight in pigs fed the high CP diet was probably due to an increase in muscular serosal tissue mass. Visceral organ hypertrophy with an increase in dietary CP content has also been reported by others (Schoknecht and Pond, 1993; Le Bellego and Noblet, 2002). The jejunal protein, peptidases and disaccharidases

concentration were not affected by dietary treatments suggesting that feeding a low CP, AA-supplemented diet to weaned pigs does not impair digestive capacity of SI. This observation supports the results of others that low dietary CP content has no negative effect on nutrient digestibility (Pierce et al., 2007; Heo et al., 2008).

Evaluation of the effects of dietary CP level on gastrointestinal health in piglets has been limited to indicators at the gut lumen level such as ammonia N concentration, digesta VFA concentration, biogenic amines and digesta pH. In Experiment 1 and 2, it was demonstrated that low dietary protein content reduced the luminal number of ETEC K88 in the small and large intestine of weaned pigs and reduced the incidence and severity of PWD. However, aetio-pathology of PWC involves considerable interaction between intestinal gut epithelium and ETEC (Nataro and Kaper, 1998). Hence, evaluation of the role of dietary CP content on development of PWC at the intestinal level would increase our understanding on gut health benefits and/or potential mechanism of action of low CP, AA-supplemented diet. The objective of the manuscript 5 was to determine the effect of dietary CP content on intestinal response of weaned pigs to ETEC challenge. The results show that adherent ETEC was detected in 60% and 20% of pigs fed the high and low CP, AA-supplemented diet, respectively. Jejunal villi of pigs fed the high CP diet tended to have a lower number of goblet cells with sialic mucins compared with those of pigs fed the low CP diet. Sialic mucins are acidic, more mature and more beneficial in maintaining gut health compared with the neutral mucins (Roberton and Wright, 1997; Nieuw et al., 1998). On the contrary, diet had no effect on mucin histochemistry in the crypts suggesting that the trend for higher number of acidic mucins

in the villus of pigs fed the low CP diet compared with the high CP diet was due to mucin loss rather than synthesis.

Sodium-coupled glucose transporter 1 mediates glucose transport across the intestinal brush border membrane and also serves as passive transporter of salt and water (Loo et al., 1999; Wright et al., 2007). It is the basis for the WHO oral rehydration therapy for treating diarrhea (Turk et al., 1993; Wright, 1993). The hypothesis was that the initiation of diarrhea will drive SGLT1 expression up as part of the host adaptive response to diarrhea. In agreement with our hypothesis, pigs fed the high CP diet had higher expression of SGLT1 in their jejunum compared with those fed the low CP diet at 20 h after ETEC challenge. Although not statistically significant, Na^+ and Cl^- concentration were 18 and 20%, respectively, higher in pigs fed the high CP diet than those fed the low CP diet. Taken together, these results are in agreement the results of Manuscript 1 and 2 that low dietary CP content reduced the incidence of diarrhea in piglets.

Toll-like receptors are members of innate immunity. Toll-like receptor 4 and TLR5 recognize LPS and flagellin, respectively, (Hayashi et al., 2001; Smith et al., 2003; Hawlisch and Kohl, 2006) and may thus play a role in initiating immunological responses to PWC. Dietary treatment had no effects on the expression of TLR4 and TLR5 suggesting that these receptors might not contribute much to the pathogenesis of PWC.

Overall, the studies demonstrated that feeding a low CP diet supplemented with crystalline AA according to the ideal protein pattern are beneficial in improving the structure and health the GIT of piglets during the immediate post-weaning period as indicated by the small intestine morphology and hind gut ammonia N concentration, and

in minimizing the incidence and severity of PWD. The studies also demonstrated that low CP diets supplemented with Lys, Met, Thr, Trp, Ile and Val reduced the impact of infection on growth performance of weaned pigs. These observations suggest that in the absence of in-feed antibiotic, low CP, AA-supplemented diets may help to improve the health status of piglets and minimize the effects of PWC, especially when pigs are housed under suboptimal conditions.

CHAPTER 10

CONCLUSSION AND FUTURE DIRECTIONS

CONCLUSION

Based on the results obtained in this thesis, it can be concluded that:

1. Supplementation of a low CP diet with crystalline AA according to the ideal protein pattern improved the indicators of GIT health and reduced the risk of PWD. Specifically, FC score, VH:CD ratio, and indices of proteolytic fermentation such as ammonia N concentration were reduced when low CP, AA-supplemented diets were fed to weaned pigs.

2. Feeding a low CP diet to weaned pigs reduces the impact of ETEC K88 challenge on growth performance suggesting a greater benefit of low CP, AA-supplemented diet under conditions that pose intestinal health challenge to piglets.

3. The results showed that the low CP, AA-supplemented diet reduced luminal ETEC K88 count in the small and large intestine of piglets as well as the percentage of pigs with *E. coli* K88 compared with the high CP diet. These results support the hypothesis that feeding a low CP diet, supplemented with AA to piglets may help to minimize the occurrence and severity of PWD by reducing ETEC proliferation in the GIT.

4. The low CP diet, supplemented with AA increased the prevalence of butyrate producing bacteria and minimized damage to the gut architecture in weaned pigs challenged with ETEC K88 compared with the high CP diet.

5. Enterotoxigenic *E. coli* K88 challenge increased the serum concentration of IL-1β in weaned pigs fed the high CP diet compared with those fed the low CP diet only at 8 h after challenge indicating a relative short-term effect of infection on pro-inflammatory cytokine. This might be due to sub-clinical rather than clinical induction of PWC.

6. Compared with the high CP diet, the low CP, AA supplemented diet reduced the weight of the SI without affecting the protein concentration and activities of brush border enzymes in the jejunum of piglets. Thus, the results demonstrated that digestive enzyme development is not impaired when a low CP, AA-supplemented diet is fed to nursery pigs.

7. After ETEC challenge, the number of jejunal adherent ETEC and the percentage of animal with mucosa-associated ETEC were higher in pigs fed the high CP diet than those fed the low CP diet. The result indicates a greater colonization of jejunal mucosa by ETEC in pigs fed the high CP diet.

8. Concomitant with the increase in number of adherent ETEC in the jejunum of piglets fed the high CP diet was an increase in the expression of SGLT1. The increased expression of SGLT1 in the jejunum of pigs fed the high CP diet might be a direct result

of the host adaptive response to ETEC infection and/or diarrhea giving the fact that gene expression was determine at 20 h after ETEC K88 infection.

9. Dietary CP level had no effect on the expression of TLR4 (recognizes LPS) and TLR5 (recognizes flagellin) in the jejunum of pigs challenged with ETEC. This suggests that TLR4 and TLR5 may not be sensitive indicators of innate immune response to ETEC infection or that the relative short-term effect of ETEC infection observed on serum IL-1β concentration at 8 h after challenge probably influenced jejunal expression of TLR4 and TLR5 such that the effect of diet was not observed at 20 h after challenge.

10. Overall, low CP diets fortified with crystalline Lys, Met, Thr, Trp, Ile and Val according to the recommended ideal protein pattern offers a potential viable nutritional tool for enhancement of GIT health in piglets during the immediate post-weaning period.

FUTURE DIRECTIONS

1. Additional research should be conducted to determine the effect of Ile and/or Val supplementation to a low CP diet in addition to Lys, Met, Thr and Trp on immediately post-weaning and subsequent growth performance of piglets. Supplementation of a low CP diet with crystalline AA can increase the feed cost significantly considering the fact that Ile and Val are not available commercially. Thus, future studies should evaluate the economics of feeding a low CP, AA-supplemented diet to piglets. In essence, will the implementation of a low dietary CP content feeding program in a commercial farm

reduce the overall cost of production putting into consideration the potential health problems associated with feeding a high CP diet?

2. Future studies should evaluate the effect of dietary CP content on stomach and pancreatic digestive enzymes development in weaned pigs. Effects of low CP, AA-supplemented diet on nutrient absorption and transport in weaned pigs should also be evaluated.

3. Studies should be conducted to explore potential synergistic effects of feeding a low CP, AA-supplemented diet and other feed additives with gut health benefits to piglets. For example, the observed prevalence of butyrate producing bacteria in the colon digesta of pigs fed the low CP diet on the hind gut butyric acid production should be investigated further through the addition fermentable carbohydrates to the low CP diet.

4. Potential roles of low CP, AA-supplemented diet in modulating infection-associated responses at the intestinal level should be investigated further. The scope of such studies should include the effects of low CP-AA supplemented diet on pro- and anti-inflammatory cytokines, inflammatory cells, members of the innate and adaptive immune system, gut associated lymphoid tissue and proteins involved in fluid and electrolytes secretions.

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