DIETARY MEANS FOR ENHANCED GASTROINTESTINAL HEALTH AND FUNCTION IN WEANED PIGS: AN EVALUATION OF CARBOHYDRASE ENZYMES TARGETING NON-STARCH POLYSACCHARIDES

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By

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DEDICATION

My Dear Wife Alice and our Adoring Daughter Mary Faith Gituanjah

My Parents Richard and Mary Kiarie

My Siblings (Evans, Leonard, Michael and Jane)

In memories of my friend and paternal grandmother

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FOREWORD

This thesis was prepared following a manuscript format. There are five manuscripts corresponding to five chapters. Manuscripts I and IV are published in the Journals of Animal Science and Nutrition, respectively. Manuscript II has been accepted in the Canadian Journal of Animal Science. Manuscript III has been submitted to the Journal of Animal Science. Manuscript V is under preparation. All manuscripts are formatted to meet the guidelines for the Journal of the Animal Science.

ABSTRACT

A major challenge for the pig industry is to formulate starter diets that primarily fit the digestive capacity, maintain GIT health and promote growth without recourse to in-feed antimicrobials. Experiments were conducted to evaluate the efficacy of carbohydrase enzymes (CE) targeting non-starch polysaccharides (NSP) in enhancing gut health and function in piglets. First, an experiment was conducted to evaluate the effects of adding CE in piglet diets on growth performance, GIT bacterial activity and nutrient digestibility. Pigs fed diets containing CE had a higher ileal lactobacilli count, total organic acids concentrations, NSP digestibility and low ammonia compared with control. The effectiveness of CE targeting NSP was further evaluated using enterotoxigenic *E. coli* (ETEC) in a challenge model to evaluate the impact on gut health and function. Two approaches for the ETEC challenge were adopted; an *in situ* small intestine segments perfusion model and an *in vivo* model. Initially, a pilot study was conducted to establish and validate the *in situ* model. In the pilot study, conventional anti-diarrhea agents; fumaric acid, ZnO, egg yolk antibodies against ETEC K88 fimbriae and carbadox, attenuated fluid losses in ETEC-infected jejunal segments. Following the establishment of the *in situ* model, four experiments were conducted to study the effects NSP hydrolysis products (HP) from various feedstuffs (i.e. wheat, soybean meal, canola meal and flaxseed) on ETEC-induced secretory diarrhea. The results demonstrated that HP protected against ETEC-induced fluid and electrolyte losses. A further study was conducted to investigate the response of piglets fed diets containing HP and EYA singly or in combination upon oral challenge with ETEC. Feeding HP and EYA alone or in combination attenuated ETEC-enteritis symptoms such that piglets fed additives showed

less pronounced acute phase responses and superior performance. Piglets fed diets containing additives had lower gastric pH, fewer ETEC adhered to ileal mucosa and lower incidence of diarrhea. Overall, reduction of intestinal pathogens or toxic bacterial metabolites contributes to enhanced GIT health and function. These novel results expand the scope of enzyme technology in animal nutrition within the paradigm of dietary approaches to gut health and function.

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LIST OF ABBREVIATIONS

AGP	Antibiotic growth promoters
ADFI	Average daily feed intake
ADG	Average daily gain
BCVFA	Branched chain volatile fatty acids
CD	Crypt depth
CE	Carbohydrase enzymes
DF	Dietary fiber
ETEC	Enterotoxigenic *Escherichia coli*
EYA	Egg yolk produced against ETEC K88 fimbriae
FOS	Fructo-oligosacharides
FS	Flaxseed
GIT	Gastrointestinal tract
GR	Growth rate
Hp	Haptoglobin
HP	NSP hydrolysis products
IL-1β	Interleukin 1β
IL-10	Interleukin 10
IL-6	Interleukin 6
MEm	Metabolizable energy for maintenance
MOS	Manno-oligosaccharides
NSP	Non-starch Polysaccharides

OA	Organic acid
PUN	Plasma urea nitrogen
PWC	Post weaning collibacillosis
PWD	Post weaning diarrhea
SDP	Spray dried plasma
VCR	Villi height to crypt depth ratio
VFA	Volatile fatty acid
VH	Villi height

CHAPTER ONE

GENERAL INTRODUCTION

Newly weaned pigs are simultaneously subjected to nutritional (e.g. loss of sow's milk), psychological (e.g. mixing and moving) and environmental (e.g. change in ambient temperature) stressors (Lallès et al., 2004; Main et al., 2004). It is difficult to unravel the contributions made by each of these factors to the growth stasis observed during the weaning period. However, post-weaning anorexia and attendant nutritional stress in concert with immature digestive and immune systems predisposes the piglet to gastrointestinal tract (GIT) disturbances (Whittemore and Green, 2001; Kelly and King, 2001).

To minimize the effects of weaning, baby pigs are often fed diets fortified with sub-therapeutic levels of antibiotic growth promoters (AGP) (Anderson et al., 1999). However, long-term use of AGP has been linked to the potential problem of increasing transferable resistance of bacteria to antimicrobial drugs (Doyle, 2001; Adjiri-Awere and Van Lunen, 2005). Because swine producers commonly rely on AGP to optimize production, there is considerable concern that the loss of AGP will severely impact the industry, especially during stressful periods like weaning (Casewell et al., 2003). As a consequence, considerable efforts are being made to seek alternative or replacement strategies for controlling enteric bacterial diseases and maintenaning functional piglet GIT (Close, 2000; Doyle, 2001; Pettigrew, 2006; Stein and Kil, 2006).

Currently, several compounds have been assessed and promoted as alternatives for AGP. These includes acidifiers, minerals (zinc and copper salts), prebiotics,

probiotics and last but not least immune active products (Close, 2000; Doyle, 2001; Pettigrew, 2006; Stein and Kil, 2006). While there is some evidence that these additives may positively influence weaning transition, including controlling enteric diseases, the majority of them have elicited some concerns which have hampered wider application. For instance, for minerals to be effective they ought to be included in the diet at pharmacological levels, which leads to excess loading in the environment (Close, 2000). Acidifiers have problems which range from handling, equipment corrosion, deleterious effects on parietal cells maturation and development of resistant zoonotic bacteria (Partanen and Mroz, 1999; Richard and Foster, 2003; Bosi et al., 2006). Most of the commercial probiotics are derived from products designed for the human market (Stein and Kil, 2006) and there are concerns about survivability in the GIT ecology prevailing at weaning. Another problem of probiotics is the perception that they are genetically modified (Sanders and Klaenhammer, 2001). Nevertheless, recent research (e.g. Huang et al., 2004) has evaluated probiotics isolated from the piglet normal microbiota or (e.g. Bhandari et al., 2007) non-pathogenic colicinogenic *E. coli* isolates which if fully developed present tremendous opportunity in controlling infectious diarrhea since the GIT ecology prevailing at weaning supports proliferation of such bacteria (Mathew et al., 1996).

Prebiotics appear to be promising but these products are included in weaner diets at the expense of much needed nutrients in addition to their cost (Mathew, 2002). Furthermore, since they are largely carbohydrate in nature, it follows that they might also be generated in the GIT *in situ* from complex dietary NSP if starter diets are supplemented with appropriate cabohydrase enzymes (CE) preparations (Pluske et al.,

2002). Immune active products, especially antibodies developed against pathogenic antigens-hold great promise because they are specific and they by-pass the need for rather immature GIT immune system to exert their effects (Marquadt et al., 1999). In this context, egg yolk antibodies (EYA) from chickens hyperimmunized with *E. coli* K88 fimbriae have been shown to be effective in controlling post weaning diarrhoea (Imberechts et al., 1997; Marquardt et al., 1999; Owusu-Asiedu et al., 2002, 2003a, b). Because hen eggs are such a rich source of immunoglobulins that are easily purified, they present tremendous opportunity for prevention and perhaps treatment of common infectious diarrhea of farm animals.

Carbohydrase enzymes targeting dietary NSP are routine additives in piglet diets to improve nutrient utilization (Partridge, 2001). To achieve this end, CE breakdown NSP to release encapsulated nutrients; in this process a variety of hydrolusis products (HP) are released (Slominski, 2000; Chesson and Stewart, 2001; Meng et al., 2005). Since these HP will not be absorbed by the host, they could support development of a healthy gut microflora or their carbohydrate moieties may act as surrogate attachment sites for pathogens commensurate to exogenous prebiotics (Chesson and Stewart, 2001; Pluske et al., 2002). However, whether HP would modulate post weaned piglet GIT disturbances remains to be determined. Characterizing protective effects of HP against enteric pathogens prevalent during the weaning transition would aid in further designing and development of new generation of CE for baby pig diets.

Therefore, the **main objective** of this research was:

To evaluate the efficacy of CE targeting NSP in enhancing gut health and function in weaned pigs.

To achieve the main objective the following **specific objectives** were formulated:

i.   To investigate the effect of a CE supplement on GIT bacteria activity in weaned pigs.

ii. To evaluate the effects of HP on net absorption of fluids and electrolytes in enterotoxigenic *E. coli* (K88) infected piglet jejunal segments.

iii. To investigate the response of piglets fed diets containing HP and EYA upon oral challenge with enterotoxigenic *E. coli* (K88).

CHAPTER TWO

LITERATURE REVIEW

2.1 The Weaned Pig and its Potential for Growth

Naturally the pig would become nutritionally independent of its dam when at 15-20 kg BW and weaning would occur at 70 d of age (Whittemore and Green, 2001). However, over the past 50 years, the weaning age in commercial intensive swine production systems has been decreased to 17-35 d in conventional and 12-14 d in off-site segregated early weaning systems (Nabuurs, 1998; Maxwell and Carter, 2000; Main et al., 2004). Without doubt, advances in nutritional knowledge as well as management and in particular the manufacture of specialized nurseries have made early weaning achievable (Whittemore and Green, 2001).

The growth performance of the young pig from birth to 8-9 wk of age is critical in determining subsequent weight-for-age relationships and, as such, ultimate weight at slaughter (Whittemore, 1998; Kings et al., 1999; Le Dividich and Séve, 2001). Using published literature, Le Dividich and Séve (2001) modeled the growth rate (GR) patterns of pigs during both suckling and weaning phases (from birth to 18-22 kg) as shown in Figure 2.1. Clearly, the GR of the piglet plateau half-way through the suckling period and drastically reduces following weaning at 21 d of age. This pattern of piglet growth is typical in commercial swine production irrespective of weaning age (Whittemore, 1998; Kings et al., 1999).
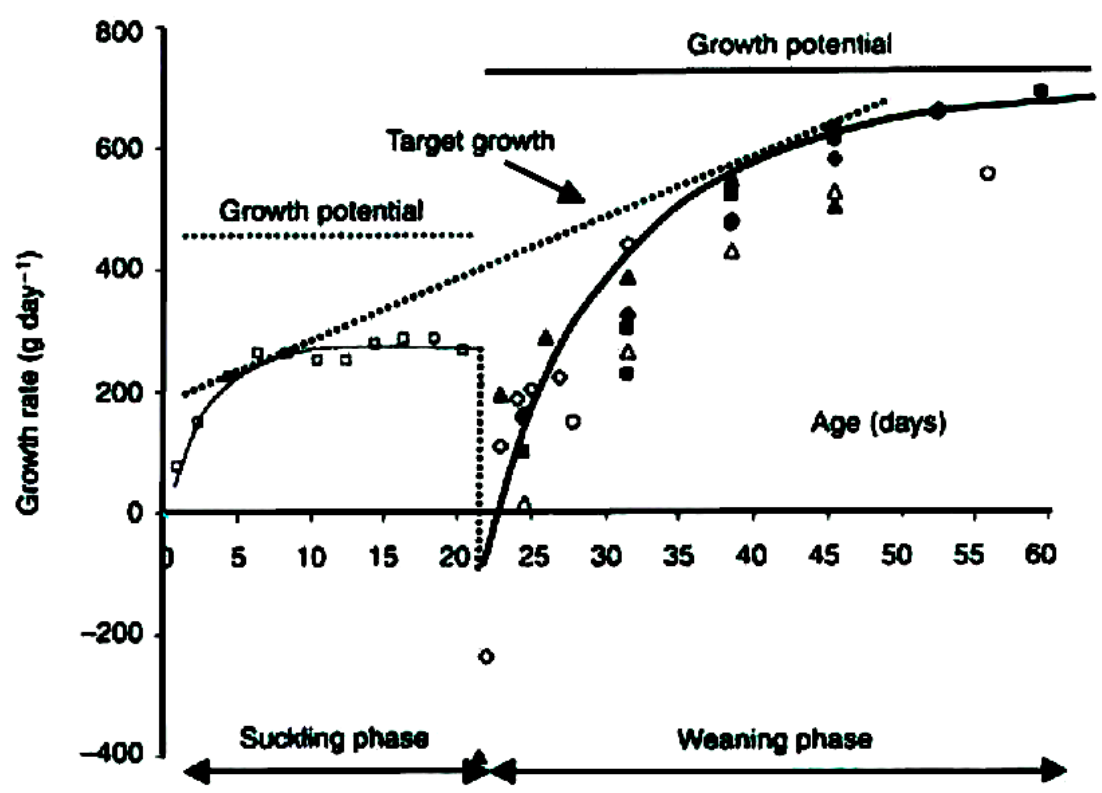
Figure 2.1. Growth rate pattern of piglets during the suckling and weaning phases (Le Dividich and Séve, 2001) (Used with permission of CAB International, March 26, 2008).

In contrast, live weight gains as high as 400-550 and 700-800 g/d have been recorded during the suckling and weaning phases, respectively, in artificial rearing studies (Hodge, 1974; Harrell et al., 1993). This suggests that the biological growth potential of both suckling and weaned pigs is much higher than the growth observed under commercial production. However, it is difficult to achieve GR commensurate to artificial rearing systems in commercial systems, but, a target GR superior to what is commonly observed is achievable if attention is paid to the periods where the deficit in GR is most pronounced (Le Dividich and Séve, 2001; Williams, 2003). Indeed, Whittemore (1998) pointed out that, given appropriate conditions, weaned pigs will grow at rates substantially above the commercial norm. In the present review the weaning phase will further be described with the aim of identifying dietary strategies for enhancing piglet performance during this phase. However, it is noteworthy that the performance of the neonatal piglet after weaning is highly dependent on its weight among other factors and readers are refereed to excellent reviews on neonatal pig development and survival (Harell et al., 1993; CABI, 1995; Whittemore, 1998; King et al., 1999).

2.2 The Weaning Process and Piglet GIT Biology

The process of weaning and its effect on the GIT biology is depicted in Figure 2.2 Newly weaned pigs are simultaneously subjected to nutritional (e.g. loss of sow's milk), psychological (e.g. mixing and moving) and environmental (e.g. change in ambient temperature) stressors (Funderburke and Seerley, 1990; Whittemore and Green, 2001; Lallès et al., 2004; Main et al., 2004). It is difficult to unravel the contributions made by each of these factors to the weaning growth stasis discussed earlier.
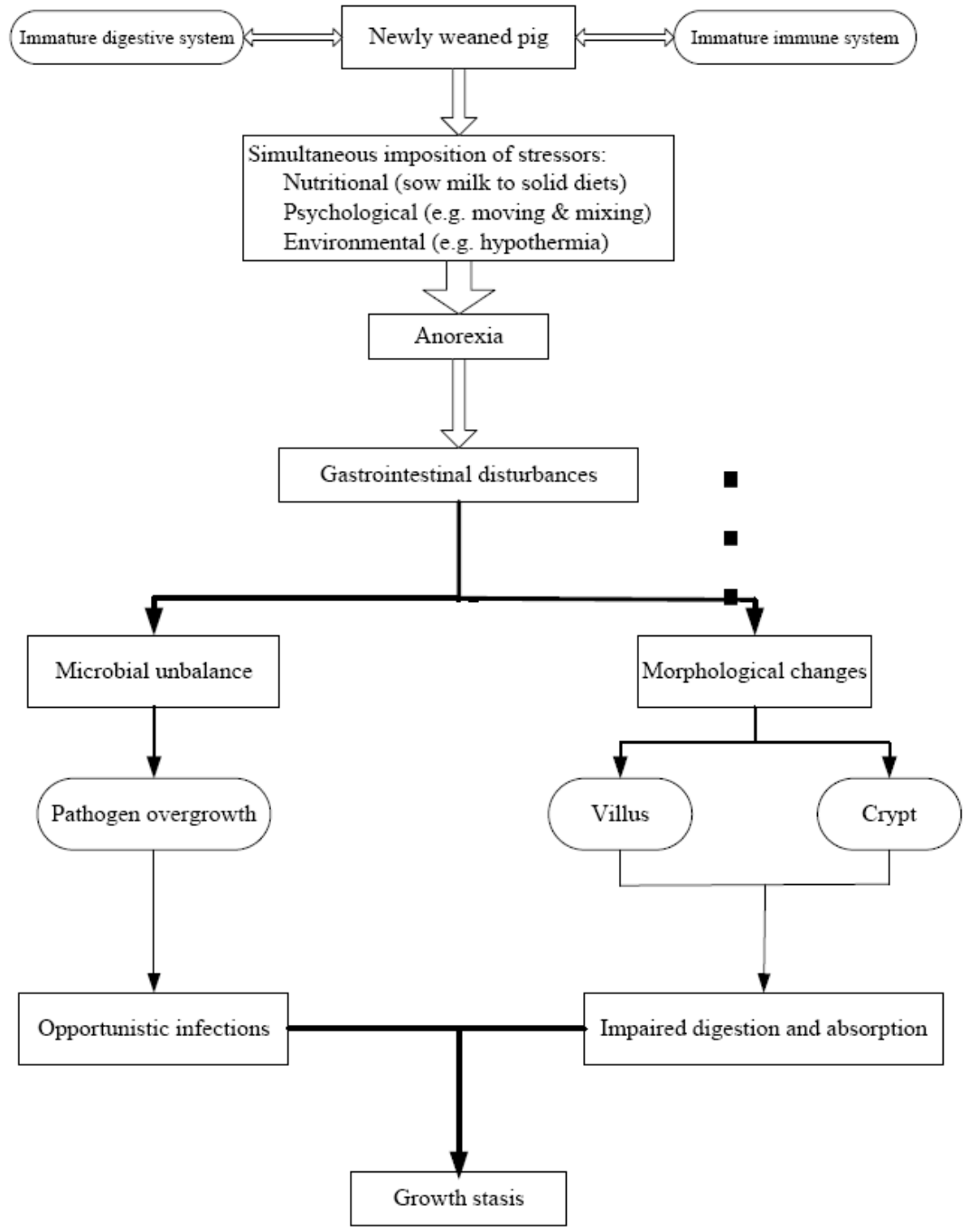
Figure 2.2. Review diagram of piglet post weaning challenges

However, there is an obvious nutritional stress when the pig's diet of sow's milk is abruptly changed to a solid diet (Whittemore and Green, 2001). Indeed it has been documented that, energy requirements for maintenance are only met 3 d later, and it can take 8-14 d for piglets to recover their pre-weaning level of energy intake (Figure 2.3; Le Dividich and Séve, 2000; Brooks et al., 2001).

Weaning anorexia and under-nutrition are associated with marked GIT disturbances characterized by reduced digestive and absorptive capacity and enteric diseases which persist for up to 14 d after weaning (Pluske et al., 1997; Lallès et al., 2004). Understanding the GIT biology of the weaned pig is a prerequisite for developing dietary strategies to ease weaning transition. Subsequently, the following sections focus on recent insights into GIT physiological and structural elements, microbiology and immunology as a basis for dietary management of GIT function and health in pigs around weaning.

2.2.1 Physiological and Structural Elements

The introduction of a solid diet at weaning to the GIT still adapted to milk is accompanied by adverse changes in physiology and morphology of the GIT until the piglet develops the mechanisms to digest this new diet (Kelly and King, 2001). Basically, this adaptation may be considered in two main parts. The first part is the stomach and the small intestine. This part of the digestive tract must be adapted to digest the solid food by changing enzymatic secretion, secreting more acid and adapting to the flow rate of the digestive content.
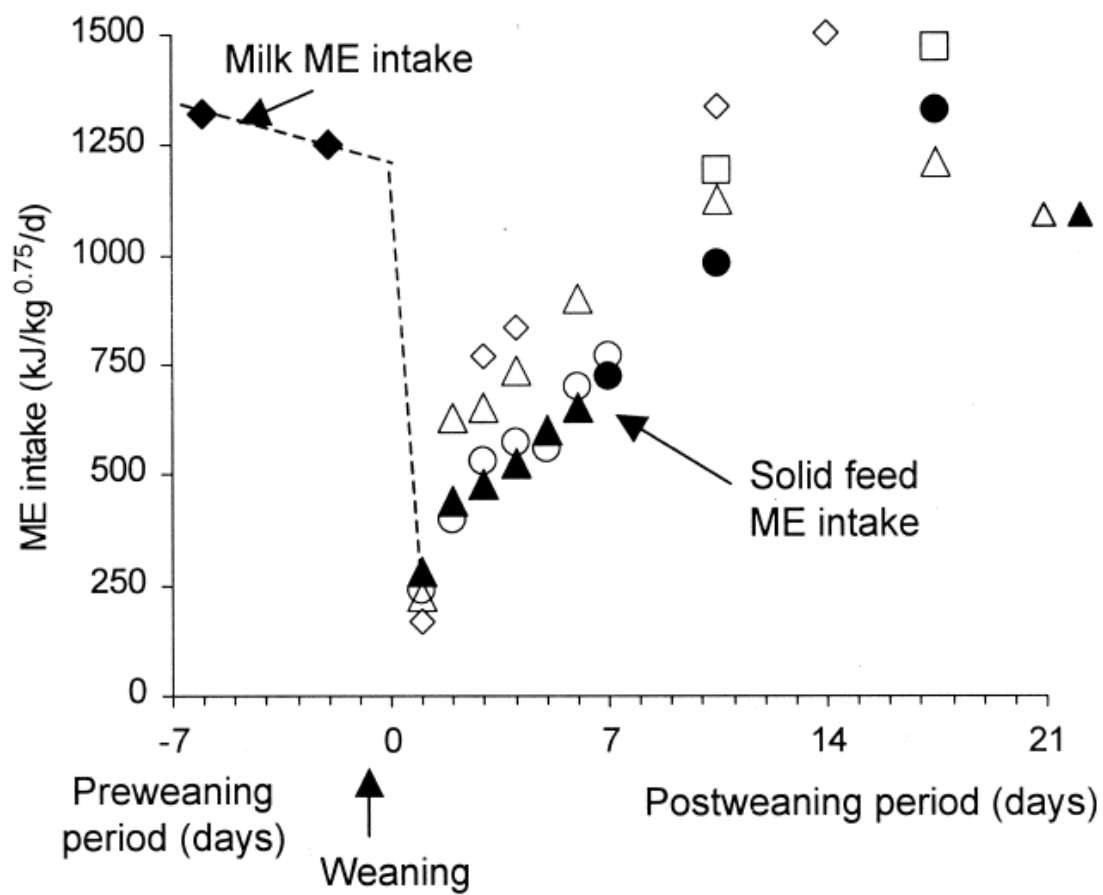
Figure 2.3. Effect of weaning between 3 and 4 wk of age on voluntary metabolizable energy intake in piglets (Le Dividich and Séve, 2000) (Used with permission of Elsevier Limited, March 25, 2008).

The second part is the hindgut. In the adult pig, the hindgut serves as a fermentation vat for the organic matter, especially the fiber fraction of the diet exiting the ileal-cecal junction. Milk has no fiber and thus little or no fermentation occurs in the hindgut during suckling. When a solid diet is ingested for the first time, digestibility is reduced and a higher proportion of the undigested diet reaches the large intestine. The undigested material supplies fermentative substrates to the microflora in the large intestine, thereby promoting physiological and functional development (Aumaître et al., 1995; Kelly and King, 2001). The stomach, small intestine and large intestine are very important for optimum digestion of the weaning diet (van Beers-Schreurs et al., 1998). However, the most studied and important changes in this period take place in the small intestine (Kelly and King, 2001).

2.2.1.1 Brief Description of the Small Intestine Epithelium

The intestines display various functions including absorption of nutrients, absorption and secretion of electrolytes (and water), secretion of mucin and immunoglobulins and selective barrier protecting against harmful antigens and pathogens (Lallés et al., 2004). The intestinal epithelium is organized in a folded structure to maximize the contact surface between the external and internal media. The structural units of this system are the villi and the Lieberkühn crypt (Buddle and Bolton, 1992). The major cells lining the intestinal epithelium are enterocytes; others are endocrine cells, immune cells and goblet cells (Kelly and King, 2001). Enterocytes are enlongated to maximize their absorptive surface and goblet cells secrete mucus to protect the epithelium.

The intestinal epithelium is renewed via the migration of enterocytes from the crypts to the top of the villi (Kelly and King, 2001). Normally, enterocytes show secretory functions when they are in the crypt and later develop an absorptive function when they migrate to the villi. It follows that net absorption in the small intestine depends on the villi-crypt ratio (Buddle and Bolton, 1992). During the migration, the enterocyte develops its principal functions: digestive and absorptive (Dahlqvist and Nordström, 1965; Yen, 2001). Once on the top of the villi, enterocytes are eliminated by different mechanisms: mechanical forces, pancreatic enzymes, bile, pepsin, bacterial aggressions, e.t.c. (Clarke, 1973). However, the extent of cell proliferation in the crypts and enterocyte loss from the villi are modified by the type of microbial flora present and the type of diet fed (Pluske et al., 1997; Willing and van Kessel, 2007). Thus, the renewal of the intestinal epithelium is a consequence of a dynamic equilibrium between production of enterocytes in the crypt and desquamation in the villi (Clarke, 1973). The renovation of enterocytes is slower in the neonatal piglet (7-10 d) than in adult pigs (3-4 d), thus, piglets need a longer time for the villi to recover their original villous height after injury (Buddle and Bolton, 1992). This difference is due to the shorter villi and deeper crypts in adult pigs (Moon, 1971). Within different parts of the small intestine, the ileum presents the fastest renewal rate because villi are shorter (Buddle and Bolton, 1992).

2.2.1.2 Changes at Weaning

Post-weaning changes in intestinal tissue, including changes in villi and crypt architecture and depressed activities of many brush-border digestive enzymes are well documented (see review by Pluske et al., 1997). Many changes in intestinal physiology occur in piglets upon weaning (Boudry et al., 2004). Pre-weaning villi and crypt integrity

are usually observed again 3 to 5 d after weaning. However, long-lasting changes are also recorded for up to 2 wk after weaning (Pluske et al., 1997).

Weaning induces villi atrophy and crypt hyperplasia (Pluske et al., 1997). Villi atrophy is caused by either an increased rate of cell loss or a reduced rate of cell renewal (Pluske et al., 1997). Deeper crypts reflect a higher enterocytes production to maintain villi height (Smith, 1992). The ratio of villi height to crypt depth (VCR) is a useful criterion for estimating the likely digestive and absorptive capacity in the small intestine and it is logical to expect that the reduction in VCR can induce transitory absorption problems (Kelly and King, 2001). Indeed, reduction in enzyme activities of the intestinal brush border membranes has been linked to a decrease in VCR (Kelly et al., 1991). Enterocytes obtain part of their nutrients from the intestinal lumen and the lack of nutrients due to transitory anorexia can induce cellular damage (Nuñez et al., 1996), loss of intestinal barrier integrity, and inflammatory reactions (McCracken et al., 1999). Thus, on weaning, the new diet and the low feed intake induce impaired nutrient absorption and damage in the epithelial barrier. When this happens, a third factor acquires a dominant role: the intestinal microbiota.

2.2.2 Microbiology

2.2.2.1 Normal Microbiota

The pig GIT harbors a numerically dense and metabolically active microbiota comprising mainly bacteria. Porcine intestinal microflora is established within 48 h after birth via ingestion of maternal feces, and involves complex successional changes until dense, stable populations colonize the GIT (Ewing and Cole, 1994; Mackie et al., 1999; Gaskins, 2001). Colonization is a process by which a population of bacteria in the GIT

becomes stable in size over time without the need for periodic reintroduction (Gaskins, 2001). A distinction between indigenous (autochthonous) and non-indigenous (allochthonous) bacteria is required for an ecological understanding of colonization, succession and mechanisms of host interactions. Autochthonous bacteria are those that have co-evolved with the host and colonize all habitats and niches in the GIT, whereas allochthonous bacteria may pass through specific microhabitats, being derived from food, water or another gut habitat, and do not colonize the tract (Pluske et al., 2002; Inoue et al., 2005; Konstantinov et al., 2006).

The pig GIT displays an increasing gradient of indigenous microbes from the stomach to the colon. In addition, a characteristic radial distribution of organisms is evident within each gut compartment (Gaskins, 2001). With respect to radial distribution, four microhabitats have been described; the intestinal lumen, the unstirred mucus layer, the deeper mucus layer in the crypt and the surface of the intestinal epithelium (Gaskins, 2001). The stomach and proximal small intestine (duodenum) contain relatively low numbers of bacteria (10^3-10^5 cfu/g or ml of contents) due to low pH and/or rapid digesta flow (Ewing and Cole, 1994). Whilst the piglet is still suckling, the dominant bacteria within the stomach and small intestine tend to be *Lactobacillus* and *Streptococcus* (Jensen, 1998). In the proximal small intestine, digesta flow rate and the rate of bacterial washout exceeds the maximal growth rates of most bacterial species and the bacteria that are present typically adhere to the mucus or epithelial cell surface (Gaskins, 2001). In contrast, the distal small intestine harbors a more diverse and numerically greater (10^8 cfu/g or ml of contents) population of bacteria. The large intestine is the major site of microbial colonization because of the long residence time of the digesta. The luminal

contents of the colon support in excess of 400 different bacterial species with numbers as high as 10^{10} and 10^{11} cultivable cfu/g (wet weight) of digesta (Ewing and Cole, 1994). The hindgut flora is considered both diverse and stable, with the many species and strains appearing to coexist without one or few ever becoming dominant. Further information pertaining to porcine GIT microbiota composition and diversity can be found in reviews by Stewart (1997), Mackie et al. (1999), Gaskins (2001), Jensen (2001) and Leser et al. (2002).

The normal microbiota in the pig GIT is responsible for a plethora of functions including intestinal development and functionality (as evidenced by differences seen between gnotobiotic and conventional animals), effects on nutrient digestion and absorption, mucus secretion, immune development, cytokine expression and exerting colonization resistance against pathogens (van der Waaij et al., 1971; Gaskins, 2001; Zoetendal et al., 2004). In addition, microbial fermentation within the GIT is very important for the pig GIT health (Williams et al., 2001). The main products of fermentation include OA, which are known to play an important role in water and electrolyte absorption and pH control (Williams et al., 2001; Pluske et al., 2002).

Adherence of bacteria to the intestinal mucosa can be of benefit to both the host and bacterium. For the host, these mechanisms promote colonization with commensal organisms and help to prevent the attachment of pathogens (competitive exclusion), and for the bacterium, adhesion offers an attachment surface from which they can withstand processes such as mucous flow and peristalsis (Kelly and King, 2001). Arguably, understanding how epithelial cells in the GIT differentiate between pathogenic and non-pathogenic bacteria offers tremendous insight into the molecular basis for such processes,

and ultimately could provide strategies for the control of pathogens and (or) regulation of GIT microbiota to optimize production. The intestinal microbiota is normally maintained in equilibrium and evolves with the age of the animal as discussed earlier. However, abrupt changes such as weaning (Dunsford et al. 1989), fasting periods, changes in diet (Brunsgaard, 1998; Hopwood and Hampson, 2003) and total parenteral nutrition (Ganessunker et al, 1999) can tip this equilibrium with the consequence of pathogens becoming dominant.

2.2.2.2 Changes at Weaning

Weaning diet and associated anorexia is expected to impact on GIT microbiota mainly by changing/depleting substrate available for microbial fermentation. As a consequence, during the first week post-weaning the microbiota becomes especially unstable (Gaskins, 2001). For instance, major quantitative and qualitative changes in microbial activity have been described in the ileal digesta of weaned pigs using conventional (Figure 2.4, panel A, Mathews et al., 1996) and molecular methods (Figure 2.4, panel B, Inoue et al., 2005). The consequence of this microbiota disruption in the period immediately following weaning is that piglets become susceptible to overgrowth of potential disease-causing pathogenic bacteria (Hopwood and Hampson, 2003; Pluske et al., 2002).

2.2.3 Immunology

2.2.3.1 Intestinal Defense Mechanisms

The intestinal defense system includes complex interactions between epithelium, external mucous layer and immune system. The epithelium acts as a physical barrier between the lumen and the lamina propria (Gaskins, 2001).
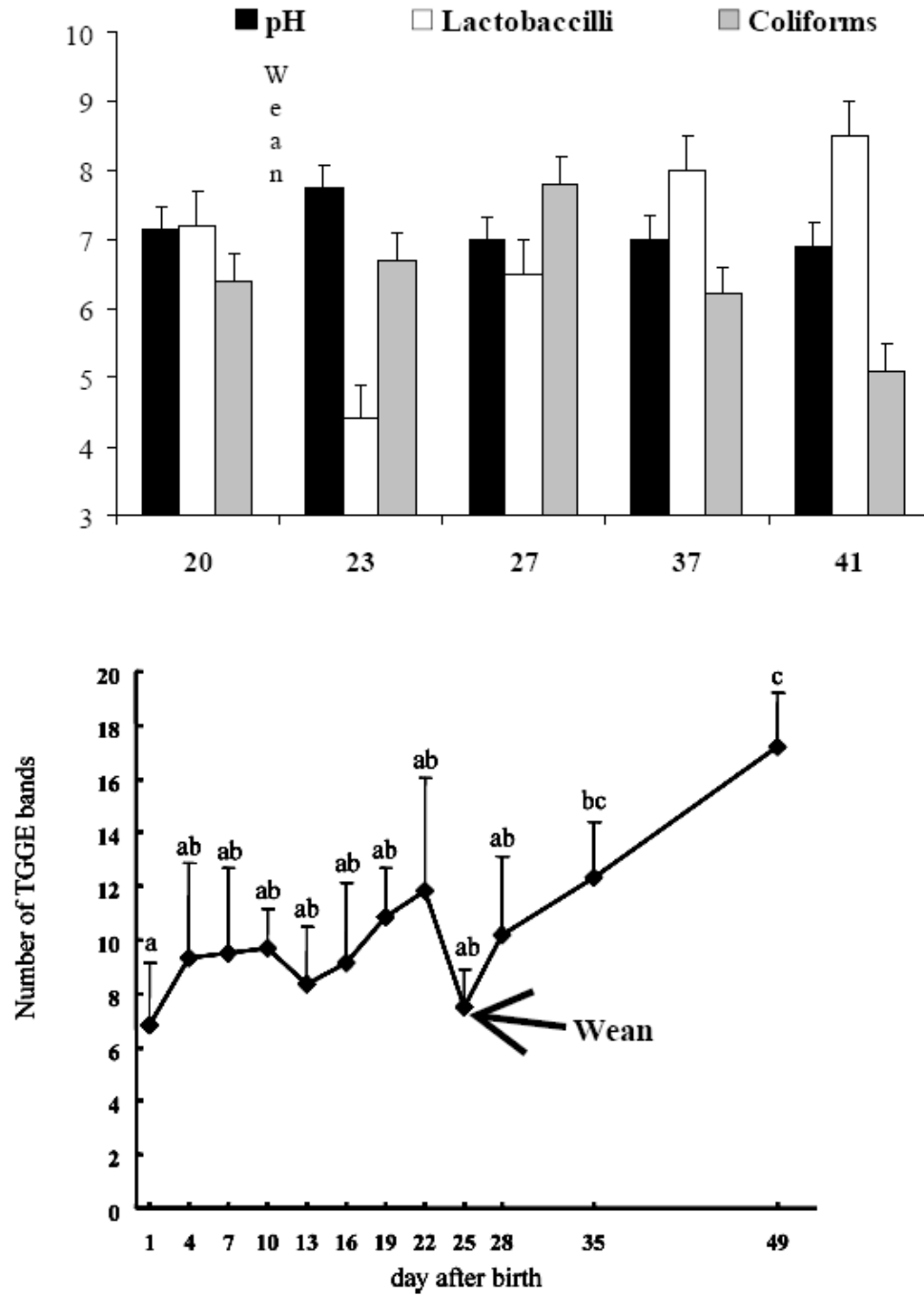
Figure 2.4. Effect of weaning on ileal digesta pH, lactobacilli and coliforms (panel A, Mathews et al., 1996) and microbial diversity, expressed as number of bands obtained by Temperature Gradient Gel Electrophoresis (TGGE) (panel B, Inoue et al., 2005). (Used with permission of Elsevier Limited, March 25, 2008).

The components of this barrier are epithelial cells, tight junctions between cells and the basal membrane of the epithelium. The epithelium also possesses specialized cells with defensive functions such as intraepithelial immune cells (paneth cells that secrete antimicrobial peptides and lymphocytes) which regulate immune function through secretion of cytokines (Gaskins, 2003).

The mucous layer which has protective, lubricant and transport functions is secreted by specialized epithelial cells called goblet cells and is composed mainly of water (95%) but its characteristic properties are due to mucin (Gaskins, 2001; 2003). The carbohydrate moiety from mucin acts as selective binding sites for bacteria.

The GIT immune system consists of lymphoid cells in organized sites like peyer patches and mesentery lymph nodes, and lymphocytes spread over the stromal tissues in the lamina propria and the epithelium (intra-epithelial lymphocytes) of the intestine (Stokes, 2001). This immune system associated with the GIT is referred to as the gut-associated lymphoid tissue and accounts for the 50% of the body lymphoid tissue (James, 1993). Peyer patches are formed by multiple follicles (B-cells) surrounded by inter-follicular zones (T-cells). In the lamina propria, plasma cells (mature B-cells) are mostly situated in the crypts and T-cells (CD4+ and CD8+) in villi (Vega-Lopez et al., 1993; Olivier et al., 1994). The elements of the GIT immune system work together to protect the host against mucosal invaders and to render the host tolerant against ubiquitous dietary antigens and the beneficial flora of the mucosa (Stokes, 2001; Gaskins, 2003).

2.2.3.2 Immature GIT Immune System at Weaning and Immune Activation

The piglet is profoundly immunodeficient at birth and is highly dependent upon a supply of both specific and non-specific immune factors present in maternal colostrum

and milk for immune protection, development and survival (Stokes, 2001). Evidence suggests that the cells and structures for the young pig GIT immune system develop in a highly-programmed sequence which take 7 to 9 wk to complete (King et al., 2003; Bailey et al., 2005). In view of the forgoing, it is logical to conclude that the early-weaned pig has a relatively immature GIT immune system.

The evolution of the intestinal immune system is clearly affected by the microbiota, as shown by data from microbial effects on germfree animals (Gaskins, 2001). Conventional animals vs. germfree present higher cell turnover rate, higher lamina propria cells, higher IgA secretion, thicker muscular wall and bigger peyer patches with different lymphocyte populations (Gaskins, 2003). All these characteristics are present in adult animals so it is logical to deduce that microbiota plays a principal role in intestinal maturation. Moreover, these differences occur despite the fact that both conventional and germ free animals are exposed to dietary antigens.

Weaning exposes the piglet to new microbial and dietary antigens. After weaning, increases in different populations of T-cells in lamina propria have been described (King et al., 2003). In addition, McCracken et al. (1995) described changes related to acute-phase responses: higher plasma concentrations of pro-inflammatory cytokines, fibrinogen, glucagon and increased liver weight. In recent experiments, gene expression for pro-inflammatory and anti-inflammatory cytokines in the GIT was shown to be up regulated soon after weaning (Pie´ et al., 2004).

Stokes et al. (2004) proposed two hypotheses to explain this sudden activation of the immune system at weaning. Firstly, anorexia compromises the integrity of the epithelial barrier allowing luminal antigens to penetrate. Secondly, the immune system is

immature with no ability to distinguish harmful and innocuous antigens and thus displays over reaction. In particular, anorexia increases para-cellular permeability rather than trans-cellular permeability (Verdonk et al., 1999) and a negative correlation has been described between villous height (indicator of epithelial damage) and CD8+ and CD4+ cells counts in lamina propria (Spreeuwenberg et al., 2001). Furthermore, it is well established that there is a tendency for increased energy requirement for maintenance (MEm) during the first week post-weaning (Diemen et al., 1995, Gentry et al., 1997, Moon et al., 1997; Sijben et al., 1997). Averaged over these studies MEm requirement during week 1 post-weaning was 461 vs. 418 $kJ \cdot kg^{-0.75} \cdot d^{-1}$ against the average of the subsequent five weeks. Given the enormous size of the intestinal immune system as discussed earlier, an elevated MEm further strongly suggests that the weaning process activate the GIT immune system.

2.3 Weaning Process and Post-Weaning Collibacillosis

The GIT disturbances experienced by weaned pigs are manifested in a general malaise referred to as post-weaning diarrhea (PWD) (Van Beers-Schreurs and Bruininx, 2002). Post-weaning diarrhea is a consequence of many factors associated with the weaning process and their complexity in precipitating PWD has been highlighted before (Medec et al., 1998; Pluske et al., 2002; van Beers-Schreurs and Bruininx, 2002). An important aspect of PWD is proliferation of viral and bacteria enteric pathogens which have been implicated as important causative agents (Bertschinger, 1999; Fairbrother et al., 2005). Proliferation of pathogens in the small intestines of weaned pig is often in tandem with changes described earlier with respect to GIT biology (morphology, microbiology and immune system).

Enterotoxigenic *E. coli* is among the most prevalent bacterial pathogens in the GIT of the piglet upon weaning and has been implicated in the pathogenesis of post-weaning collibacillosis (PWC) in piglets (Pluske et al., 2002; Fairbrother et al., 2005). Post weaning collibacillosis is a disease of the small intestine and is a major cause of mortality and morbidity worldwide and has been estimated to be responsible for as much as 50% of the economic losses seen in the production of weaned pigs (Cutler et al., 2007). In herds with PWC, up to 2% mortality in weaned pigs can be seen, but of greater economical significance is the morbidity and reduction in growth performance in the pigs that survive these infections (Fairbrother et al., 2005; Cutler et al., 2007).

Although digesta flows relatively quickly through the small intestine, the ETEC that proliferate in the condition possess fimbriae, or pili, that attach to the enterocytes lining the small intestinal villi or to the mucus covering the villi (Fairbrother et al., 2005). The ETEC causing PWC mostly carry the F4 (K88) or F18 fimbriae (Fairbrother et al., 2005). After colonizing the small intestine, ETEC provoke hyper-secretory diarrhea through the release of specific enterotoxins. Secretion of Cl^-, Na^+, HCO_3^-, and water into the lumen is induced by the actions of a heat-labile toxin binding irreversibly to the mucosal cells and activating the adenyl cyclase-cyclic AMP system (Argenzio, 1992). A second heat-stable toxin, with subtypes a and b, inhibits the absorption of Cl^- and Na^+ from the lumen into the epithelial cell via the guanyl cyclase-cyclic GMP system.

Colonization of the small intestine and diarrhea usually last between 4 and 14 d, with the strains being spread between animals primarily by the fecal-oral route, and also by aerosols (Bertschinger, 1999). It is common for ETEC to appear in the feces of pigs in increased numbers in the first week after weaning in both healthy and diarrhoeic pigs,

although the numbers of ETEC in diarrhoeic pigs is higher (Kenworthy and Crabb, 1963; Hampson et al., 1985). Pigs displaying PWC harbor massive numbers of ETEC in the small intestine, whilst there is minimal change in numbers of other bacteria (Smith and Jones, 1963).

2.4 Management of Weaning Transition

In view of the foregoing discussion, weaning presents several unique problems not experienced in other phases of the pig growth. Furthermore, piglet performance in the period immediately after weaning has been shown to determine the days the pigs will take to attain market weight (Tokach et al., 1992). That post-weaning growth check may increase housing and feed costs due to an increase in days to market is an important concern in swine production (Whittemore and Green, 2001). As a consequence, for the last 50 years a lot of different strategies have been used to improve performance and to minimize casualties during this phase of the pig's life. These strategies includes: biosecurity rules, segregated early weaning systems, vaccination and selection against specific pathologies, improved housing with environmental control, well trained staff and nutritional strategies related to the presentation of the feed with modified ingredients and additives (Madec et al., 1998; NRC, 1998; Hemsworth and Barnett, 2000; Evans, 2001; Henry, 2001; Jørgensen et al., 2003). In the present review, further insights on dietary strategies, specifically additives, will be described. However, the other strategies are very important and do not exclude the application of dietary strategies simultaneously, and can be obtained from reviews by Madec et al. (1998); NRC (1998); Hemsworth and Barnett (2000); Evans (2001); Henry (2001) and Jørgensen et al. (2003).

2.5 Nutritional Strategies to Improve GIT Function and Health at Weaning: Feed Additives

From a dietary standpoint, a number of strategies exist to ease the weaning transition and improve the piglet's performance and health. Some of these strategies are mentioned briefly as follows: 1) Phase feeding, a means of gradually adapting the piglet to low-fat, low-lactose, high carbohydrate, dry diet consisting of cereal grains and various sources of protein (Goodband et al., 1993). 2) Feed processing and composition, e.g. fermented liquid feed (Canibe and Jensen, 2003), amino acid supplemented low protein diets (Nyachoti et al., 2006). 3) Novel ingredients, e.g. cooked rice (Pluske et. al., 2002), spray dried plasma (van Dijk et al., 2001) and milk products (Patterson, 1987). 4) Enteric nutrients e.g. amino acids such as glutamine and glutamate (Domeneghini et al., 2004; 2006; Yi et al., 2005), arginine (Ewtushick et al. 2000) and L-tryptophan (Koopmans et al., 2006). 5) Feed additives (Jensen et al., 1998; 2003). In the present review current insights on some of the major feed additives for managing gastrointestinal health and function in weaned pig will be presented.

2.5.1 Antibiotics Growth Promoters and Mode of Action

The era of antibiotic growth promoters (AGP) began between 1946 to 49 with the recognition of substantial growth responses to the inclusion of streptomycin and dried mycelial mass recovered from the fermentation of *Streptomyces aureofaciens* in the feed of pigs and chickens (Anderson et al., 1999). Since then, AGP have been generalized all over the world to support animal performance and in the prevention of GIT disturbances (Anderson et al., 1999). As a consequence, 90% of starter diets in North America contained AGP by the end of the last century (Doyle, 2001). A meta analysis of 453

experiments in the US involving a total of 13, 632 weanlings (7 to 25 kg) showed that AGP improved ADG by an average of 16% and G:F by 6.9% (Cromwell, 2001).

There are ten AGP registered for use in the swine industry in Canada (Health Canada, 2002). Of these, five are also registered for use both in therapy and disease prevention, prophylaxis and/or control. There is very little publicly available data on the quantities of antimicrobial drugs used in food animals in Canada, and there is currently no mechanism by which this data could be collected (Health Canada, 2002). As a consequence, one of the recommendations of the Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health was to design and implement a national monitoring program of antimicrobial use in food animals, and to make the data publicly available (Health Canada, 2002).

Despite widespread (temporal and spatial) use of AGP in swine feed their mode of action is still contentious. However, considerable evidence suggests that AGP modify gut microbial populations or activity (Visek, 1978; Tollefson et al., 1997). For example, feeding antibiotics does not induce a growth-response in germfree animals (Coates et al., 1963), while infecting germfree animals with gastrointestinal bacteria from normal animals results in growth depression (Coates, 1963). Furthermore, the growth response to feeding antibiotics is enhanced in conventional animals raised under conditions with greater microbial load (Hays, 1991; Tollefson et al., 1997). In this context, four mechanisms have been suggested to underlie the effects of AGP on animal growth: 1) inhibition of sub clinical infections, 2) reduction of growth-depressing microbial metabolites, 3) reduction of microbial use of nutrients, and 4) enhanced uptake of

nutrients through a thinner intestinal wall (Francois, 1962; Visek, 1978; Anderson et al., 1999).

Anderson et al. (1999) observed that the range of antibiotics used in animal feeding are of different chemical structures and act on microbial population through different mechanisms. However, their effect is linked, at least in part, to their reduction of intestinal microbial mass in a specific way (Anderson et al., 1999). Anderson et al. (2000) surmised that the perceived advantage of AGP is that they decrease the energetic costs associated with constitutive low level inflammation caused by enteric pathogens. Indeed, a recent experiment using modern molecular techniques suggests that AGP may influence GIT microbiota in a specific way (Collier et al., 2003). In the aforementioned study, analysis of porcine GIT microbiota using molecular techniques showed that AGP decreased total bacterial mass and produced higher homogeneity in bacterial populations (Collier et al. 2003). Furthermore, the sudden withdrawal of AGP in Sweden was accompanied with increase of enteric infections (Casewell et al., 2003).

2.5.1.1 Concerns on AGP

In the recent years, the use of AGP by the poultry and livestock industries has come under increased scrutiny because of the potential risk of bacteria acquiring resistance to specific antibiotics to the detriment of human health (Health Canada, 2002). Antibiotic resistance is a property of bacteria that confers the capacity to grow in the presence of antibiotic at levels that would normally suppress growth of or kill susceptible bacteria (Adjiri-Awere and Van Lunen, 2005). Bacteria that produce a particular antibiotic need mechanisms to protect themselves from that antibiotic and consequently, contain genes inherited from their forerunner that confer intrinsic antibiotic resistance

(Koch, 2000; Adjiri-Awere and Van Lunen, 2005). This form of antibiotic resistance, however, is not a major source of concern for human and animal health. What is of concern is acquired genetically-based resistance expressed by resistance genes that enable bacteria to survive an antibiotic treatment. These specialized genetic elements appear to have evolved in parallel with, and in response to, antibiotic use in animals and people over the past 50 years (Adjiri-Awere and Van Lunen, 2005). Indeed, the problem of antibiotic resistance as result of AGP concept is not new and was raised in the Swann report (Swann et al., 1969).

Antimicrobial use at a sub-therapeutic level over long periods is liable to selection and preservation of resistant bacteria (McEwen, 2002). In this context, a four year study linked ineffectiveness of ciprofloxacin in treating patients with campylobacteriosis to the use of fluoroquinolones in poultry diets (Smith, 1999). As a follow up study, McDermott et al. (2002) demonstrated that feeding broilers diets containing fluoroquinolones at levels approved by Food and Drug Administration resulted in rapid and persistent development of *Campylobacter jejuni* resistant isolates. Interestingly, the minimum inhibitory concentration of fluoroquinolones increased from 0.25 to 32 μg/ml during the 5 d of treatment (McDermott, 2002). Lagoons in swine facilities have also been shown to carry antibiotic resistant bacteria (Chee-Sanford et al., 2001). Antibiotic resistant strains of *Enterococcus* have been isolated from pork (Klein et al., 1998; Hayes et al., 2003). Multi drug-resistant *Salmonella* have also been isolated from swine feces (Gebreyes and Altier, 2002) and commercial pork samples (Chen et al., 2004). These examples indicate that antimicrobial resistant human pathogens could be transferred to vehicles of infection (meat, soil) and then to humans.

In view of the foregoing, there is evidence to suggest that feeding AGP to animals may pose a threat to human health. However, it is still contentious as to the extent of risk imposed to human health (Phillips et al., 2004). Arguably, if antimicrobial resistant human pathogens contaminate meat or other food, ideally standard proper handling and cooking procedures would kill them. Furthermore, Cromwell (2001) surmised that the high level of antimicrobial resistance in humans likely resulted from antibiotics prescribed directly to humans.

2.5.1.2 Of Imminent Ban and Search for Alternatives

With the assumption that all AGP use in livestock feeding is imprudent, and that the human health hazard is real, the EU adopted the 'precautionary principal', and a widespread ban of AGP in livestock feeds took full effect in January 2006. A comprehensive report released by the World Health Organization (2003) on the status of AGP concluded that there have been no serious negative effects on animal performance, health, food safety, and consumer prices upon banning of AGP in Denmark. There is currently very little regulatory activity regarding AGP use in North America, although commercial and consumer pressure in this region may force producers to voluntarily remove AGP from animal feeds (Dibner and Richards, 2005).

Because swine producers commonly rely on antibiotics to optimize production, there is considerable concern that the loss of AGP will severely impact the industry especially during stressful periods like weaning (Casewell et al. 2003). Indeed, the ban of AGP in Sweden revealed that these agents had important prophylactic activity and their withdrawal is now associated with a deterioration in weanling health, including increased diarrhea, weight loss and mortality due to *E. coli* and *Lawsonia intracelluralis* infections

(Casewell et al., 2003). As a consequence, efforts are being made to seek alternative or replacement strategies for controlling enteric bacterial diseases and maintenance of functional piglet GIT (reviews, Close, 2000; Doyle, 2001; Wenk, 2002). While research must intensify to find additional, acceptable growth promoting agents, it is also important that producers and scientists understand the differences between the mechanisms of alternative products and antibiotics, as well as their individual limitations. A better understanding of these products will then likely result in their more effective use in current swine production systems (Mathews, 2002). In the next parts of the present review, the main alternatives (additives) to AGP will be discussed.

2.5.2 Minerals

Feeding pharmacological levels of Zn and Cu has been shown to increase the performance of weanling pigs (NRC, 1998). Growth-promoting properties of pharmacological levels of zinc oxide and copper sulfate during post-weaning period are associated with improved feed intake (NRC, 1998). It has also been reported that feeding pharmacological levels of Zn maintains the stability of the intestinal microbiota and diversity of coliforms during the first 2 week post-weaning (Melin et al., 1996), improves gut morphology (Carlson et al., 1999) and attenuates ETEC diarrhea (Roselli et al., 2005). Feeding high levels of Cu was reported to reduce the number of streptococci in fecal samples (Fuller et al., 1960). However, there are concerns about the high rate of excretion of these elements into the environment (Pettigrew, 2006). As a consequence, their use is becoming regulated by law and limited to nutritional doses since as components of many enzymes; Zn and Cu are involved in many metabolic processes and affect immune function (Rink and Kirchner, 2000). In this respect, there is growing

interest in the potential role of organic minerals which are both more biologically active and bio-available; hence less risk of potential environmental pollution (Close, 2000).

2.5.3 Acidifiers

Gastric acidification in suckling pigs is mainly due to the presence of lactic acid, resulting from bacterial fermentation of lactose (Cranwell, 1976). Cranwell et al. (1985) demonstrated that piglets achieved maximal gastric acid output at the age of 5 to 6 weeks and that exposure to creep feed was important in this process. It follows that at weaning, the piglet not only looses lactose induced acidity but the ensuing anorexia exacerbate the ability of physiologically immature gut to produce enough HCl to keep stomach pH at an optimum of 3.5 (Lallès et al., 2007). Furthermore, diets fed to young pigs often have a high buffering capacity, which can further reduce stomach acidity. At low gastric pH, digestion of proteins and populations of beneficial bacteria (lactobacilli) are maximized and harmful bacteria are inhibited (Doyle, 2001).

Therefore, acidifiers (organic acids, OA) added to feed can have a beneficial effect to weaned pig. Organic acids usually contain one to seven carbon atoms and they are used as food preservatives in their free or salt form (Doyle, 2001). Acidifiers may have two mechanisms of action when fed to pigs (Partanen and Mroz, 1999). Firstly, acidifiers can act through pH reduction. The second mechanism is the antimicrobial activity of the acid molecule, independently of the pH. Most OA are in un-dissociated forms and thus able to diffuse across cell membranes of pathogens, destroying their cytoplasm or inhibiting growth (Roth and Kirchgessner, 1998).

A meta-analysis of 35 experiments on growth promoting effects of OA in weaned piglets found that the acids generally improved performance but the magnitude of the

effect varied with the type of acid used and other components of diet (Partanen and Mroz, 1999). Fumaric acid usually caused a greater weight gain than formic or citric in young piglets while formic acid was more effective in fattening pigs (Partanen and Mroz, 1999). It is likely that the antimicrobial effects of the OA ions are responsible for the beneficial effects of these acids (Roth and Kirchgessner, 1998). In contrast, Jensen (1998) concluded that data from many experiments showed inconsistent effects of OA on gut pH and flora. The later argument has been demonstrated in experiments monitoring enteric diseases in OA fed piglets. For instance, various OA were able to reduce the incidence and severity of ETEC diarrhea (Tsiloyiannis et al., 2001; Owusu-Asiedu et al., 2003b) while in other studies OA did not have a positive effect on diarrhea score in ETEC K88 challenged pigs (Risley et al., 1993). Inconsistent results may be due to the variety of diets with different buffering capacities that were used in these experiments. Furthermore, bacteria are known to develop acid-resistance when exposed to acidic environments for some time (Richard and Foster, 2003).

Nevertheless, OA (such as fumaric, formic, lactic) are commonly added to swine feed in many European countries (Doyle, 2001). Two problems may occur at higher OA levels in swine feed: (i) unpalatability, leading to feed refusal (Partanen and Mroz, 1999) and (ii) acidic feed is corrosive to cement and galvanized steel in swine housing. A recent concern in the use of OA in feeds is that of development of acidic resistance *Salmonella* enterica *serovar typhimurium* and *Escherichia coli* after exposure to OA (Richard and Foster, 2003). Because OA are used in food preservation, then the fate of OA as additives in animal feed may in future be uncertain. Furthermore, a recent experiment by Bosi et al. (2006) showed that calcium formate decreased the number of HCl-secreting parietal cells

and increased somatostatin-producing cells (somatostatin has a suppressive action on HCl secretion), and reduced H^+/K^+ ATPase gene expression. Thus, OA exert a negative feed-back on gastric acid secretion, an effect which may not be desirable in weaned piglets.

2.5.4 Immune Active Products

Immunologically active compounds affect the working of the immune system and may enhance resistance to disease. These substances include antibodies, cytokines, spray-dried plasma (SDP), and other compounds (Doyle, 2001). Some or all of the growth-promoting effects of AGP in feeds may result from their action against sub-clinical infections or competitive intestinal bacteria (Doyle, 2001). Therefore, it has been suggested that addition of antibodies or other immunoactive compounds to feed may accomplish the same purpose. As discussed elsewhere in this review a characteristic of weaned pig is an immature immune system. In this context, oral passive immunization of piglets with antibodies specific to enteric pathogens or their antigenic ligands has been proposed as a potential prophylactic and therapeutic approach to controlling pathogens prevalent at weaning (Marquardt et al., 1999). An advantage of using oral antibodies as a strategy in controlling enteric disease such as PWC is that, as a passive immunotherapy, it passes the requirement of the induction of the immune system (Fairbrother et al., 2005).

Protection against intestinal colonization by ETEC may be attained by addition to the feed of egg yolk from hens immunized with specific antigens (Marquardt et al., 1999). To this end, egg yolk (EYA) from chicken immunized with K88 fimbriae has been shown to be effective in controlling PWD (Imberechts et al., 1997; Marquardt et al., 1999; Owusu-Asiedu et al., 2002, 2003a, b) although some studies failed to show similar effects (Zuniga et al., 1997; Chernysheva et al., 2003; 2004). In those studies reporting

positive effects, piglets fed diets supplemented with EYA showed reduced incidence and severity of diarrhea following experimental challenge with ETEC. *In vitro* studies have shown that purified antibodies against ETEC K88 fimbrial antigens from chicken egg yolk block the binding of ETEC to immobilized mucus obtained from weaned pigs (Yokoyama et al., 1992; Jin et al., 1998). Because hen eggs are such a rich source of immunoglobulin that is easily purified, they present tremendous opportunity for prevention and perhaps treatment of common infectious diarrhea of animals.

Spray-dried plasma incorporated into the diet stimulates growth performance mostly through an increase in feed intake (van Dijk et al., 2001); small intestinal alterations as well as in reducing incidence and severity of post-weaning diarrhea (Owusu-Asiedu et al., 2002). Part of the positive effects of SDP has been associated with the action of insulin-like growth factor 1 on growth, antibodies against enteric pathogens and improved immuno-competence through the provision of immunoglobulins (van Dijk et al., 2001). With respect to immunoglobulins, Pierce et al. (2005) demonstrated that piglets fed high molecular weight fraction (containing immunoglobulins) performed better than those fed whole plasma or low molecular weight fractions. Owusu-Asiedu et al. (2002) demonstrated that SDP contained substantial titers of K88 and F-18 antibodies which reduced the incidence and severity of ETEC induced diarrhea. As a consequence, SDP lend itself as an important additive in weaner diets to complement the immature immune system. Ideally, antibodies in SDP depend on the nature of antigens exposed to the source pigs. Caveat with SDP would be that it may contain antibodies specific to bacterial strains present on one farm but which are different from those on other farms (Normantiene et al., 2000).

Another option related to immune active compounds is the inclusion of fatty acids such as conjugated linoleic acid (Bassaganya-Riera et al., 2001; Patterson, 2006) prostaglandins and leucotriens precursors (Harbige et al., 2001) or inclusion of adjuvants that improve defense mechanisms against specific antigens (Cheeke, 2000). All these kind of products present important possibilities of development but require more efforts in the future.

2.5.5 Probiotics

Fuller (1992) defined a probiotic or direct fed microbial as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Most of the commercial probiotics for human and livestock consumption are lactic acid producing bacteria i.e. lactobacilli, gram-positive cocci and bifidobacteria genuses (Roselli et al., 2005) and yeast (Stein and Kil, 2006). The rationale for probiotic use is that they are able to restore normal microflora (Fuller, 1992). As already discussed, the numbers of the enteric lactic acid producing bacteria are reduced during the immediate post-weaning period enabling pathogens and *E. coli* in particular to increase in concentration (see Figure 2.4). It is, therefore, not surprising that there is a substantial interest in providing newly weaned pigs with lactic acid producing bacteria to restore the balance in the intestinal tract (Stein and Kil, 2006).

There are several proposed mechanisms by which probiotics may protect the host from the intestinal disorder (Gibson, 1995). Firstly, probiotic microorganisms produce several inhibitory substances such as OA, hydrogen peroxide and bacteriocins. These substances may limit growth of harmful bacteria in the gut. Another proposed mechanism is that of competitive exclusion in which attachment of probiotic microorganisms on the

intestinal epithelial surfaces prevent pathogens such as *E. coli* to attach (Stein and Kil, 2006). Thirdly, probiotics may prevent the utilization of nutrients by pathogenic bacteria. Fourthly, it has been postulated that probiotics can protect against intestinal disease by stimulation of specific and nonspecific immunity (Roselli et al., 2005; Bontempo et al., 2006).

2.5.5.1 Probiotic in Starter Diets

During the weaning period, the most promising effects of the use of probiotics are related to the competitive exclusion of pathogenic bacteria (Lallés et al., 2007). These effects could be a result of their positive influence on gut microbiota balance, intestinal epithelium integrity, appropriate maturation of the gut associated lymphoid tissue and function of the neuro-endocrine system (Metzler et al., 2005). Only a few probiotics have been tested for their capacity to prevent intestinal diseases in piglets. Oral administration of *Streptococcus faecium* to gnotobiotic piglets challenged with various pathogenic strains of *E. coli* increased weight gain and reduced severity of diarrhea and colonization by pathogenic bacteria in the gut compared to control animals (Underdahl, 1983). Feeding *Bifidobacterium lactis* reduced concentrations of fecal rotavirus and *E. coli* as well as severity of diarrhea in piglets (Shu et al., 2001). Similarly, feeding piglets diets containing *Bacillus toyoi* or *Bacillus licheniformis*, reduced the incidence and severity of diarrhea, as well the number of enterococci and coliforms, especially ETEC in the intestines (Adami et al., 1999; Kyriakis et al., 1999). Administration of live yeast (*Saccharomyces cerevisiae spp. boulardii*) to weaned pigs for 3 to 4 weeks improved growth performance post weaning, villus height, epithelial cell proliferation and the numbers of macrophages at various sites of the small intestine (Bontempo et al., 2006).

The potential of probiotics in managing post-weaning stress in pigs has also been studied by supplementing creep feed as well as gestating and lactating sow diets with probiotics. In this context, a study on the interaction between intestinal physiology, dietary supplementation with the probiotic *E. coli* strain Nissle 1917 and ETEC challenge was recently reported (Schroeder et al., 2006). Before being weaned at 21 d of age the piglets were creep-fed a diet with or without the probiotic supplement for 10 d and challenged with ETEC 24 h post-weaning. In that study probiotic-fed piglets showed reduced severity of diarrhea compared to those fed probiotic-free creep feed (Schroeder et al., 2006). In other studies, piglets weaned from sows fed gestating (d 90 antepartum) and lactating (d 28 postpartum) diets supplemented with *E. faecium* and fed the same probiotic upon weaning showed decreased incidence of diarrhea, level of cytotoxic (CD8 +) T-cells in the jejunal epithelium and fecal β-haemolytic and O141 serovars of *E. coli* in the first week post-weaning compared to controls (Scharek et al., 2005; Taras et al., 2006).

Largely, the forgoing review highlights some of the studies in which at least probiotics supplementation induced positive results. However, there are many more studies in which effects of probiotics were not apparent and in some instances somewhat inconsistent (see Stein and Kil, 2006). In theory, a probiotic culture ought to be able to establish itself in the GIT of the animal in order to have an effect (Fuller, 1992). In this context, it is well known that different species of animals including human display differences in microbial profiles perhaps as a consequence of unique GIT ecology (Gaskins, 2001). Furthermore, in pigs microbial profile differ by age. Most commercial probiotics are designed for the human use and those available for piglets are not

necessarily designed for the unique GIT ecology at weaning; perhaps the reason why their effectiveness in easing weaning transition has been somewhat inconsistent. In the recent years, researchers have been selecting favorable commensal strains from the piglet GIT; the few studies conducted thus far appear to suggest that this may be a successful strategy.

For instance, a mix of four lactobacilli isolated from weaned pigs reduced *E. coli* and anaerobe counts in the gut, and decreased diarrhea (Huang et al., 2004). In another study, supplementation of a diet based on fermentable fiber with *Lactobacillus. sobrius* improved the ADG of weaned pigs orally challenged with ETEC K88 and reduced ileal ETEC abundance (Lallés et al., 2007). Beneficial effects of this strain were also demonstrated when it counteracted intestinal permeability disturbances induced by ETEC on porcine IPEC-1 intestinal cells (Lallés et al., 2007). However, feeding piglets *Lactobacillus rhamnosus* GG (used in human subjects) reduced growth and increased fecal excretion of ETEC upon oral ETEC challenge (Lallés et al., 2007).

Equally interesting is the research on collicinogenic *E. coli* and its potential for controlling *E. coli* associated diarrhea. Colicins are a class of bacteriocins produced by, and effective against, *E. coli* and closely related bacteria (Fredericq, 1971). Pore forming colicins, such as Colicin E1 (ColE1), bind to their target bacteria and kill them by disrupting the ionic gradient of the cell (Fredericq, 1971). Recent research on colicins has focused on either selecting colicin producing but non-pathogenic *E. coli* strains as probiotics or extraction of colicin for inclusion in baby pig diets. In this context, studies at the University of Manitoba (Animal science) shows that colicinogenic *E. coli* were effective in suppressing ETEC K88 growth *in vitro* and severity of ETEC induced

diarrhea *in vivo* (Bhandari et al., 2007; Setia, 2007). Research in other laboratories has focused on isolation, purification and inclusion of colicin in starter diets (Cutler et al., 2007). For instance, feeding weaned pigs a colicin supplement resulted in less ETEC-induced diarrhea upon oral challenge with ETEC F18 (Cutler et al., 2007). The use of collicinogenic *E. coli* appear particularly attractive in weaned pigs because the GIT ecology of weaned pigs support proliferation of *E. coli* (see figure 2.4) and therefore the question of the carrier organism surviving through the GIT does not arise. Development of such products in parallel with unique prebiotics to provide their nutrients *in vivo* hints tremendous opportunities within the paradigm of nutritional influences in bacterial enteric diseases (Pluske et al., 2002).

2.5.6 Prebiotics

2.5.6.1 Definition and Scope

The term prebiotic was introduced by Gibson and Roberfroid (1995) who exchanged "pro" for "pre," which means "before" or "for." They defined prebiotic as ''non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health,'' and emphasized that this criterion was fulfilled only by some indigestible but fermentable carbohydrates. A consensus report commissioned by the European Union defined the prebiotic effect as a food-induced increase in numbers and/or activity of predominantly bifidobacteria and lactic acid bacteria in the human large intestine (Van Loo et al., 1999). Excluded in these definitions is the claim that prebiotics may act as receptor analogues which interfere in the specific interaction between surface adhesins or lectins of bacterial pathogens and the oligosaccharide component of

glycoconjugate receptors present on the brush border (Voragen, 1998). Recently, the definition of prebiotic was refined as ''selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host well-being and health'' (Gibson et al., 2004).

Of the possible prebiotics considered thus far, the non-digestible oligosaccharides have received the most attention (Gibson et al., 2004). Fructo-oligosacharides (FOS, inulin and oligofructose) are the most extensively studied of the oligosaccharides used for food and feed purposes (Buddington, 2001; Chesson and Stewart, 2001; Verdonk et al., 2005). They are produced either by extraction from food sources (onion, artichoke), controlled hydrolysis of naturally occurring fructans or by the transglycosylation of sucrose (Chesson and Stewart, 2001). Other oligosaccharides (Table 2.1) are also produced commercially either by partial hydrolysis (chemical or enzymatic) of an appropriate polysaccharides or by an enzyme-catalyzed transglycosylation (Voragen, 1998; Chesson and Stewart, 2001).

Table 2.1. Commercial and other sources of oligosaccharides used to manipulate mammalian flora

Oligosaccharide	Method of manufacture
β-fructo-oligosaccharides	Hydrolysis of inulin or transfructosylation of sucrose
α-galacto-oligosaccharides	Extracted from legumes or transgalactosylation of sucrose
α-gluco-oligosaccharides (isomalto-oligosaccharides)	Hydrolysis of starch or transglucosylation of maltose
β-gluco-oligosaccharides	Trasglucosylation of cellobiose, or laminaribiose
Manno-oligosaccharides	Hydrolysis of yeast cell wall
Xylo-oligosaccharides	Hydrolysis of xylans

Partially adapted from Chesson and Stewart, 2001

Comprehensive reviews by Flickinger et al. (2003), Patterson and Burkholder (2003), and Verdonk et al. (2005) summarize experiments in which different types of prebiotics were supplied in solid feed, formula or drinking-water to pigs alone or in combination with a probiotic. Effects on piglet performance vary from little or no effect (Farnworth et al., 1992; Howard et al., 1993), mixed (Houdijk, 1998) to positive effects (Russell et al., 1996; Shim and Choi, 1997; Estrada et al., 2001; He et al., 2002).

Effect of prebiotics on the GIT microbial activity has also been studied. Some studies in pigs evaluating bacterial populations showed that supplementation had little effect on size and activity of microbial populations (Farnworth et al., 1992; Houdijk et al., 1998). Some studies found enhanced intestinal bifidobacteria populations (Howard et al., 1993; Klein et al., 2001). Others reported modulation of the intestinal flora (Nemcova´et al., 1999) and speeding up of recovery following ETEC infection (Oli et al., 1998). Indeed, supplementation of inulin-type fructans to the diet or drinking-water resulted in fewer cases of ETEC induced diarrhea, reduced mortality and decreased number of pigs shedding the pathogen compared to controls (Bunce et al., 1995; Oli et al., 1998).

Konstantinov et al. (2003) studied the changes in time of the predominant fecal bacterial community in weaning pigs that were fed diets containing inulin-type fructans and/or sugar beet pulp using denaturing gradient gel electrophoresis analysis. Piglets fed diets containing sugar beet pulp or inulin-type fructans plus sugar beet pulp showed a higher bacterial diversity and a more rapid stabilization of the bacterial community compared to those fed the control diet.

With respect to competitive exclusion, manno-oligosaccharides (MOS), a preparation of the outer layer of the yeast cell wall has been evaluated (see Newman, 1994; Cromwell et al., 2001: Pettigrew, 2006). Several pathogens, including some *E. coli*, are thought to attach to mannose units on the mucosal surface (Gaskins, 2001). It is perceived that the yeast cell wall fragment containing a mannose unit in the lumen of the intestine may bind to the pathogens, preventing the pathogens from binding to the intestinal wall. As a result, the potential pathogens flow out of the intestine, and beneficial microorganisms such as lactobacilli are given opportunity to attach, this process is commonly referred to as "competitive exclusion" (Newman, 1994). As a consequence, inclusion of MOS in pig diets may promote overall health and growth by decreasing pathogenic bacteria (Connolly, 2001) and immunomodulation (Newman, 1994; Pettigrew, 2006).

With respect to weaned pigs, various researchers (Le Mieux et al., 2001; Hancock et al., 2002; Davis et al., 2002) have reported numerical improvements in performance when MOS were fed alone or in combination with other antimicrobials such as Cu and Zn. In contrast, Burkey et al. (2005) did not find any beneficial effect of MOS in piglets challenged with *Salmonella typhimurium*. In that study, piglets fed MOS showed similar levels of serum acute phase proteins to piglets fed control diets whilst piglets fed diets containing carbadox an (antibiotic) showed lower circulating serum acute phase proteins suggesting less severe enteritis.

Few studies have described the effects of prebiotics on the host defense system and gut integrity. Herich et al. (2002) demonstrated that the combination of oligofructose and probiotics fed to pigs before and after weaning increased the number of CD4^{+} T

lymphocytes compared to the control diet. In another study, inulin reduced the *in vitro* association of *E. coli* to jejunal organ tissue and of *Salmonella* spp. to ileal tissue (Naughton et al., 2001). Rossi et al. (2001) showed that inulin reduced adhesion of pathogenic coliforms to intestinal porcine mucosa *in vitro*. Howard et al. (1993) concluded that oligofructose improved the morphological and the cellular kinetics of the epithelial mucosa in the large intestine. Spencer et al. (1997) investigated the effect of supplementation of spray-dried animal plasma and inulin-type fructans on the morphology of the small intestine in weaned pigs. Inulin-type fructans did not affect crypt depth but did increase the villous height and VCR.

A recent comprehensive review (Lallès et al., 2007) on the effect of the weaning process and its impact on the GIT physiology, microbiology and immunology identified manipulation of carbohydrate (prebiotic) composition of the weaner diet as the most promising way to improve weaned pig gut health.

2.5.7 Carbohydrase Enzymes Targeting Cell Wall NSP

2.5.7.1 Enzyme Technology in Animal Nutrition

The 1990's saw a steady rise in the use of certain biotechnological products as feed additives. This whole area and its potential impact on farm animal nutrition, physiology and health was reviewed by Bonneau and Laarved (1999). Amongst the biotechnological additives, feed enzymes have made most progress and impact in the past decade, following the extensive and increasing use of crystalline amino acids, many of which are also produced from industrial fermentation (Partridge, 2001). In this context, three types of enzymes derived from fungi and bacteria currently dominate the animal feed market: carbohydrases, proteases and phytase (Partridge, 2001). The use of these

exogenous enzymes in improving the farmed animals' performance has been extensively studied and reviewed (e.g. Simon, 1998; Bedford and Schulze, 1998; Bonneau and Laarved, 1999; Bedford, 2000; Slominski, 2000; Partridge, 2001; Meng, 2005). Literature on enzyme use in animal production converges to suggest that the principal rationale of enzyme technology application is to improve the nutritive value of feedstuffs viz:

i.   To breakdown anti-nutritional factors that are present in many feed ingredients.

ii. To increase availability of starches, proteins and minerals that are either encapsulated within fiber-rich cell walls and, therefore, not as accessible to the animal's own digestive enzymes, or bound up in a chemical form that the animal is unable to digest (e.g. phosphorous as phytic acid).

iii. To breakdown specific chemical bonds in raw materials that are not usually broken down by the animal's own enzymes, thus releasing more nutrients.

iv. To supplement the enzymes produced by young animals where, because of the immaturity of their own digestive system, endogenous enzyme production may be inadequate.

As discussed earlier the weaner pig has always provided a unique set of gastrointestinal functional and health challenges that need to be overcome in order to maximize the young animal's growth potential. Among the principal nutritional limitations experienced by weaner pigs in which application of enzyme technology may influence are inadequate acid secretion, endogenous enzyme secretion, and feed intake (Partridge, 2001).

There is recent interest in the use of supplementary CE targeting cell wall NSP to create oligosaccharides *in situ*, which might then influence GIT microbial activity (see

reviews, Chesson and Stewart, 2001; Pluske et al., 2002). This has been coined the 'pre-pro-biosis' concept (Partridge and Tucker, 2000). This rather new paradigm is hard to conceptualize as has been quoted by others (Pluske et al., 2002). However, an insight of the common ingredients in weaner pig diet and application of CE would perhaps suffice to hint at potential opportunities for *in situ* prebiosis.

2.5.7.2 Carbohydrates Content of the Weaner Pig Diet

The choice of the weaner diet ingredients must primarily fit its digestive capacity, maintain gut health and promote feed intake (Le Dividich and Séve, 2001). However, by virtue of the ingredients available and the economics of feeding, the diets for weaner pig will contain cereal, legume and oil seed feedstuffs in which carbohydrates comprise between 40% and 80% of DM (Table 2.2).

Ingested carbohydrates can be broadly divided into: simple sugars and starch which are readily available or targeted by the endogenous enzymes and dietary fiber (DF). Chemically, DF is the sum of NSP and lignin which are resistant to mammalian endogenous enzymes (Bach Knudsen, 1997). The NSP are major constituents of the DF and comprise a large variety of polysaccharide molecules, often in association with phenolic lignified polymers, protein and starch, that have glycosidic bonds other than α-(1-4), (1-6) bonds of starch.

Table 2.2. Carbohydrate contents (g/kg DM) and major NSP in common feedstuffs

Ingredient	Carbohydrate[1]	Total NSP	Major NSP
Corn	830	119	Cellulose
Wheat	814	78-129	Arabinoxylans, xyloglucan
Barley (hulled)	823	167	Mixed-linked β-glucan
Oat (hulled)	787	232	Mixed-linked β-glucan
Soybean meal	400	148	Galacturonans, arabinans, galactomannan
Canola meal	454	171	(Arabino) β-1, 4 galactan
Flaxseed meal	493	271	Pectic polymers, arabinoxylans

[1]Include lignin

Partially adapted from Bach Knudsen (1997); Meng, (2005).

The building blocks of plant cell wall polysaccharides are pentoses (arabinose and xylose), hexoses (glucose, galactose and mannose), 6-deoxyhexoses (rhamnose and fucose), and uronic acids (glucuronic and galacturonic acids) (Pluske et al., 2001). These monomers are chemically linked to each other to build the various NSP in the plant cell walls of cereals, legumes and oilseeds (Table 2.2). The NSP are also divided into the soluble (in weak alkaline or water) and the insoluble fractions (Pluske et al., 2001).

The major NSP of plant cell walls comprise of cellulose (linear β-glucan chains), non-cellulosic polysaccharides (arabinoxylans, mixed-linked β-glucans, mannans, galactans, xyloglucan) and pectic polysaccharides (polygalacturonic acids, which may be substituted with arabinan, galactan and arabinogalactan) (Bach Knudsen, 1997; Pluske et al., 2001). The non-cellulosic and pectic polysaccharides, as well as the aromatic polymer lignin, interact with the cellulose fibrils, creating a rigid structure strengthening the plant cell wall. A typical arrangement for cereals is given in Figure 2.5 (Bach Knudsen, 2001).

As the lignin-polysaccharide complexes are hard, they stiffen the walls thus preventing biochemical degradation and physical damage of the walls (Liyama et al., 1994). The physical and chemical location of polysaccharides within the plant cell wall has a large influence on the physico-chemical properties of cell wall polysaccharides and consequently the action in the GIT (Bach Knudsen, 2001).
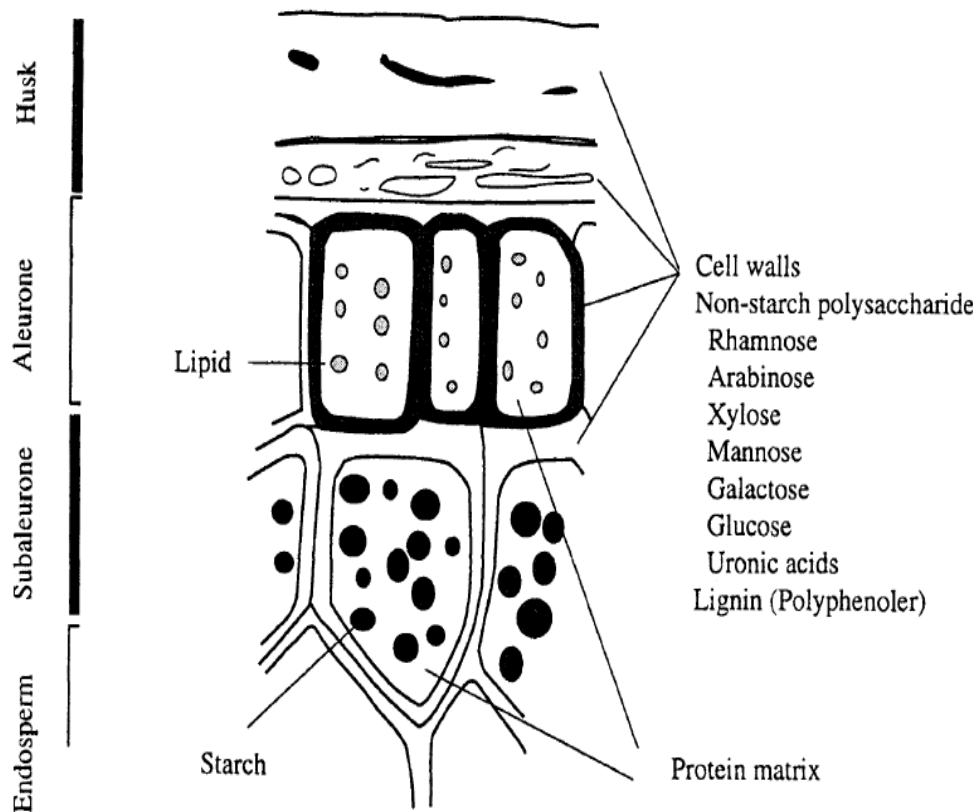
Figure 2.5. A typical cell wall materials of a cereal grain (Bach Knudsen, 2001) (Used with permission of Elsevier Limited, March 25, 2008).

2.5.7.3 Anti Nutritive Effects of NSP

Effects of NSP on a non-ruminant animal entails complex interactions with the gut epithelium, the mucus and the microflora a consequence, of which NSP influences the physiological and functional development of the GIT (Pluske et al., 2001). Certainly, a comprehensive review of this important subject is outside the scope of the foregoing review as it has been reported elsewhere (e.g. Bach Knudsen and Jørgensen, 2001; Pluske et al., 2001; Montagne et al., 2003).

The effects of the NSP on the digestibility of AA, endogenous losses and nutrient digestibility in pigs are well documented (Low, 1989; Nyachoti et al., 1997; Mosenthin et al., 1999; Pluske et al., 2001). In general terms, these authors report that NSP in particular increases intestinal transit time, delays gastric emptying, delays glucose absorption, increases pancreatic secretion, and slows absorption.

Thus, NSP have been recognized as "anti-nutritive" due to these negative influences on digestion of energy and nutrients including starch, protein and lipid in non-ruminant animals (Slominski, 2000; Souffrant, 2001). Furthermore, the physical entrapment of starch and protein by cell wall polysaccharides has been suggested as another important factor by which NSP exert their anti-nutritive properties (Theader et al., 1989). As a consequence, different treatments of fiber sources, e.g. dehulling of legume seeds, heating, or supplemental microbial enzyme targeting NSP are routinely used to improve the nutritional value of fiber-rich sources used in non-ruminants animals (reviewed by Gdala, 1998; Partridge, 2001).

2.5.7.4 Carbohydrase Enzymes Targeting NSP

Generally, the enzyme systems available for the animal feed industry are derived from microbes (fungi and bacteria) through traditional submerged liquid fermentation (Bailey and Ollis, 1986) or solid state fermentation (Mitchell and Lonsane, 1992). Plant cell wall polysaccharides are the most abundant and heterogeneous organic compounds found in nature (Bedford and Schulze, 1998). As a result of heterogeneity in plant cell wall structure, a plethora of polysaccharidases have evolved, each with their own characteristics (Bedford and Schulze, 1998). For instance, hydrolysis of xylan requires the presence of an enzyme system comprising a β-1, 4 endoxylanase (which cleaves the internal linkages of the xylan backbone), a α-D-xylosidase (which hydrolyses short xylo-oligosaccharides from the non-reducing end to release xylose), and enzymes designed to release the substituents (Bedford and Schulze, 1998). Furthermore, an endoxylanase from different fungi and bacteria species display a wide range of optimal physical requirements. For instance, Sunna and Antranikian (1997) analyzed characteristics of endoxylanase from 31 fungal and 31 bacterial species (in some cases each species producing as many as 5 xylanases). The range of pH for fungal xylanase ranged from 2 to 7 and temperature from 30 to 80ºC while corresponding values for bacteria were 4.5 to 10 and 30 to 105ºC, respectively. Furthermore, some endoxylanases require lengths of unsubstituted xylan in order to bind and hydrolyze the backbone, while others are not so demanding (Bastawde, 1992; Sunna and Antranikian, 1997). As a result there are differences between enzyme sources in their dependence on the accessory or debranching enzymes (e.g. acetyl xylan esterase, arabinosidase) for depolymerization of the xylan backbone (de Vries and Visser, 2001).

Regardless of the catalytic properties, the goal of these enzyme systems is to provide sugars for the organisms that produce them. Such sugars may be in the form of oligomers of 3 to 4 sugars length or single sugars, depending on the requirements of the organism involved. While these characteristics are designed and set by the evolution of the organism, the efficiency of such enzymes in animal feed applications depends very much on a completely different set of criteria which depend on the mechanism of action of the enzyme in animal feed usage (Bedford and Schulze, 1998).

2.5.7.4.1 The Mode of Action

As reviewed by Simon (1998), CE targeting NSP may have several modes of action: partial hydrolysis of NSP, decrease in digesta viscosity, rupturing of NSP-containing cell walls and thereby making the encapsulated nutrients available for digestion. Partial hydrolysis of soluble and insoluble NSP and rupturing of NSP-containing cell walls has been associated with reduced recovery of NSP when feedstuff samples were incubated with CE in *in vitro* studies (Castanon et al., 1997; Meng et al., 2005). Studies investigating efficacy of NSP hydrolyzing enzymes, reported not only improvement of nutrient digestibilities but also of NSP (Li et al., 1993; Mosenthin et al., 1994; Dierick and Decuypere, 1996; Diebold et al., 2004; Omogbenigun et al., 2004; Sterk et al., 2007). Although there are some studies in which no effect of added CE on nutrients digestibility was recorded (see review by Partridge, 2001), if the added CE in the diet do achieve the intended purpose it does so by partially hydrolyzing its' substrate i.e. dietary NSP.

2.5.7.4.2 Potential for In situ Generation of Oligosaccharides

In commercial food production, oligosaccharides are extracted from natural sources as is the case for the galacto-oligosaccharides from soybean, but they can also be obtained biochemically (Voragen, 1998). Indeed, in the food industry, enzymatic hydrolysis processes are applied for production of a whole array of oligosaccharides from plant cell wall polysaccharides (Voragen, 1998; Vázquez et al., 2000). Figure 2.6 schematically show the potential production of oligosaccharides from different plant cell wall polysaccharides (Van Laere et al., 1997 queted by Voragen, 1998). As outlined by Voragen (1998), the concept for manufacturing oligosaccharides from suitable polysaccharides is simple: starting from a polysaccharides rich feedstock followed by controlled hydrolysis of some of the heterocyclic ester bonds of the main chain backbone by an exogenous enzyme to give compounds of lower degree of polymerization. For example, the enzymatic hydrolysis process is widely applied in commercial production of fructo-oligosaccharides from inulin (Chesson and Stewart, 2001). Manufactured sources of oligosaccharides may allow dose to be more precisely defined and controlled, but only a very limited range of oligosaccharide structures is presently available. Most are linear, of short chain length and contain only one or two different sugar units and are specially manufactured for the ever-increasing human market.

polysaccharide	enzyme	oligosaccharide structure

arabinan — endo-arabinanase

(arabino)-galactan — endo-galactanase

(arabino)-xylan — endo-xylanase

homogalacturonan — polygalacturonase

rhamnogalacturonan — rhamnogalacturonase

xyloglucan — endo-glucanase

$\bullet$: α-L-arabinofuranose, ∇ : ß-glucopyranose, $\square$: ß-galactopyranose, $\blacklozenge$: ß-xylopyranose,
$\mathbf{O}$: α-L-rhamnopyranose $\blacksquare$: α-D-galacturonopyranose

Figure 2.6. Production of oligosaccharides by enzymatic hydrolysis of plant polysaccharides (Van Laere et al., 1997 as quoted by Voragen, 1998). (Used with permission of Elsevier Limited, March 25, 2008).

Plant and plant by-products used by the feed industry contain almost an unlimited range of polysaccharides (Table 2.2). The use of specific CE, singly or in combination, against a range of polysaccharides can generate very large numbers of oligomer mixtures (Chesson and Stewart, 2001). However, the CE typical of most commercial products offers little opportunity for the control of a limited hydrolytic process. A mixed polysaccharidases preparation typically will declare up to three major activities and guarantee a minimum value for each (Chesson and Stewart, 2001). Other activities in the mixture are not usually identified or amounts guaranteed and may vary significantly between batches. It is often these minor activities which define the fine structure of the oligosaccharides released from branched chain polysaccharides such as arabinoxylans or xylogucan (Chesson and Stewart, 2001). For instance, Meng et al., (2005) showed that incubating wheat with xylanase reduced recoveries of arabinose and glucose, which suggested presence of other activities in the declared xylanase preparation

It appears that the inherent heterogeneity of polysaccharides within and between sources will influence the nature of oligosaccharides produced. The NSP listed in Table 2.2 represent general classes in which there is commonality of overall structure but considerable variation in fine structure. For instance, while the underlying structure of most arabinoxylans is similar i.e. a β-1,4 linked backbone of D-xylose residues, in practice the variety is enormous due to differences in backbone size and in type and degree of substitutions from the backbone, all of which depend on the source of arabinoxylan (Sunna and Antranikian, 1997). Even when the source of polysaccharide is constant, fine structure can vary with age, environmental conditions and between varieties (Chesson and Stewart, 2001). In this context, partial degradation of arabinoxylan

with an endoxylanase (single cloned) resulted in mixtures of up to 12 oligosaccharides, with each of 12 samples of wheat studied differing in the presence and relative proportions of these oligomers (Austin et al., 1999). Such an observation suggested that even when the enzyme has only one activity, it is likely that polysaccharides will uniquely release mixture of oligomers.

Regardless of whether a crude or cloned enzyme product is used to hydrolyze NSP in typical diets for non-ruminants, it follows that oligosaccharides must be generated on every occasion when polysaccharide-degrading enzymes are used as feed additives. In this context, Apajalahti and Bedford (1998) showed that addition of xylanase in wheat based broiler diets resulted in 5-fold increase in concentrations of short-chain xylo-oligomers (<10 degree of depolymerization) in the caecum. There is apparently no recorded instance where the *in situ* generation of oligosaccharides has been monitored in piglets fed diets containing CE. However, since oligosaccharides will be among the hydrolysis products released when CE degrades NSP a question has been raised as to whether the presence of such products would influence GIT ecology of the weaned pigs (Chesson and Stewart, 2001; Pluske et al., 2002). This is because the resulting NSP hydrolysis products (NSPHP) are not absorbed by the host but will be available to modulate intestinal microflora (Chesson and Stewart, 2001; Pluske et al., 2002). *In situ* production of oligosaccharides by the use of CE targeting dietary NSP could support the development of a healthy gut microflora or their carbohydrate moieties may act as surrogate attachment sites for pathogens commensurate to exogenous prebiotics (MOS) discussed earlier in the present review (see section 2.5.6).

2.5.7.4.3 Carbohydrase Enzymes and Piglet GIT Health

In view of the foregoing, it can be deduced that in the process of depolymerizing NSP to liberate nutrients CE will generate HP which are non-digestible by endogenous enzymes. While not optimized for the purpose, if the generation of oligomers can modulate the gut flora to the benefit of the host, some evidence of this might be expected from *in vivo* trials made with feed enzymes. However, the effects of CE on intestinal health are well characterized in poultry than in pigs.

Thus, Sinlae and Choct (2000) demonstrated that broilers fed a wheat-based diet with xylanase had a negligible number of *Clostridium perfringens* compared with the control birds. Fernandez et al. (2000) reported that broiler chickens fed a wheat-based diet supplemented with xylanase showed less *Campylobacter jejuni* in the caecum following experimental infection. Interestingly, this was associated with an increased number of neutral and sulfated mucins in goblet cells. Furthermore, Hampson et al. (2002) reported that 22-week-old laying hens experimentally infected with a virulent strain of *Brachyspira intermedia* and fed xylanase in a wheat-based diet showed reduced proliferation of this spirochaete in the caecum. More recently, Jia et al. (2007) observed similar digesta *Clostridium perfringens* count in birds fed wheat-based diets upon experimental *Clostridium perfringens* challenge. Surprisingly, birds fed diet containing CE showed improved feed efficiency compared to control suggesting nutrient sparing effects in birds fed diets with CE which presumably may have resulted from less pronounced acute phase response activation characteristic of the gastroenteritis (Gaskins, 2001).

Such studies are scarce in piglets and where GIT health status has been monitored, there was significant reduction in the frequency and severity of diarrhea in piglets fed diets supplemented with enzyme mixtures (Inborr and Ogle, 1988; Partridge and Tucker, 2000). However, in no case was the cause of any episode of diarrhea determined. This is important because the diarrhea seen after weaning can be osmotic in nature rather than being of bacterial origin which might be influenced by the presence of oligosaccharides (Pluske et al., 2002). Pluske et al. (2002) suggested that future studies in this area require alignment of dietary work and age of the pig with assessments of microbial populations to truly study the effects of enzymes and oligosaccharides on enteric bacterial diseases.

2.6 Post Weaning Collibacillosis as a Model for GIT Dysfunction

The study of a specific bacterial disease, particularly if it causes economic loss, offers a means of assessing the usefulness of nutritional interventions/strategies on the survival of that particular pathogen in the GIT, and its subsequent effects on production, morbidity and mortality (Madec et al., 1998; 2000; Jones et al., 2001; Pluske et al., 2002). One such disease is PWC caused by hemolytic ETEC as discussed earlier in the present review (see section 2.3). In the small intestine, ETEC fimbriae attach to glycoprotein receptors expressed in the brush border of cells lining the intestinal villous (Jorgensen et al., 2003; Fairbrother et al., 2005). The most common fimbriae associated with *E. coli* causing PWC is K88, renamed F4. The receptor for F4 disappears a few weeks after weaning, offering only a brief window of opportunity for this pathogen to attach and proliferate.

The act of weaning is an essential precipitating factor for PWC, regardless of the age at weaning. All of the factors involved with weaning create an environment suitable for the proliferation of *E. coli* in the small intestine (see sections 2.2.1, 2.2.2 and 2.2.3) (Pluske et al., 2002; Fairbrother et al., 2005). As a consequence, researchers have used PWC to evaluate strategies for managing weaning process. One approach widely used is the experimental inoculation of newly weaned pigs with a strain of ETEC, in an attempt to replicate what happens clinically in the field (see Madec et al., 2000). Another approach is use of an *in situ* model of secretory diarrhea in which jejunal segments of anaesthetized piglets are infected with ETEC to quantitatively assess the effect of enterotoxin-producing pathogens on net absorption in the small intestine (see Nabuur et al., 1993). Recently, *in vitro* cell culture models have also been used in which case cell lines (enterocytes) are inoculated with ETEC and the response studied (see Rosselli et al., 2005).

2.7 Summary of the Literature Review

The young pig has a high potential for growth. The intestine is an important compartment of the GIT; being a major site of digestion, nutrient absorption and hydro-mineral exchange homeostasis, harboring a complex microbiota and a highly evolved mucosal immune system. Undoubtedly, all these aspects of GIT physiology, microbiology and immunology contribute interactively to the gut health balance. Weaning of piglets is often accompanied by a severe growth check and diarrhea. It is well established that this process is multi-factorial. However, post-weaning anorexia and attendant nutritional stress are major factors in the process which in concert with

immature digestive and immune system predisposes the piglet to GIT disturbances manifested in growth check and infectious diarrhea.

Recent data indicate alterations in GIT architecture, transiently-increased mucosal permeability, disturbed absorptive-secretory electrolyte balance and reduced brush border enzyme activities after weaning. Pigs coexist with a diverse and dense commensal microbiota in their GIT. The microbial colonization of the porcine intestine begins at birth and follows a rapid succession during the neonatal and weaning period. Following the withdrawal of sow's milk the young piglets are highly susceptible to enteric diseases partly as a result of the altered balance between developing beneficial microbiota and the establishment of intestinal bacterial pathogens. The intestinal immune system of the newborn piglet is poorly developed at birth and undergoes a rapid period of expansion and specialization that is not achieved before early (commercial) weaning.

The major dietary additives focused on improving gut health in post weaning transition are reviewed. Important additives are AGP, which albeit many years of usage are increasingly being eliminated or minimized. Some of the major alternatives to AGP which have been assessed thus far include acidifiers, minerals, immune active products, prebiotics and probiotics. While there is some evidence that these additives may positively influence weaning transition including controlling enteric diseases majority of them have elicited some concerns. For instance, for the minerals to be effective they ought to be supplemented at pharmacological levels which lead to excess mineral loading in the environment. Acidifiers have problems which range from handling, equipment corrosion, deleterious effects on parietal cells maturation and development of resistant zoonotic bacteria. For probiotics, there are concerns about survivability of the culture in

the prevailing GIT ecology at weaning; largely most of commercial probiotics derive from products designed for the human market. Prebiotics appear promising but quite often these products are included in the weaner diets at the expense of the much needed nutrients in addition to the cost of buying them. Immune active products especially antibodies developed against pathogenic antigens hold a great promise because they are specific and they by pass the need for rather immature GIT immune system to exert their effects.

The potential of CE targeting dietary NSP hold even greater promise because these additives are routinely added in the weaned diet to improve nutrient utilization. To achieve this end CE breakdown the NSP to release encapsulated nutrients, in the process a variety of HP are released. Since these products will not be absorbed by the host, they are capable of modulating GIT microbiota including pathogens in similar fashion as the prebiotics. However, whether HP would modulate post weaned piglet GIT disturbances remains to be determined.

CHAPTER THREE

MANUSCRIPT I

Growth performance, gastrointestinal microbial activity and nutrient digestibility in early-weaned pigs fed diets containing flaxseed and carbohydrase enzyme

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3.1 ABSTRACT

The effects of ground flaxseed (FS) and a multi-carbohydrase enzyme (CE) supplement on piglet performance, gastrointestinal microbial activity and nutrient digestibility were investigated in a 28-d trial. Enzyme supplement provided 500 units pectinase, 50 units cellulase, 400 units mannanase, 1,200 units xylanase, 450 units glucanase and 45 units galactanase per kg of diet. Ninety-six pigs were weaned at 17 d of age (BW, 6.1 ± 0.4 kg, mean $\pm$ SD) and based on a 2 x 2 factorial arrangement allotted to 4 diets in a completely randomized design to give 6 pens per diet (4 pigs per pen). The diets contained wheat, barley, peas, soybean meal and canola meal with 0 or 12% FS and fed without or with CE. Flaxseed was included by changing the levels of the other ingredients to balance the diets for DE and nutrients. Diets had similar nutrient contents and met the NRC (1998) nutrient specification with exception of DE, CP and AA, which were 95%, 94% and 97%, respectively. Diets were fed in a 2-phase feeding program (2-wk/phase). Feed intake and BW were measured weekly and 1 pig per pen with BW close to the pen average was bled weekly to evaluate plasma urea nitrogen (PUN). On d 28 fresh fecal samples were collected from each pen and 1 pig per pen with BW close to the pen average was killed to evaluate intestinal microbial activity and nutrient digestibility. Dietary effect on piglet performance was only evident in wk 3 when FS diets reduced ($P = 0.005$) ADG and G:F, tended to depress ADFI ($P = 0.070$) and increased PUN ($P = 0.027$). An interaction between FS and CE was observed on ileal digesta viscosity ($P = 0.045$) such that CE increased viscosity in FS diet but had no effect in non-FS diet. Flaxseed and CE interacted in influencing ileal ammonia content ($P = 0.049$) such that in absence of FS, inclusion of CE in non-FS diet reduced ammonia compared to diet without CE. Flaxseed

and CE affected other ileal parameters independently; pigs fed FS diets had lower (P = 0.003 to 0.033) anaerobic spore count, organic acids, DM, CP and non-starch polysaccharide (NSP) digestibility than pigs fed non-FS diets whereas pigs fed CE supplemented diets had higher (P = 0.009 to 0.008) lactobacilli count, lactate, DM and NSP digestibility than pigs fed unsupplemented diets. In conclusion, FS reduced ileal microbial activity, nutrient digestibilities and piglet performance in wk 3. Multi-carbohydrase supplement increased ileal DM and NSP digestibilities as well as lactobacilli counts and lactate.

Key words: Carbohydrase enzyme, Early-weaned pig, Flaxseed, Growth performance, Intestinal microbial activity, Nutrient digestibility

3.2 INTRODUCTION

Weaning expose piglet to nutritional and environmental stresses resulting in reduced performance and in some instances diarrhea and death (Cromwell, 2002). Growth performance at this stage is optimized by use of in-feed antibiotics (Pluske et al., 2002). Because of concerns over residues in meat products and bacterial resistance to antibiotics, alternatives to in-feed antibiotics are required (Cromwell, 2002).

Dietary manipulation has been proposed as a strategy for optimizing performance of newly weaned pigs (Pluske et al., 2002). Flaxseed (FS) is a rich source of α-linolenic acid (ALA) and lignans that possess broad anti-microbial activity (Pauletti et al., 2000; Kankaanpää et al., 2001). It can be speculated that including FS in weaned pig diets could potentially modulate intestinal microbiota (Smith et al., 2004). However, there are few studies assessing effects of FS on intestinal microbial activity in weaned pigs as most of

swine research on FS has primarily focused on changing fatty acid profile in finishing pigs (e.g. Mathews et al., 2000).

A concern in using FS in piglet diets is the high content of mucilaginous NSP that could increase digesta viscosity and thus impair nutrient utilization (Bhatty, 1993). However, supplemental CE long recognized to be effective in hydrolyzing NSP in feedstuffs for swine (Kim et al., 2003; Omogbenigun et al., 2004) may allow inclusion of FS in piglet diets. Furthermore, it has been suggested that CE may partially hydrolyze NSP in the intestinal tract to yield substrates capable of modulating microbial activity (Vahjen et al., 1998). Therefore, we studied the effects of supplementing starter diets with flaxseed and a multi-carbohydrase enzyme on growth performance, gastrointestinal microbial activity and nutrient digestibility in early-weaned pigs.

3.3 MATERIALS AND METHODS

Experimental Diets

The diets were based on wheat and barley as the cereal source and protein from pea, soybean and canola meals with 0 or 12% ground FS and fed with or without a multi-CE supplement. The CE preparation supplied per kilogram of complete diet: 500 units of pectinase, 50 units of cellulase, 400 units of mannanase, 1,200 units of xylanase, 450 units of glucanase and 45 units of galactanase. Diets were formulated for 2-phase feeding program of early-weaned pigs: phase I, 1 to 14 d and phase II, 15 to 28 d, corresponding to NRC (1998) nutrient specifications for pigs weighing 5 to 10 kg and 10 to 20 kg BW, respectively. The diets for phase I were fortified with dried whey, lactose and oat grouts to ease transition from the sow milk. Diets were formulated to similar nutrients content NRC (1998) with exception of DE, CP and AA, which were 95%, 94% and 97%,

respectively (Table 3.1). Prior to diet mixing, FS was ground in a hammer mill to pass through a 3-mm screen. Ground FS was added in the diet by changing the levels of wheat, barley, wheat middlings, peas, soybean and canola meals to balance the diets for DE and nutrients. Chromic oxide (0.3%) was added to each diet as an indigestible marker and diets were provided in mash form. Flaxseed used in the present study was provided by Flax Council of Canada, Winnipeg, MB, Canada.

Pigs and Housing

The experimental protocol was approved by the University of Manitoba Animal Care Committee (protocol # F05-004) and followed the principles established by the Canadian Council on Animal Care (CCAC, 1993). A total of 96 Cotswold piglets (48 barrows and 48 gilts) 17 ± 1 d old and 6.1 ± 0.4 (mean $\pm$ SD) kg BW with no prior exposure to creep feed were obtained from the University of Manitoba Glenlea Swine Research Farm. Based on their weaning BW the piglets were randomly assigned within gender to pens (n = 4 piglets per pen) containing either 4 barrows or gilts. The pens were equipped with a feeder, a nipple type drinker, plastic-covered expanded metal floors and a wall partitioning between pens that allowed visual contact with pigs in adjacent pens. Room temperature was initially set at 29.5°C and gradually reduced by 1.5°C per wk. Four diets in a 2 x 2 factorial arrangement were allotted in a completely randomized design to give 6 pens per treatment. Pigs had unlimited access to feed and water.

Table 3.1. Composition of the basal diets, as fed basis

Item	Flaxseed[2], %:	I 0	I 12	II 0	II 12
Phase[1]:		I		II	
Ingredients, %					
Wheat		28.0	22.5	35.2	42.0
Barley		7.15	6.36	24.0	12.0
Flaxseed		-	12.0	-	12.00
Soya bean meal		21.0	17.5	14.00	10.00
Peas		8.0	7.7	8.20	8.00
Canola meal		7.0	7.0	6.40	5.50
Wheat middlings		5.55	4.55	6.58	7.00
Dried whey		12.0	13.0	-	-
Oat Grouts		5.0	4.0	-	-
Lactose		2.0	2.0	-	-
Canola oil		1.0	-	2.20	-
Limestone		0.93	0.89	1.02	1.02
Biofos[3]		0.35	0.29	0.39	0.31
Vitamin-mineral premix[4]		1.0	1.0	1.0	1.0
L-lysine-HCl		0.47	0.54	0.52	0.62
Threonine		0.21	0.28	0.20	0.28
Chromic oxide		0.30	0.30	0.30	0.30
Calculated Nutrient Content					
DE, Kcal/kg		3,262	3,265	3,266	3,260
CP (N x 6.25), %		22.0	22.0	20.0	20.0
Total Lysine, %		1.32	1.31	1.12	1.12

[1]Phase I, (1 to 14-d); phase II, (15 to 28-d).
[2]Included by changing the levels of other feedstuffs to balance the diets for energy and nutrients.
[3]Ca, 21%; P, 17%, Feed rite, Winnipeg, MB, Canada.
[4]Provided per kilogram of complete diet: vitamin A, 8,255 IU; vitamin D3, 1,000 IU; vitamin E, 10.9 IU; vitamin B12, 0.115 mg; vitamin K, 1.1 mg; Niacin, 36.8 mg; Choline chloride, 781.2 mg; Biotin, 0.25 mg, Folic acid, 0.75 mg, Mn (as MnO), 55 mg; Zn (as ZnO), 50 mg, Fe (as $FeSO_4.H_2O$), 80 mg, Cu (as CuO), 5 mg; Se (as $NaSeO_3$), 0.1 mg, I (as Ca $(IO_3)_2$), 0.28 mg. The premix did not contain additional copper, zinc, antibiotics or probiotics.

Performance Monitoring and Sample Collection

Experimental diets were fed for a 28-d period during which individual BW and feed disappearance were monitored weekly. On d 7, 14, 21, and 28 blood samples (10 mL) were collected from 1 pig per pen whose BW was close to the pen average via jugular vein puncture into vacutainer tubes coated with lithium heparin (Becton Dickinson & Co, Franklin Lakes, NJ). The samples were immediately centrifuged at 2,000 x *g* for 10 min at 5°C to recover plasma, which was immediately stored at -20°C until used for urea N analysis.

Freshly voided feces were collected on d 28 for determination of nutrient digestibility, pH and ammonia. Fecal pH was determined prior to acidifying samples with 0.1 *N* HCl (1:1 wt/vol). Samples were frozen at -20˚C until required for analysis. After 28 d, pigs were put under feed restriction for 3 d prior to slaughter; feeding time was tailored to the estimated slaughter time, allowing 45 min per pig for anesthetizing and getting samples. The daily feed allowance was calculated to provide 2.6 times the maintenance energy, which was assumed to be 110 kcal of DE per kg $BW^{0.75}$ (NRC, 1998) and based on the pen average BW on d 28. Feed intake was restricted to maximize recovery of the digesta at the terminal ileum (Houdijk et al., 2002).

At the end of the 3 d restricted-feeding period, 1 pig per pen with BW close to the pen average was sacrificed to evaluate digesta pH, selected bacterial population, viscosity, organic acids, nutrient digestibilities and ammonia content. On the slaughtering day pigs were fed at the designated time; 2-h later pigs were held under general anesthesia by inhalation of 5% isoflurane (Pharmaceutical Partners of Canada, Inc., Richmond Hill, ON, Canada) via a facial mask.

The abdominal cavity was exposed by midline incision and ileal-cecal junction located; the ileum with its content was immediately clamped at 30 cm and 5 cm anterior to the junction, freed of mesentery, cleaved and flushed with physiological saline (Bimeda-MTC Animal health, Inc., Cambridge, ON, Canada) to remove excess blood. Ileal luminal contents were emptied into a sterile plastic sample bag (Fisher scientific, Fairlawn, NJ) and gently mixed. Approximately 5-g sample was aseptically transferred into a second sterile bag and placed on ice and transported (within an h) to the laboratory for selected bacterial enumeration. The viscosity and pH of the remaining ileal samples were determined after which the samples were acidified (0.1 *N* HCl, 1:1 wt/vol) and stored at -20˚C until required for analysis. The entire caecum and mid colon (30 cm) were cleaved and flushed with physiological saline and contents from each segment emptied into separate sterile bags. The pH was determined and the samples were acidified (0.1 *N* HCl, 1:1 wt/vol) prior to storage at -20˚C until required for analysis. Following digesta sampling, pigs were killed with an intercardiac overdose (110 mg/kg) of sodium pentobarbital (Bimeda-MTC Animal Health, Inc., Cambridge, ON, Canada).

Digesta and Fecal pH Measurements

The pH of the ileal, cecal and colon digesta were measured on undiluted samples. Fecal pH was measured on fecal homogenate (feces: deionized water, 1:1 wt/vol). All pH measurements were made with an electronic pH meter (Accumet Basic, Fisher Scientific, Fairlawn, NJ), which was standardized with certified pH 4 and 7 buffer solutions.

Ileal Digesta Viscosity Measurements

Ileal digesta samples from some pigs were too dry and it was difficult to obtain more than 0.1 ml supernatant sample for viscosity measurement. Therefore, all ileal

samples were diluted 1:1 (wt/vol) with deionized water to enable comparison between dietary groups. Briefly, within 30 min of collection, 1-g of thoroughly mixed ileal contents were diluted 1:1 (wt/vol) with deionized water, vortex mixed and centrifuged at 12,000 x g for 8 min. The supernatant fraction (0.5 mL) was placed in a Brookfield digital viscometer (Model DV-II+ Version 3.0, Brookfield Engineering Laboratories Inc., Stoughton, MA), in which viscosity was measured at a shear rate of 60/s at 38ºC. The viscometer was rinsed with deionized water and wiped clean between samples. The viscosity values were recorded as apparent viscosity in milliPascal seconds (mPa·s).

Laboratory Analysis

 Bacterial enumeration. Approximately 1-g ileal digesta sample was added to sterile 0.1% peptone (99 mL) massaged for 1 min (Stomacher Lab-Blender 400, Seward medical, London, UK) and serially diluted in sterile 0.1% peptone (9 mL). For *Esicherichia coli*, dilutions (10^4 to 10^8) were plated on Petrifilm^{TM} *E. coli/*Coliform plates (3M Canada Inc., London, ON, Canada). Typical colonies were counted following incubation at 35°C for 24 h. Lactobacilli were enumerated using MRS medium (Becton Dickson & Co., Franklin Lakes, NJ) following incubation at 32°C for 24 h. To determine aerobic and anaerobic spore formers, 5 mL portions of the diluted samples (10^1 to 10^4) were heated in a thermostatically controlled water bath at 80°C for 15 min. Samples were cooled in an ice bath, plated using Trypticase soy agar (Becton Dickson & Co., Franklin Lakes, NJ) and incubated at 35°C for 24 to 48 h. Anaerobic spore formers were incubated in jars containing anaerobic gas generating kits (BBL GasPak Plus, Becton Dickson & Co., Franklin Lakes, NJ). All plating was performed in duplicate and results recorded in colony forming units (CFU) per g of wet digesta.

Chemical analyses. Digesta and fecal samples were freeze-dried and, along with air-dried diet samples (phase II diets only), finely ground in Smart Grind coffee grinder (Applica Consumer Products, Inc., Miami Lakes, FL). Diets, ileal and fecal samples were analyzed for chromic oxide, DM, CP and NSP constituent sugars. Chromic oxide was determined using the procedure of Williams et al. (1962). Dry matter was determined by oven drying at 105°C for 12 h. Crude protein (N x 6.25) content was determined using a Leco NS 2000 Nitrogen Analyzer (LECO Corporation, St. Joseph, MI).

Non-starch polysaccharide levels were determined by gas-liquid chromatography (component neutral sugars) and by colorimetry (uronic acids) as described by Slominski et al. (2006). Briefly, 100-mg of feed or 50-mg of digesta or feces sample was boiled with 2 mL of dimethylsulfoxide for 1 h and incubated at 45°C overnight with a sodium acetate buffer solution (pH 5.2) containing the starch-degrading enzymes amylase, pullulanase, and amyloglucosidase (Sigma Chemical Co., St. Louis, MO). Ethanol was then added and the mixture was left for 1 h at room temperature before being centrifuged. The supernatant was discarded and the dried residue was dissolved in 1 mL of 12 *M* sulfuric acid and incubated for 1 h at 35°C. Six milliliters of water and 5 mL of myoinositol (internal standard) solution were added and the mixture was boiled for 2 h. The resulting hydrolysate was used to determine uronic acids and component sugars. For the component sugars, 1 mL of the hydrolysate was neutralized with 12 *M* ammonium hydroxide, reduced with sodium borohydride, and acetylated with acetate anhydride in the presence of 1-methylimidazole. Component sugars were separated using SP-2340 column and Varian CP 3380 gas chromatograph (Varian Canada, Inc., Mississauga, ON, Canada). Uronic acids were determined using the procedure described by Scott (1979).

Plasma samples were analyzed for PUN using a Nova Stat profile M blood gas and electrolyte analyzer (Nova Biomedical Corporation, Waltham, MA). Ammonia content in the digesta and fecal samples was analyzed as ammonia-N using the method described by Novozamsky et al. (1974). Briefly, an aliquot (5 g) of digesta or fecal samples was diluted with 0.1 N HCl (1:5, wt/vol), vortexed and centrifuged for 10 min at 2,000 x g. The supernatant (50 µL) was transferred to 10-mL test tube and 1.5 mL of a solution containing 200 mL 0.05% sodium nitroprusside and 10 mL of 4% EDTA was added and 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 a test tube rack wrapped with black plastic sheets and placed in complete darkness for 30 min followed by the reading of absorbance at 630 nm. Ammonia-N concentrations were determined by calculating the concentrations from a regression equation of the standard curve (range: 25 to 200 mg/L). To obtain the final ammonia concentrations in the sample, values calculated from the standard curve were corrected for dilution.

Organic acids (volatile fatty acids; VFA, lactate, and branched chain volatile fatty acids; BCVFA) were assayed in the ileal, cecal and colon digesta. Approximately 5-g of the digesta sample was resuspended with 25 mL of 0.1 N HCl (1:5, wt/vol) in 125 mL conical flask, tightly sealed with parafilm and loaded onto a controlled environment incubator shaker (New Brunswick Scientific, Inc., Edison, NJ) set at 180 rpm (at room temperature) for 2 h. The resulting digesta fluid was then assayed for OA using gas chromatography according to Erwin et al. (1961). Briefly, an aliquot of 2.5 mL of digesta fluid was mixed with 0.5 mL of 25% meta-phosphoric acid in a centrifuge tube and the mixture frozen overnight. Thawed samples were mixed with 200 µL of 25% NaOH and

vortexed; followed by addition of 320 µL of 0.3 M oxalic acid. The samples were then centrifuged for 20 min at 3,000 x g and supernatant (2 mL) transferred to GLC vial. The OA were determined using a glass column packed with 80/120 Carbopack B-DA/4% CABOWAX 20M (Supelco, Bellefonte, PA) in Varian model 3400 gas chromatograph. To obtain the final OA concentrations in the sample, gas chromatography readings were corrected for dilution. All analyses were performed in duplicate.

Calculation and Statistical Analyses

The apparent nutrient digestibilities were calculated as described by Opapeju et al. (2006). Bacterial enumeration data were transformed to $\log_{10}CFU/g$ before statistical analysis. Data were analyzed as a completely randomized design with 2 x 2 factorial treatments arrangement using the GLM procedures of SAS (SAS Inst., Inc., Cary, NC). Gender effect tested non-significant and was excluded from the model. Subsequently, the final model included the main effects of FS, C and associated two-way interactions. For the performance data (ADG, ADFI and G:F) the pen was the experimental unit, but for the other response criteria, pig within diet was the error term. Treatment effects were determined with orthogonal contrasts for a 2 x 2 factorial arrangement. Treatment differences were considered significant at $P < 0.05$ and trends $(0.05 > P < 0.10)$ were discussed. Where appropriate, main effect means and associated standard errors or P-values were reported in the text.

3.4 RESULTS

The analyzed CP and NSP contents for the phase II basal diets are shown in Table 3.2. The CP values were similar among the diets. The total NSP content in the FS diets was slightly more than in the non-FS diets.

Table 3.2. Analyzed CP and non-starch polysaccharides (NSP) contents of basal diets (%)[1]

Item Flaxseed[2], %:	0	12
DM	92.9	92.9
CP (N x 6.25)	18.4	18.5
NSP component sugars		
Arabinose	2.27	2.27
Xylose	2.93	3.28
Mannose	0.19	0.16
Galactose	0.78	0.79
Glucose	3.42	3.83
Uronic acid	0.64	1.96
Total NSP[3]	10.2	12.3

[1]Phase II (d 15 to 28)
[2]Included by changing the levels of other feedstuffs to balance the diets for energy and nutrients.
[3]Includes arabinose, xylose, mannose, galactose, glucose, and uronic acid

Growth Performance and PUN

Final BW, ADG, ADFI and G:F are shown in Table 3.3. The effect of dietary treatment on piglet performance was only evident in wk 3. Pigs fed FS diets had lower (P < 0.005) ADG and G:F (268 vs. 353 g/d and 0.55 vs. 0.62, respectively) and tended to have lower ADFI (481 vs. 561 g/d, P = 0.070) compared to pigs fed non-FS diets. The effect of FS and C on G:F tended to interact (P = 0.080) such that CE increased G:F in pigs fed FS diet but had no effect in pigs fed non-FS diet. Flaxseed and CE did not influence (P > 0.10) piglet growth performance in the entire experiment (d 1 to 28).

Flaxseed and CE affected PUN measurement on d 7 and 21 only (Table 3.3). On d 7, there was an interaction (P = 0.050) between FS and CE, such that CE reduced PUN in pigs fed FS diet but not in pigs fed non-FS diet. On d 21, FS and CE affected PUN independently, with both treatments resulting into higher PUN, (4.71 vs. 3.92 mmol/L for pigs fed FS and non-FS diets, respectively; P = 0.027 and 4.56 vs. 4.07 for pigs fed diets with CE and diets without CE, respectively; P = 0.090).

Table 3.3. Effect of flaxseed and carbohydrase addition on growth performance in nursery pigs[1]

Flaxseed[2], %:		0		12			P-value[3]		
Item Carbohydrases[4]:	-	+	-	+	SEM[5]	FS	C	FS x C
Initial BW, kg	6.0	6.1	5.9	6.2	0.4	-	-	-
Final BW, kg	12.4	12.9	12.1	12.6	0.8	0.668	0.554	0.968
ADG, g/d								
d 1 to 7	55.4	64.6	84.5	64.90	25.6	0.575	0.819	0.579
d 8 to 14	127	115	149	157	27.6	0.262	0.950	0.710
d 15 to 21	340	367	250	285	27.2	0.005	0.259	0.873
d 22 to 28	389	419	401	403	27.5	0.933	0.563	0.616
ADFI[6], g/d								
d 1 to 7	137	139	161	165	20.9	0.239	0.903	0.953
d 8 to 14	283	279	269	277	22.4	0.744	0.938	0.772
d 15 to 21	517	606	469	493	41.9	0.070	0.193	0.453
d 22 to 28	687	687	653	702	47.5	0.837	0.613	0.613
G:F								
d 1 to 7	0.35	0.37	0.46	0.29	0.125	0.928	0.555	0.446
d 8 to 14	0.45	0.40	0.53	0.56	0.079	0.159	0.922	0.604
d 15 to 21	0.63	0.61	0.52	0.58	0.026	0.005	0.749	0.080
d 22 to 28	0.57	0.61	0.61	0.59	0.031	0.901	0.771	0.296
PUN[7], mmol/L								
d 7	4.92	5.02	4.93	3.53	0.405	0.085	0.165	0.050
d 14	5.27	5.53	5.44	5.42	0.678	0.952	0.837	0.856
d 21	3.93	3.91	4.21	5.21	0.419	0.027	0.090	0.104
d 28	4.58	4.60	4.84	4.73	0.459	0.914	0.774	0.617

[1]Values are means of 6 observations per diet; dietary treatments were imposed at weaning.
[2]Included by changing the levels of other feedstuffs to balance the diets for energy and nutrients.
[3]Effects: FS = flaxseed; C= carbohydrase; FS x C = interaction.
[4]Provided per kilogram of complete diet: 500 units of pectinase, 50 units of cellulase, 400 units of mannanase, 1,200 units of xylanase, 450 units of glucanase and 45 units of galactanase.
[5]Pooled SEM.
[6]As fed basis.
[7]Plasma urea nitrogen.

Digesta and Fecal Microbial Activity

Pigs fed diets with CE had higher ileal lactobacilli count (8.94 vs. 8.25 $\log_{10}$ CFU/g, P = 0.009; Table 3.4) compared to pigs fed diets without CE. Pigs fed FS diets had lower ileal anaerobic spore formers counts than pigs fed non-FS diets (2.69 vs. 3.09 $\log_{10}$ CFU/g, P = 0.033). *Escherichia coli* and aerobic spore formers counts as well as lactobacilli to *Escherichia coli* ratio in pigs were similar (P > 0.10) among the diets. Except for lactobacilli, pigs fed FS diet supplemented with CE had numerically lower concentrations of all bacteria enumerated compared to pigs fed the other diets.

Flaxseed and CE affected ileal digesta ammonia content interactively (P = 0.05; Table 3.5). In the absence of FS, pigs fed diet with CE had lower ileal ammonia than those on the diet without CE. Although variability did not permit statistical differences in the cecal ammonia content, feeding FS diets resulted in a 36% increase in ammonia (441 vs. 325 mg/L; SEM 69.36) compared with the non-FS diets. At the fecal level, pigs fed FS diets tended to have lower fecal ammonia (324 vs. 404 mg/L, P = 0.067) compared to pigs fed non-FS diets.

The ileal, cecal, and colon pH were not affected by dietary treatment (P > 0.10; Table 3.5). However, FS and CE affected fecal pH independently. Pigs fed FS diets had a lower fecal pH (6.5 vs. 6.8, P = 0.008) than pigs fed non-FS diets whereas pigs fed diets with CE had higher fecal pH (6.7 vs. 6.5, P = 0.050) than pigs fed diets without CE. Flaxseed and CE did not interact (P > 0.10) in influencing VFA, lactate and total OA concentrations in the ileum (Table 3.5).

Table 3.4. Effect of flaxseed and carbohydrase addition on ileal microbial populations in nursery pigs[1, 2]

	Flaxseed[3], %:	0		12			*P*-value[4]		
Item	Carbohydrase[5]:	-	+	-	+	SEM[6]	FS	C	FS x C
Lactobacilli		8.01	9.12	8.50	8.76	0.454	0.774	0.009	0.088
Escherichia coli		6.47	6.84	6.97	5.64	0.638	0.588	0.459	0.198
Lactobacilli: *E. coli* ratio		1.24	1.33	1.23	1.55	0.174	0.861	0.320	0.601
Aerobic spore formers		3.39	3.27	3.28	3.03	0.148	0.242	0.221	0.657
Anaerobic spore formers		2.93	3.22	2.81	2.59	0.165	0.033	0.858	0.137

[1]Values are means of 6 observations per diet; dietary treatments were imposed at weaning.
[2]Log_{10} colony forming units per gram of ileal contents, wet basis.
[3]Included by changing the levels of other feedstuffs to balance the diets for energy and nutrients.
[4]Effects: FS = flaxseed; C= carbohydrase; FS x C = interaction.
[5]Provided per kilogram of complete diet: 500 units of pectinase, 50 units of cellulase, 400 units of mannanase, 1,200 units of xylanase, 450 units of glucanase and 45 units of galactanase.
[6]Pooled SEM.

Table 3.5. Effect of flaxseed and carbohydrase addition on digesta and fecal microbial activity in nursery pigs[1]

Item	Flaxseed[2], %: C[4]:	0 -	0 +	12 -	12 +	SEM[5]	FS	C	FS x C
Ileum									
Ammonia, mg/L		90.4	59.0	72.5	75.9	8.34	0.951	0.109	0.049
Lactate, mmol/L		9.20	17.4	4.38	6.75	2.64	0.009	0.061	0.288
VFA[6], mmol/L		7.71	11.7	5.66	5.45	2.21	0.045	0.152	0.210
Total OA[7], mmol/L		16.9	29.1	10.0	12.2	1.50	0.006	0.073	0.205
pH		7.8	7.9	8.2	8.1	0.253	0.239	0.840	0.599
Caecum									
Ammonia, mg/L		291	360	435	447	98.1	0.252	0.684	0.777
Lactate		2.84	5.86	3.80	2.74	1.870	0.452	0.492	0.149
VFA[6], mmol/L		99.1	107	104	102	8.349	0.978	0.740	0.535
BCVFA[8], mmol/L		2.54	3.45	3.47	3.59	0.454	0.259	0.275	0.399
Total OA[7], mmol/L		104	116	111	108	8.597	0.952	0.642	0.366
pH		6.7	6.5	6.7	6.9	0.285	0.611	0.998	0.479
Colon									
Ammonia, mg/L		507	504	524	519	115	0.802	0.673	0.706
Lactate, mmol/L		6.11	5.45	4.95	3.55	1.127	0.201	0.385	0.753
VFA[6], mmol/L		73.4	64.2	76.6	77.8	9.601	0.331	0.794	0.544
BCVFA[8], mmol/L		5.38	5.02	7.21	7.98	1.776	0.192	0.910	0.753
Total OA[7], mmol/L		84.8	74.7	88.5	91.2	11.40	0.348	0.804	0.627
pH		7.4	7.1	7.3	7.2	0.273	0.947	0.541	0.727
Fecal									
Ammonia, mg/L		378	430	289	360	40.93	0.067	0.149	0.811
pH		6.7	6.8	6.3	6.6	0.104	0.008	0.050	0.281

[1]Values are means of 6 observations per diet; dietary treatments were imposed at weaning.
[2]Included by changing the levels of other feedstuffs to balance the diets for energy and nutrients.
[3]Effects: FS = flaxseed; C= carbohydrase; FS x C = interaction.
[4]Provided per kilogram of complete diet: 500 units of pectinase, 50 units of cellulase, 400 units of mannanase, 1,200 units of xylanase, 450 units of glucanase and 45 units of galactanase.
[5]Pooled SEM.
[6]Volatile fatty acids; sum of acetate, propionate and butyrate.
[7]Total organic acids; sum of VFA, lactate and BCVFA.
[8]Branched chain volatile fatty acids; sum of isobutyrate, isovalerate and valerate.

Pigs fed FS diets had lower ileal concentration of VFA (5.56 vs. 9.71 mmol/L, P = 0.045), lactate (5.57 vs. 13.3 mmol/L, P = 0.009) and total OA (11 vs. 23 mmol/L, P = 0.006) than pigs fed non-FS diets. Pigs fed diets with CE tended to have higher ileal lactate (12.2 vs. 6.79, mmol/L, P = 0.061) and total OA (20 vs. 14, P = 0.073) than pigs fed diets without CE. There was no treatment effect (P > 0.10) on large intestine total OA concentrations. In relative terms, pigs fed either FS or non-FS diets resulted in a 10 and 5 fold increase in total cecal OA than in the ileum, respectively.

Ileal Digesta Viscosity

An interaction was observed between FS and CE in digesta viscosity (P = 0.045; Table 3.6). Enzyme supplementation increased digesta viscosity in pigs fed FS diets but no effect was observed in pigs fed non-FS diets. Generally, pigs fed FS-based diets had higher digesta viscosity (3.41 vs. 1.46, P < 0.001) than pigs fed non-FS diets.

Apparent Ileal and Fecal Components Digestibility

There was no interaction (P > 0.10) between FS and CE in apparent ileal component digestibilities (Table 3.6). Pigs fed FS-based diets had lower apparent ileal digestibilities of CP (63.1 vs. 69.2%, P = 0.023), DM (46.1 vs. 58.5%, P = 0.003), xylose (1.03 vs. 17.9%, P = 0.016), glucose (12.2 vs. 28.8%, P = 0.007) and total NSP (2.34 vs. 15.6%, P = 0.008) than pigs fed non-FS diets. Pigs fed enzyme supplemented diets had higher apparent ileal DM (55. 9 vs. 49.1%, P = 0.021), arabinose (3.37 vs. -10.4%, P = 0.014), mannose (35.5 vs. 13.2%, P = 0.001), and total NSP (13.9 vs. 4.1%, P = 0.044) digestibility than pigs fed diets without CE.

Table 3.6. Effect of flaxseed and carbohydrase addition on ileal viscosity and apparent ileal and fecal nutrient and NSP digestibilities (%) of nursery pigs[1]

| Flaxseed[2], %: | 0 | | 12 | | | P-value[3] | | |
Item C[4]:	-	+	-	+	SEM[5]	FS	C	FS x C
Ileum								
Viscosity, mPa·s	1.62	1.34	3.15	3.67	0.155	< 0.001	0.523	0.045
CP (N x 6.25)	68.5	70.0	60.7	65.5	2.489	0.023	0.223	0.527
DM	56.1	60.9	41.9	51.0	2.756	0.003	0.021	0.443
Arabinose	-6.48	5.81	-14.2	0.93	5.110	0.231	0.014	0.783
Xylose	14.4	21.3	-6.36	8.42	6.360	0.016	0.104	0.541
Mannose	13.6	37.1	12.8	34.0	4.209	0.644	<0.001	0.784
Galactose	-11.8	-7.27	-27.7	-12.86	7.986	0.184	0.228	0.550
Glucose	27.9	29.6	7.46	16.9	5.500	0.007	0.327	0.489
Uronic	9.93	13.4	13.8	23.6	8.070	0.394	0.418	0.699
Total NSP[6]	11.9	19.3	-3.76	8.45	4.532	0.008	0.044	0.594
Fecal								
CP (N x 6.25)	74.1	73.3	73.3	75.9	1.235	0.464	0.486	0.183
DM	79.5	78.7	75.6	78.9	0.645	0.009	0.068	0.004
Arabinose	64.8	61.6	67.6	69.3	2.112	0.022	0.718	0.257
Xylose	53.5	53.2	65.3	69.2	1.999	0.001	0.385	0.311
Mannose	77.7	74.4	78.1	78.6	1.373	0.115	0.326	0.180
Galactose	83.6	82.9	82.9	84.8	0.999	0.164	0.687	0.190
Glucose	45.6	43.9	53.9	56.4	2.003	0.001	0.845	0.299
Uronic	42.3	30.8	58.0	60.1	5.612	0.001	0.415	0.239
Total NSP[6]	55.4	52.6	62.8	65.1	1.675	0.001	0.889	0.146

[1]Values are means of 6 observations per diet; dietary treatments were imposed at weaning.
[2]Included by changing the levels of other feedstuffs to balance the diets for energy and nutrients.
[3]Effects: FS = flaxseed; C= carbohydrase; FS x C = interaction.
[4]Provided per kilogram of complete diet: 500 units of pectinase, 50 units of cellulase, 400 units of mannanase, 1,200 units of xylanase, 450 units of glucanase and 45 units of galactanase.
[5]Pooled SEM.
[6]Includes arabinose, xylose, mannose, galactose, glucose, and uronic acid.

There was an interaction ($P = 0.004$) between FS and CE in apparent fecal DM digestibility (Table 8). Enzyme supplementation increased apparent fecal DM digestibility in pigs fed FS diet but had no effect in pigs fed non-FS diet. Fecal CP digestibility was similar among diets ($P > 0.10$). Flaxseed and CE affected fecal NSP digestibility independently. Except for mannose and galactose, pigs fed FS diets had higher fecal digestibility of arabinose (68.4 vs. 63.2%, $P = 0.022$), xylose (67.2 vs. 53.3%, $P = 0.001$), glucose (55.1 vs. 44.7%, $P = 0.001$), uronic acid (59.0 vs. 36.5%, $P = 0.001$) and total NSP (64.0 vs. 54.0%, $P = 0.001$) than pigs fed non-FS diets. Carbohydrase supplementation had no effect ($P > 0.10$) on fecal NSP constituent sugars and total NSP digestibility.

3.5 DISCUSSION

As reviewed by Simon (1998), NSP-depolymerizing carbohdrase enzymes may have several modes of action: partial hydrolysis of NSP, decrease in digesta viscosity, and rupturing of NSP-containing cell walls, thereby making the encapsulated nutrients available for digestion. Other effects include shifts in the population and activity of the microflora as a result of enzyme supplementation (Vahjen et al., 1998). Flaxseed is a rich source of components such as ALA and lignans that possess broad anti-microbial activity (Pauletti et al., 2000; Kankaanpää et al., 2001). However, the high content of mucilaginous NSP in FS may impair nutrient utilization by increasing digesta viscosity (Bhatty, 1993). We hypothesized that by inclusion of a carbohydrase blend in a simple pig starter diet containing 0 or 12% ground FS more nutrients will be made available to support the piglet growth performance and that FS components as well as enzyme-

hydrolysis products from partial NSP breakdown would modulate microbial activity in the gastrointestinal tract leading to a healthier piglet.

Flaxseed effect on the overall growth performance in the present study is in agreement with the study reported by Van Kessel et al. (2006). In that study feeding piglets a wheat-soybean meal basal diet or the basal diet with either 5 or 10% ground FS exhibited similar growth performance. However, as demonstrated in wk 3 of the present study and studies with chicken (Rodríguez et al., 2002) FS can exert depressive effects on growth performance of young non-ruminant animals. This may be attributable to the presence of various anti-nutritional factors in this oilseed, such as mucilage, linatine (a vitamin B6 antagonist) and cyanogenic glycosides (Bhatty, 1993). The levels of these anti-nutritional factors were not determined in the present study as well as in the study by Van Kessel et al. (2006). It is rather difficult to explain why the depressive effects of FS occurred in the third wk and not earlier. However, it should be pointed out that it takes several days for the weaned pigs to recover from weaning effects (Pluske et al., 2002) and it is likely that weaning effects may have confounded diet effects on growth performance during this period.

In agreement with studies by Högberg and Lindberg (2004) and Zijlstra et al. (2004), enzyme supplementation did not have consistent effects on growth performance variables. This is surprising given that a previous study in this laboratory using the same multi-carbohydrase enzyme showed consistent improvement in growth performance of piglets fed wheat-soybean-based diets (Omogbenigun et al., 2004). However, contrary to the present study pigs in the previous study were given a commercial starter diet for a 1-wk adaptation period immediately after weaning before exposure to experimental diets.

Along with other experimental variations; lack of an adaptation period in the present study may have contributed to the differences between the two studies. Nevertheless, multi-carbohydrase supplementation in FS-based diet improved gain efficiency in wk 3. This indicated potential for the carbohydrase blend in overcoming depressive effects of FS in starter diets.

Carbohydrase supplementation reduced PUN in pigs fed FS diet and not in pigs fed the non-FS diet in wk 1. In addition, pigs fed FS diets tended to have lower PUN than pigs fed non-FS diets in wk-1. Plasma urea N has been used as an indicator for AA breakdown as a result of less than optimal systemic AA supply for protein synthesis (Coma et al., 1996) and of muscle protein breakdown to release AA for synthesizing acute phase proteins in the liver as a response to immune system activation (Wannemacher, 1977). In this context, McCracken et al. (1995) demonstrated that metabolic responses during the initial days post weaning were due to diet dependent and independent factors. Thus, we cannot clearly establish what in the present study may have caused the observed differences in PUN in wk 1. In the third wk, however, pigs fed FS diets had higher PUN levels than pigs fed non-FS diets, which coincided with reduced ADG for pigs in this treatment group. This indicated that FS may have adversely affected dietary AA availability leading to less than optimal systemic AA supply for protein accretion resulting in increased AA catabolism concomitant with growth depression. However, FS effect on PUN was not detected in wk 4 possibly because the digestive system at this age was capable of overcoming any adverse effect of FS on dietary AA availability.

Lactic acid bacteria such as lactobacilli and streptococci dominate microbial populations in the swine small intestine (Pluske et al., 2002). Increasing concentrations of lactate in ileal digesta should therefore reflect an increased population and activity of these microbes (Pluske et al., 2002). In the present study pigs fed enzyme-supplemented diets had higher ileal lactate and total OA concentrations as well as high ileal mannose, arabinose and xylose digestibility. This indicated that enzyme hydrolysis products containing arabinose, xylose and mannose sugar residues may have supported lactic acid bacteria activity. Indeed, pigs fed enzyme supplemented diets had high ileal lactobacilli count compared to pigs fed unsupplemented diets. These observations support recent interest to use carbohydrase enzymes to generate carbohydrate fragments capable of supporting beneficial bacterial groups in the gastrointestinal tract (Pluske et al., 2002). Similarly, an increased molar proportion of lactate in the ileum was reported when piglets were fed cereal-based diets supplemented with xylanase and β-glucanase (Högberg and Lindberg, 2004). Overall, these findings are of significant importance in the management of weaned pigs since lactate has been shown to have antibacterial effects on *Escherichia coli* and *Salmonella* species (Nout et al., 1989), and lactobacilli has been shown to inhibit adhesion of enterotoxigenic *Escherichia coli* to the ileal epithelium (Hillman et al., 1995). Although a statistical difference was not apparent, numerically lower ileal *Escherichia coli* count in pigs fed enzyme supplemented diets (6.24 vs. 6.72 $\log_{10}$ CFU/g) than pigs fed unsupplemented diets in the present study is indicative of suppression of this organism. Furthermore, the reduction in ileal digesta ammonia as was observed in enzyme-supplemented non-FS diet is a further indication of a healthier gut as high

concentration of ammonia is thought to have negative influences on gut health (Lin and Visek, 1991).

Except for the enzyme-supplemented non-FS diet, the levels of lactobacilli in the present study were close to those reported for ileal digesta of growing (33 kg BW) pigs fed diets with or without FS (Smith et al., 2004). In that study, ileal levels for lactobacilli in pigs fed either a wheat-peas-soybean basal diet or basal diet plus 20% ground FS were 7.97 and 8.13 $\log_{10}$ CFU/g, respectively. Thus, pigs fed FS diets exhibited reduced ileal OA concentration, anaerobic spore formers count and NSP digestibility perhaps indicating pre-cecal suppression of microbial activity. Indeed there tended to be an interaction in lactobacilli count such that in the absence of FS pigs fed C supplemented diet had higher ileal lactobacilli count. A reduction in ileal microbial activity in FS fed pigs albeit high viscosity would appear to contrast studies in poultry (Langhout et al., 2000) and piglets (McDonald et al., 2001) associating high intestinal viscosity with high bacterial activity. However, the present data appears to indicate that other chemical components in the FS may have influenced microbial activity in the gastrointestinal tract. For instance, the aforementioned studies used several sources of viscous polysaccharides such as guar gum, carboxymethylcellulose, pearl barley and citrus pectin to elevate intestinal viscosity, sources which are distinctly different from FS especially in terms of chemical composition. In this context, FS is a rich source of components such as ALA and lignans that possess broad anti-microbial activity (Pauletti et al., 2000; Kankaanpää et al., 2001). Indeed, ALA from FS has been exploited in poultry to control protozoa responsible for coccidiosis (Allen et al., 1997). Similarly, the observed suppression of anaerobic spore formers in the present study is indicative of FS potential in reducing

piglets' intestinal pathogens such as *Clostridium perfrigens*, a common etiological agent in swine enteric diseases which is a prominent member of the anaerobic spore formers group (Moxley and Duhamel, 1999). Clearly, pathways that could suppress microbial activity, especially the pathogenic types in the ileum of FS fed piglets need further investigation.

Large intestine OA and ammonia concentrations were 5 to 10-fold higher relative to the ileum and resulted in additional fermentative activities yielding branched chain volatile fatty acids (BCVFA, sum of isobutryrate, isovalerate and valerate). This indicated that there was higher microbial activity and considerable N metabolism in the hindgut. This is not surprising given that it is well established that OM entering the large intestine is subjected to extensive fermentation owing to a large reservoir of bacteria (Ewing and Cole, 1994; Pluske et al., 2002). However, the nutritional significance of the hindgut N metabolism is inevitably reduced by the fact that the host cannot utilize either bacterial protein or the BCVFA (Dierick and Decuypere, 1996). In contrast to the ileum, dietary treatments did not have significant effects in large intestine total OA and ammonia concentrations. This implied that there are greater opportunities of utilizing FS and CE to modulate intestinal microbial activity in the ileum than in the large intestine. It further shows that the antimicrobial activity of FS may not be as effective in the large intestine probably due to the large number of bacteria in this section of the gut. In general, our results indicate that lactate was the principal OA in the ileum, while VFA dominated in the large intestine, which agreed with earlier findings (Högberg and Lindberg, 2004).

As noted by Nyachoti et al. (2006) digesta pH is not well correlated with OA concentration. Subsequently, the observed differences in the ileal OA concentrations were not reflected in ileal pH values. It has been suggested that digesta pH is dependent on the individual organic acid pK values, proportion of specific OA and the buffering capacity of dietary nutrients such as protein (Ewing and Cole, 1994). This suggestion was partly supported by the cecal pH which declined in response to more diversified OA profiles. Nonetheless, gastrointestinal tract pH values observed in the present study decreased from ileum to caecum as has been reported for weaned pigs (Högberg and Lindberg, 2004). The colon pH was higher than in the caecum, which coincided with considerably higher ammonia in the former section. The elevation of colon pH is an indication that by the time the digesta had reached the colon there was little fermentable carbohydrate remaining and protein fermentation was occurring releasing ammonia, which is known to raise pH (Ewing and Cole, 1994). Pigs fed FS diets had lower fecal ammonia and pH than pigs fed non-FS diets indicating exhaustion of fermentable protein at the fecal level. Interestingly, pigs fed CE supplemented diets had higher fecal pH. This may be indicative of increased bacterial protein degradation, more proximal completion of carbohydrate fermentation or more complete OA absorption (Houdijk et al., 2002).

Viscosity is often measured on the liquid portion of the digesta as separated by centrifugation (Zijlstra et al., 2004). The small intestine is the main site sampled for viscosity measurements in pigs and the need for dilution does not seem to be a commonly recorded problem due to the fact that there is greater water content in the upper regions of the intestinal tract (McDonald et al., 2001). However, in the present study some samples were too dry to allow for extraction of enough liquid which necessitated dilution of

intestinal contents to enable comparison between diets. Similarly, McDonald et al. (2001) diluted dry intestinal samples with water (1:1 wt/vol) to enable viscosity measurement. As dilution is expected to reduce the actual viscosity (Bedford and Schulze, 1998), the viscosity values in the present study should be viewed as an estimate of apparent viscosity for the purposes of comparison between diets rather than as an absolute quantification.

It has been suggested that CE are capable of partially degrading soluble NSP into smaller molecular weight polymers and thus decreasing digesta viscosity (Simon, 1998). However, in the present study pigs fed FS diet with CE had greater viscosity than pigs fed FS diet without CE, suggesting that CE effect on NSP mediated intestinal digesta viscosity may be dependent on the nature of the viscous NSP. In this context, viscosity has been described as a consequence of NSP dissolving in the digestive tract to form high molecular weight viscous aggregates (Bedford and Schulze, 1998). Subsequently, increased viscosity in the presence of CE as was observed in the present study is an indication of further NSP solubilization. Similarly, an enzyme mediated increase in digesta viscosity was observed in weaned pigs fed rye-based diets supplemented with pentosanase and it was associated with enzyme-catalyzed release into solution of insoluble pentosans (Bedford et al., 1992). The soluble NSP in the present study derived primarily from wheat, barley and FS as polysaccharides in canola meal, soybean meal and peas are minor contributors (Bach Knudsen, 1997). However, their relative contribution to the overall digesta viscosity was such that FS exerted more viscosity than other feedstuffs as indicated by high viscosity in FS-based diets. This is not surprising as

FS mucilage is a viscous polysaccharide with functional properties similar to those of gum Arabic (Rodríguez et al., 2002). Indeed, FS diets had slightly higher NSP content.

Pigs fed FS diets had lower apparent ileal digestibility of DM and CP, an observation that correlated well with the data discussed earlier demonstrating that FS depressed piglet performance and increased PUN in wk 3. The depressive effect of FS on nutrient digestibility could be attributable to the viscousness of the FS mucilage. Viscosity is considered to be the mechanism by which soluble fiber components, such as β-glucans, arabinoxylans, gums, mucilage and pectins reduce nutrient digestibility by interfering with interaction between digestive enzymes and their substrates (Bedford and Schulze, 1998). Therefore, it is no coincidence that FS diets had the highest ileal digesta viscosity and lowest CP and DM digestibility. The improved DM digestibility in the enzyme-supplemented diets in the present study appears to be due mainly to an increase in NSP digestibility, particularly NSP containing mannose, xylose and arabinose sugar. The improved fecal DM digestibility in enzyme supplemented FS diet could be attributable to prolonged action of the added enzymes and the presence of microorganisms in the large intestine.

The loss of 12% total NSP before the terminal ileum of pigs fed enzyme unsupplemented non-FS diet support suggestions by Gdala et al. (1997) that bacterial activity capable of degrading NSP are present in the upper small intestine. Ileal digestibility of some NSP constituent sugars among diets and total NSP for non-enzyme FS diet were negative. Negative ileal digestibility of NSP and constituent sugars has been reported in swine (Graham et al., 1986). To this end, several mechanisms have been advanced for the negative ileal NSP digestibility values: anti-peristaltic movements,

contaminating endogenous and microbial matter as well as phase separation of the digesta and the marker (Graham et al., 1986; Högberg and Lindberg, 2004). Since neither of these mechanisms was investigated it is not definitive as to the probable reasons for the negative ileal digestibility values observed in the present study. However, it is noteworthy that galactose digestibility values are consistent with findings reported by Lien et al. (1997), demonstrating that endogenous mucins accounted for 71-82% of galactose in the terminal ileum of growing pigs. Nevertheless, with the exception of mannose, galactose and uronic acid, FS diets had lower pre-cecal constituent sugars and total NSP digestibility, which coincided with lower microbial activity observed in pigs fed these diets. An increase in arabinose, xylose and mannose digestibility coincided with a tendency for higher OA concentration in ileal digesta of piglets fed enzyme-supplemented diets.

Total tract NSP digestibility was higher than in ileum and reflected increased OA and ammonia production in the large intestine. However, in contrast to the ileum and with the exception of galactose, FS diets had higher total tract constituent sugars and NSP digestibility than non-FS diets. This indicated that inclusion of FS in the piglet diet shifted microbial activity from the ileum to the hindgut, which coincided with higher DM digestibility as well as lower fecal pH and ammonia concentration in pigs fed FS diets.

In conclusion, the present study demonstrated that FS reduced ileal microbial activity, ileal nutrient digestibility and piglet performance in wk 3. However, it should be noted that diet composition is one of the major factors that can influence nutrient utilization and microbial activity in the gastrointestinal tract, mainly through the contents of anti-nutritional factors and the nature of the substrate available, respectively. Thus, to

clearly delineate effects due to FS, feedstuff levels other than FS ought to be similar among the diets. However, this could not be achieved in the present study owing to the fact that FS like wheat, barley, peas, canola and soybean also supplied energy and nutrients. Indeed FS contains 20% CP, 41% oil and 30% dietary fiber on a DM basis (Slominski et al., 2006). Carbohydrase on the other hand increased gain efficiency in FS fed pigs, ileal DM and NSP digestibility, lactobacilli and lactate concentrations as well as reducing ileal ammonia content. Although these effects were not translated into improved growth performance, it should be pointed out that the present study was conducted in a research facility. Subsequently, the effectiveness of CE on piglet intestinal microflora should be evaluated in conditions commensurate to commercial facilities to fully understand the impact on gut health and performance. Furthermore, the prospects for using CE to hydrolyze flaxseed NSP and thus allowing FS inclusion in starter diets needs to be evaluated more so in light of some FS fractions possessing anti-microbial properties.

CHAPTER FOUR

MANUSCRIPT II

A pilot study to establish *in situ* model for the enterotoxigenic *Escherichia coli* infected piglet jejunal segments using conventional anti-diarrhoea agents (fumaric acid, zinc oxide, egg yolk antibodies or carbadox)

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4.1 ABSTRACT

A pilot study was conducted to establish an *in situ* model for the enterotoxigenic *Escherichia coli* (ETEC) infected jejunal segments. Intestinal segments (10 per piglet) prepared in four anesthetized piglets were then used to evaluate the effects of conventional anti-diarrhea agents (ZnO, fumaric acid; FA, egg yolk antibody against K88 fimbriae; EYA and carbadox; AB) on net fluid and electrolyte losses and prostaglandin E_2 (PGE_2) levels upon infection with enterotoxigenic *E. coli* (ETEC). Pairs of segments (non-infected and ETEC-infected) were perfused with saline (control), FA, ZnO, EYA or AB during a 7.5 h period. Infected segments perfused with saline had higher ($P < 0.05$) net fluid and electrolyte losses and PGE_2 levels compared to segments perfused with anti-diarrhea agents.

Keywords: Anti-diarrhea agents, Enterotoxigenic *E. coli*, *In situ* model, piglets

4.2 INTRODUCTION

Enterotoxigenic *E. coli* infection results in intestinal fluid and electrolyte losses in piglets. Piglets with ETEC induced gastroenteritis suffer from depressed growth, lack of appetite, morbidity and mortality (Fairbrother et al., 2005). Colibacillosis induced by ETEC has become increasingly difficult to treat because of the growing antibiotic resistance by many strains of these bacteria (Fairbrother et al., 2005). Subsequently, non-antibiotic intervention methods for these organisms are a high priority. Many non-antibiotic alternatives to ETEC control have been tested but with varied degree of success (Pluske et al., 2002). Quite often the methodology for evaluating many of these non-antibiotic interventions on ETEC control have often led to incomparable results between studies (Pluske et al., 2002). A better approach is to utilize a method which would allow

direct application of test additives to evaluate the outcome of the experimental infection. In this manuscript, a piglet small intestinal segment perfusion method was established and utilized to evaluate the efficacy of FA, ZnO, EYA and AB in attenuating ETEC-induced fluid and electrolyte losses. In addition, intestinal tissue PGE_2 was measured to assess host response to infection.

4.3 MATERIALS AND METHODS

Animals and Surgical Procedures

The experimental protocol was approved by the University of Manitoba Animal Care Committee (protocol # F06-025) and followed the principles established by the Canadian Council on Animal Care (CCAC, 1993). Four Genesus ([Yorkshire x Hampshire] x Duroc; Keystone Pig Advancement Inc., Oakville, MB, Canada) 3-wk old piglets were obtained from the University of Manitoba Glenlea Swine Research Farm and fed a standard commercial starter diet (FeedRite, Winnipeg, MB) for 7 d before surgery. The starter diet had a minimum of 19 % and 4.8 % crude protein and crude fat, respectively and a maximum of 2.6% crude fiber.

The anesthetic and surgical procedures were as described previously (Nabuurs et al., 1993). Briefly, each piglet (fasted overnight) was tranquillized with 20 mg of ketamine and 2 mg of xylazine per kg BW. Anesthesia was induced and maintained throughout the experiment with isoflurane and oxygen via intubation. The piglet was placed in dorsal recumbency on a heated surface, to maintain body temperature. The abdominal cavity was opened and 10 jejunal segments prepared beginning approximately 300 cm caudal to the pylorus. The 300-cm mark was consistently identified in all piglets by using a 300 cm piece of string. Briefly, one end of the string was held at the pylorus

junction and the full length of the string run along the convolutions of the intestines without cutting the mesentery. For the first segment preparation, a cut was made across the 300 cm mark such that only the intestine was cut while the mesentery remained intact. A small cranial tube (inflow; i.d. 3 mm, o.d. 5 mm) was placed and a wider tube (outflow; i.d. 5 mm, o.d. 9 mm) was placed 20 cm distal (measured by 20 cm piece of string) from the first. Caudal from and adjacent to this first segment nine other segments were prepared in the same way giving a total of ten segments. Segments were labeled consecutively in numerals beginning from the first segment to the last. Between odd and even segments, 2 cm pieces of the intestine were removed during segments preparation for measurement of the circumference. The segments were completely isolated from each other and covered between 37 and 73% of the total length of the jejunum based on small intestine length for a 3 wk piglet according to Adeola and King (2006).

Bacterial Strain

A pure strain of ETEC was obtained from Dr. Carlton Gyles of the University of Guelph (Guelph, ON, Canada) and was confirmed by PCR genotyping as possessing the genes for K88 fimbrial antigen, heat-labile and heat-stable toxins (Setia, 2007). Prior to preparation of the inoculation culture, the strain was progressively made resistant to 5 μg ciprofloxacin/ mL in Luria-Bertani broth. The inoculation culture was grown overnight in Luria-Bertani broth containing 5 μg ciprofloxacin (Sigma, St. Louis, MO/ mL. After overnight incubation at 37°C with shaking, the culture was centrifuged at 3,000 x g for 10 min at 37°C and the pellet suspended in PBS to an OD_{600nm} = 1.0 corresponding to 10^8 cfu/mL.

Test solutions, Infection and Perfusion Procedure

Treatments were FA, ZnO, EYA, and AB. Egg yolk antibodies (titer of 500,000 of anti-K88) were kindly provided by a local company (Zyme Fast Inc., Oakbank, MB, Canada). Treatments were suspended in deionized water in concentrations (FA; 20, ZnO; 3,000, EYA; 5000 and AB; 55 mg/l). Physiological saline solution was also included as a treatment to serve as control. All perfusion mixtures were supplemented with 1g/L of glucose and casamino acids (acid-hydrolyzed casein) to assure bacteria growth. Fifteen min before perfusion started even numbered segments (i.e. 2, 4, 6, 8 and 10) were perfused with 5 mL of PBS and odd numbered segments were perfused with 5 mL of the ETEC suspension in PBS. In each piglet, four pairs of segments (an ETEC-infected and an adjacent non-infected) were perfused using a 4 x 4 Latin square design for FA, ZnO, EYA, and AB only. Saline was perfused in the middle two segments (5 and 6) in all piglets. Pairs of segments, one non-infected and the other ETEC-infected were perfused simultaneously with 60 ml of different treatments using syringes attached to the inflow tubes over a 7.5 h period by perfusing 4 ml of fluid every 30 min. The non-absorbed fluid passed through the outflow tubes into corresponding drainage bottles placed at the same level as the piglet's abdomen. At the end of the experiment the fluid remaining in the segments was emptied into the drainage bottles (outflow). The piglets were killed by an intracardiac injection of 110 mg sodium pentobarbital/kg bodyweight (Bimeda-MTC Animal Health, Inc., Cambridge, ON, Canada). The segments were dissected free of the mesentery and the length measured and from each segment; tissue samples were taken and stored at -80°C until required for PGE_2 analysis.

Laboratory Analysis

Prostaglandin E_2 was measured using an enzymatic immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. To normalize PGE_2 measurements, total protein content in the intestinal tissue was determined using the method of Lowry with bovine serum albumin as a standard (Hartree et al., 1972). The PGE_2 concentration was expressed as pg/mg protein. Sodium and Cl^- contents in the perfusion and the outflow fluids were determined using Dual Ion Chromatography (Dionex Canada, Oakville, ON, Canada). Osmolarity was determined using The Advanced™ Micro Osmometer (Model 3300, Advanced Instruments Inc., Norwood, MS). To determine viable counts of ETEC, outflow fluids (1 mL) and mucosal scrapings (1 g) from non-infected and ETEC infected segments were serially diluted in 0.1% peptone water. Dilutions (10^4 to 10^9) were plated on LB agar supplemented with 0.5μg of ciprofloxacin/ mL. After 24-h incubation at 37°C under aerobic conditions, cfu were counted.

Calculations and Statistical Analysis

Net fluid, total solutes, Na^+ and Cl^- absorption were calculated from the difference between the volume and concentration of inflow and outflow divided by the surface area (length x circumference) of the segment. Net fluid and electrolyte losses were defined as the difference between net absorption of non-infected and infected segments perfused with the same treatment. Bacterial enumeration data were transformed to $log_{10}CFU/g$ or mL before statistical analysis. Data were subjected to GLM procedures of SAS and means were separated means were separated by PDIFF Adjust = Tukey option.

Prostaglandin E_2 data from non-infected and ETEC-infected segments perfused with the same perfusion fluid were compared using the Student's paired t test.

4.4 RESULTS

Enterotoxigenic *E. coli* infection resulted in higher net fluid, total solutes, sodium and chloride losses ($P < 0.05$) in segments perfused with saline compared with other segments (Figure 4.1). In saline perfused segments, ETEC infection resulted in high ($P < 0.05$) levels of PGE_2 compared to non-infected segment (Figure 4.2). Overall, PGE_2 levels for the segments perfused with any of the anti-diarrhea agents were similar ($P > 0.05$) between infected and non-infected segments. Bacterial enumeration was only reported for the ETEC-infected segments because no growth was observed from samples from the out-flow fluids and mucosal scrapings of the non-infected segments. The ETEC counts in outflow and mucosal scrapping of ETEC-infected segments are shown in (Table 4.1). No ETEC counts were detected in segments perfused with C and FA after 24 h incubation at 37°C. The ETEC numbers in outflow fluid of segments perfused with EYA was higher ($P < 0.05$) than in segments perfused with S and ZnO, however, these differences were not evident ($P > 0.05$) in mucosal scrapings.

4.5 DISCUSSION

Enterotoxigenic *E. coli* is among the most common enteric pathogens causing acute secretory diarrhea in neonatal and weaned piglets (Fairbrother et al., 2005). Diarrhea due to ETEC is attributable to the action of one or more enterotoxins produced by bacteria that have colonized the small intestine.
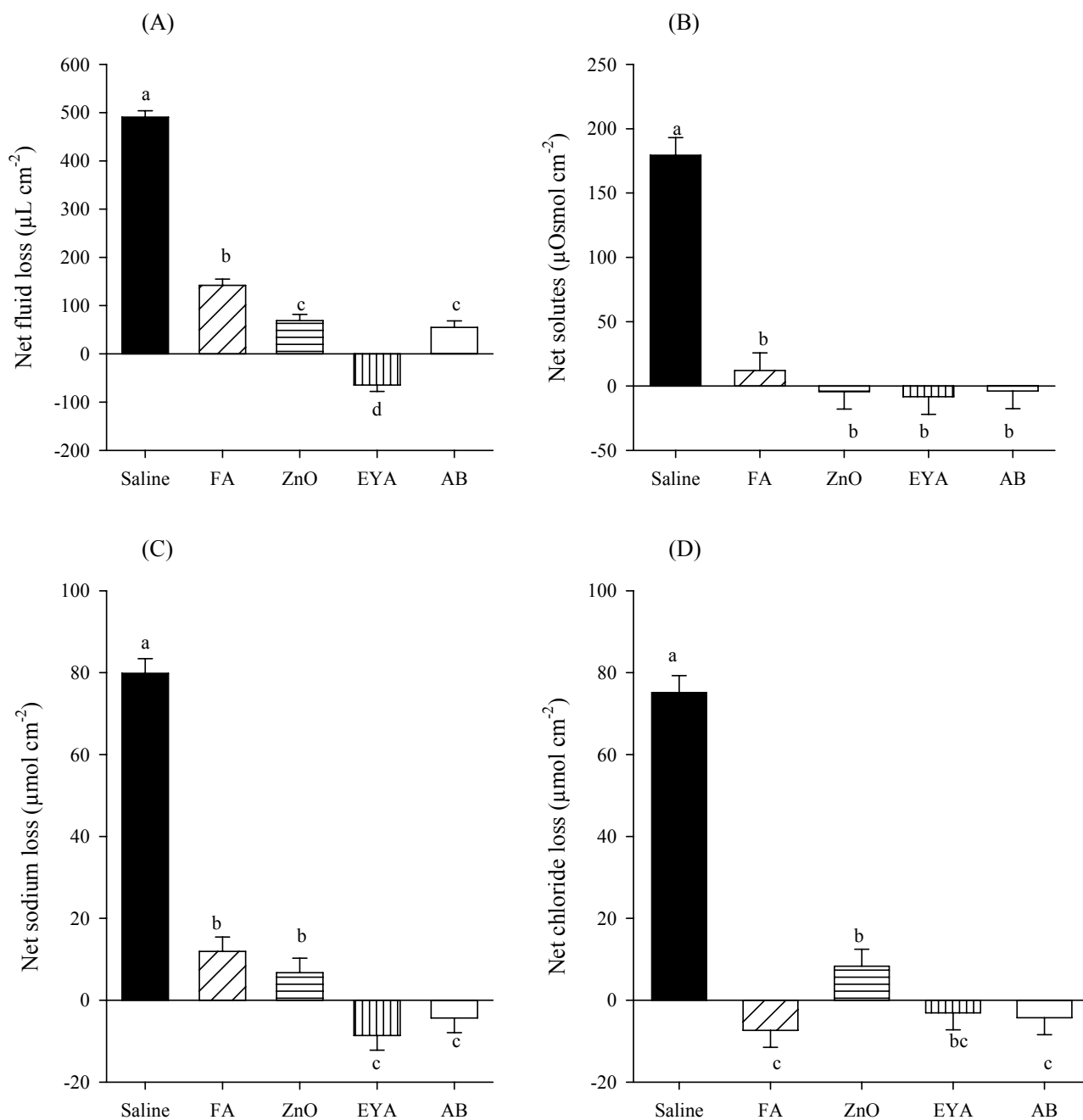
Figure 4.1. Fluid (panel A), net solutes (panel B), Na$^+$ (panel C) and Cl$^-$ (panel D) losses upon ETEC infection in piglet small-intestinal segments after perfusion with saline, fumaric acid (FA), ZnO, egg yolk antibodies (EYA) and carbadox (AB).
Values are means, with their standard errors represented by vertical bars. Within a panel mean values with different letters were significantly different (P < 0.05).
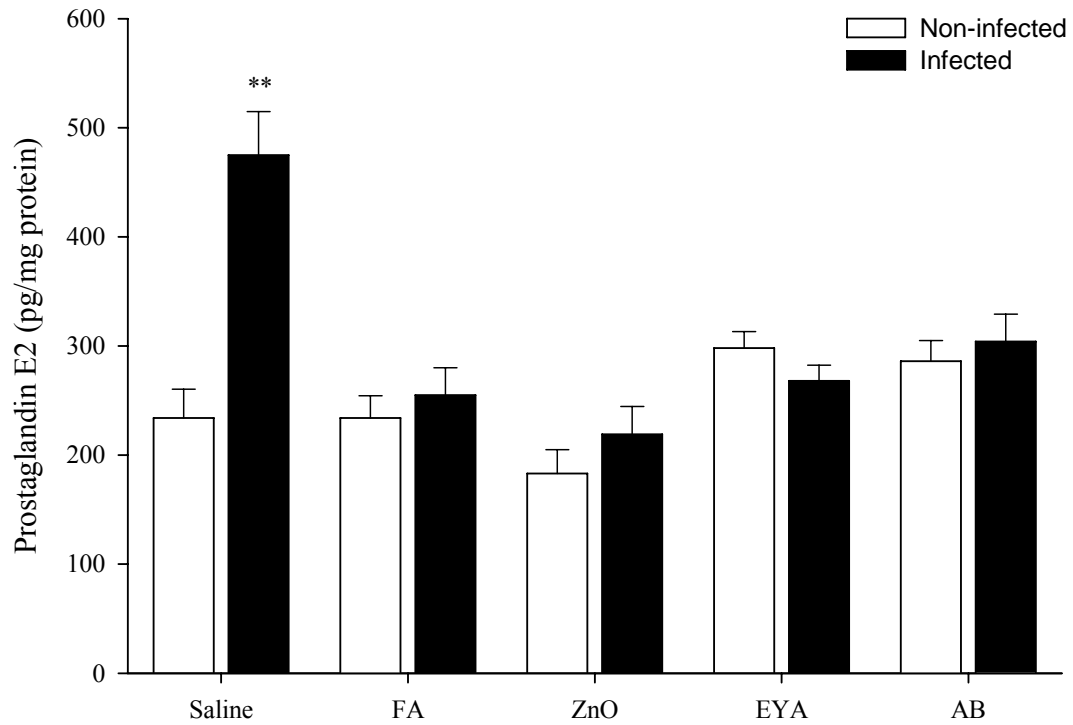
Figure 4.2. Levels of prostagland E2 in non-infected (□) and enterotoxigenic *E. coli*-infected (■) piglet jejunal segments after perfusion with saline, fumaric acid (FA), ZnO, egg yolk antibodies (EYA) and carbadox (AB).
Values are means, with their standard errors represented by vertical bars. ** Different ($P < 0.01$) from non-infected counterpart.

Table 4.1. Enterotoxigenic *Escherichia coli* count in the outflow fluid and mucosal scrapings in infected piglet jejunal segments after perfusion with saline (S), fumaric acid (FA), ZnO, carbadox (AB) and egg yolk antibodies (EYA)[1]

Item	Fluid (Log$_{10}$CFU/mL)		Mucosal scrapings (Log$_{10}$CFU/g)	
	Mean	SEM	Mean	SEM
S	5.87[b]	1.014	5.85	0.985
FA	NV[3]	-	NV[3]	-
ZnO	5.43[b]	0.850	5.45	0.900
AB	NV[3]	-	NV[3]	-
EYA	8.55[a]	0.881	5.95	0.714

[1]Means values with their SEM.
[2][ab]Mean values within a column with unlike superscript letters different ($P < 0.05$).
[3]No viable count detected after 24 h incubation at 37°C.

Studies suggest that enterotoxins also up regulate inflammation mediators such as PGE$_2$ that, through autocrine or paracrine effects, also stimulate intestinal secretion (Farthing, 2002). Effects of the anti-diarrhea agents on the outcome of ETEC infection was examined using a perfusion model considered to be suitable for quantitative assessment of the effect of enterotoxin-producing pathogens on net absorption in the small intestine (Nabuur et al., 1993). Challenge of the intestinal segments with ETEC resulted in increased fluids, solutes and electrolytes losses upon perfusion with saline. However, segments perfused with anti-diarrhea agents showed reduced or absence of fluid and electrolytes losses. These observations further demonstrated the efficaciousness of FA, ZnO, EYA and AB in controlling ETEC-secretory diarrhea as has been reported for pigs fed these additives (Tsiloyiannis et al., 2001; Savoini et al., 2002; Owusu-Asiedu et al., 2003b; Roselli et al., 2003). Furthermore, these observations suggested that the *in situ* model was successively established.

Enterotoxigenic *E. coli* infection elicited high levels of PGE$_2$ upon perfusion with saline, effects which were not observed in segments perfused with anti-diarrheal agents. Increased intestinal fluid secretion is a protective host response to enteric infections that may be mediated by PGE$_2$ (Farthing, 2002). Thus, anti-diarrhea agents might have prevented ETEC induced inflammatory mediators and likely the consequence of attenuating fluid losses as observed in the present study. Surprisingly, the number of ETEC attached on the mucosal scrapings of ETEC-infected segments upon perfusion with saline, EYA and ZnO were not different. Bruins et al. (2006) reported similar observations using the same model of secretory diarrhea.

The mechanisms of the protective effects of FA, ZnO, EYA and AB are largely unknown. Our finding that these anti-diarrhea agents attenuated fluid losses and down regulated inflammation mediators contribute to the understanding of the mechanisms through which they prevent ETEC-induced secretory diarrhea in piglets. The current trend in swine research is to minimize or eliminate usage of antibiotics such as carbadox and minerals such as ZnO in swine diets. Subsequently, evidence that FA and EYA could protect against ETEC-infection commensurate to AB and ZnO provides further implications for prevention and perhaps treatment of common infectious diarrhea in piglets without recourse to antibiotics and mineral salts.

CHAPTER FIVE

MANUSCRIPT III

Response of enterotoxigenic *Escherichia coli* (K88) infected piglet jejunal segments to products derived from hydrolysis of wheat and flaxseed non-starch polysaccharides by carbohydrase enzymes

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5.1 ABSTRACT

Enterotoxigenic *Escherichia coli* infection results in intestinal fluid and electrolyte losses in piglets. The response of ETEC-infected piglet jejunal segments to hydrolysis products derived from wheat middlings (WM) and FS NSP was investigated. Products were generated by incubating ethanol-extracted WM or FS with a blend of carbohydrase enzymes. Following incubation, slurries were centrifuged and the supernatants mixed with absolute ethanol to produce 2 product types: 80% ethanol-soluble (ES) and 80% ethanol-insoluble (EI). Products from WM and FS were studied in 2 independent experiments involving 4 piglets each in which 2 factors were studied: product type (EI vs. ES) and time of infection (before vs. 30 min after perfusion). Pairs of jejunal segments (1 non-infected and the other ETEC-infected) were perfused simultaneously with different products during a 7.5 h period. In each piglet one pair of segments was perfused with saline as a control. Net absorption of fluid, electrolytes, solutes as well as ETEC counts in outflow fluids were determined. In both experiments, ETEC-infected segments perfused with saline had the lowest ($P < 0.05$) net fluid absorption compared with segments perfused with WM and FS products. There was no interaction ($P > 0.05$) between product type and time of infection on any of the response criteria in both experiments. In the WM experiment, product type effects were such that, ETEC-infected segments perfused with ES had higher ($P < 0.05$) fluid and solute absorption than segments perfused with ethanol-insolubles. Net absorption in FS experiment was similar ($P > 0.05$) among product type. Time of infection had no effect ($P > 0.05$) on any of the response criteria in both experiments. In conclusion, hydrolysis products from WM and FS were beneficial

in maintaining fluid balance during ETEC infection, suggesting potential in controlling ETEC induced diarrhea.

Key words: Carbohydrase, Early-weaned pig, NSP hydrolysis products, Post weaning diarrhea

5.2 INTRODUCTION

Majority of the studies on the application of exogenous enzyme in swine nutrition have focused on their potential to improve the nutritive value of feedstuffs (Slominski, 2000; Partridge, 2001). For instance, CE targeting NSP in feedstuffs have been shown to be effective in improving pre-cecal nutrient digestibility in piglets (Kim et al., 2003; Omogbenigun et al., 2004). This improvement has been ascribed to the ability of CE to attenuate the negative effects of soluble NSP on digesta viscosity and the ability to partially hydrolyze NSP thus releasing nutrients entrapped in the NSP-containing cell walls (Castanon et al., 1997; Simon, 1998; Meng et al., 2005).

By hydrolyzing NSP, CE may be able to generate NSP hydrolysis products with potential functional (e.g. pre-biotic effect) properties. Specifically, such NSP hydrolysis products, which the host animal cannot utilize, may influence gastrointestinal microbial activity (Chesson and Stewart, 2001) or expression of swine enteric bacterial diseases such as post weaning diarrhea (Pluske et al., 2002). Indeed, the addition of CE to cereal based diets fed to weaned pigs resulted in reduced frequency and severity of diarrhea (Inborr and Ogle, 1988), although the cause of the observed diarrhea was not determined. In spite of this evidence, studies examining other potential benefits of exogenous enzymes in swine nutrition other their ability to improve nutrient utilization are scarce.

Therefore, the effect of NSP hydrolysis products generated from WM and FS by CE on the ability of enterotoxigenic *Escherichia coli* to infect piglet jejunal segments was studied using an *in situ* model. The NSP hydrolysis products were fractionated to low (ethanol-soluble, ES) and high molecular (ethanol-insoluble, EI) to establish whether the responses may be ascribable to molecular size of the products.

4.3 MATERIALS AND METHODS

Non-starch Polysaccharide Hydrolysis Products

All products were produced from the same batch of WM and defatted FS. Prior to incubation with CE, feedstuffs were subjected to ethanol extraction to remove free sugars and low molecular weight carbohydrate components (i.e. sucrose, oligosaccharides) (Slominski et al., 1993). Briefly, each feedstuff was mixed with 80% ethanol at 1:5 wt/vol ratio and subjected to 4 cycles of ethanol extraction in an environmentally controlled incubator shaker set at 200 rpm and 40ºC (New Brunswick Scientific, Inc., Edison, NJ). Cycles were run one after another; the first and last cycles were run for 16 h while the second and third cycles were run for 3 h. Every cycle was followed by centrifugation (1,838 x *g*, 15 min) to discard supernatant and retente mixed with fresh 80% ethanol for the next cycle. After the fourth cycle the retente was dried at room temperature under a fume hood and finely ground in a coffee grinder.

Non-starch polysaccharide hydrolysis products were prepared by mixing 50 g of either ethanol-extracted WM or FS with 0.5 g of CE blend in distilled water. The enzyme blend contained pectinase, cellulase, mannanase, xylanase, glucanase, galactanase and other activities and in earlier research has been proven effective in wheat and flaxseed NSP depolymerization as evidenced by reduced NSP recovery (Meng et al., 2005;

Slominski et al., 2006). The enzyme preparations were provided, along with the enzyme assay procedures by Canadian Bio-System Inc. (Calgary, Alberta, Canada). The slurries were incubated for 16 h in a shaker set at 200 rpm and 40ºC. Following incubation, the slurries were centrifuged at 1,838 x g for 20 min. The supernatants were then mixed with absolute ethanol in a 1: 4 vol/vol ratio and let to stand at room temperature for 1 h and then centrifuged at 1,838 x g for 20 min. The supernatant was decanted and ethanol was evaporated in a rotary evaporator to yield 80% ethanol-soluble (ES). The retente was dissolved in a small amount of water to yield 80% ethanol-insoluble (EI). Both ES and EI were then frozen at -80°C, freeze dried, finely ground and stored in sealed containers at 4°C.

Animals and Surgical Procedures

The animal description (except that 8 piglets were used in the present study), anesthetic and surgical procedures were as described previously in manuscript II.

Bacterial Strain

The bacterial strain and inocula preparation was as previously described in Manuscript II.

Test solutions, Infection and Perfusion Procedure

All test solutions were made fresh before use. Ethanol-soluble and ethanol-insoluble (0.3 g/l) from WM and FS were dissolved in a sterile isotonic saline solution. Sterile isotonic saline solution also served as an internal control to determine maximum response to infection (Kiers et al., 2006). All test solutions were supplemented with 1g/l of glucose and casamino acids (acid-hydrolyzed casein) to assure bacteria growth. Test

solutions were adjusted with $1M$ NaHCO$_3$ to pH 6.6 (average pH of the piglet jejunum lumen; Bruins et al., 2006).

Hydrolysis products (ES and EI) were tested in two independent experiments (one for WM and the other for FS) involving 4 piglets each. In each experiment two factors were studied; time of infection (before vs. 30 min after perfusion) and product type (ES vs. EI). In each piglet, infection was allotted to odd numbered segments (i.e. 1, 3, 5, 7 and 9) and even numbered segments (i.e. 2, 4, 6, 8 and 10) served as non-infected controls (Figure 5.1). Time of infection and product type combinations were allotted to 4 pairs (an odd and adjacent even numbered segment) of segments in a 4 x 4 Latin square design in each experiment (Figure 5.1). This way each of hydrolysis product type and time of infection combination was tested in a different pair in each pig within an experiment. Saline was perfused in the middle pair (segments, 5 and 6) in all piglets. Experimentation began by perfusing 4 mL of ES or EI solutions to pairs to be infected 30 min after perfusion whereas pairs to be infected before perfusion as well saline perfused pair were perfused with either 4 mL of PBS containing ETEC (10^8 cfu/mL) or PBS only (non-infected controls). After 30 min, segments to be infected at 30 min post-perfusion were infected and perfusion commenced in pairs infected at time 0. Thereafter, pairs of segments were perfused simultaneously with different test solutions in syringes attached to the inflow tubes over a 7.5-h period by perfusing 4 mL of fluid every 30 min. The non-absorbed fluid continually passed through the outflow tubes into corresponding drainage bottles placed at the same level as the piglet's abdomen.
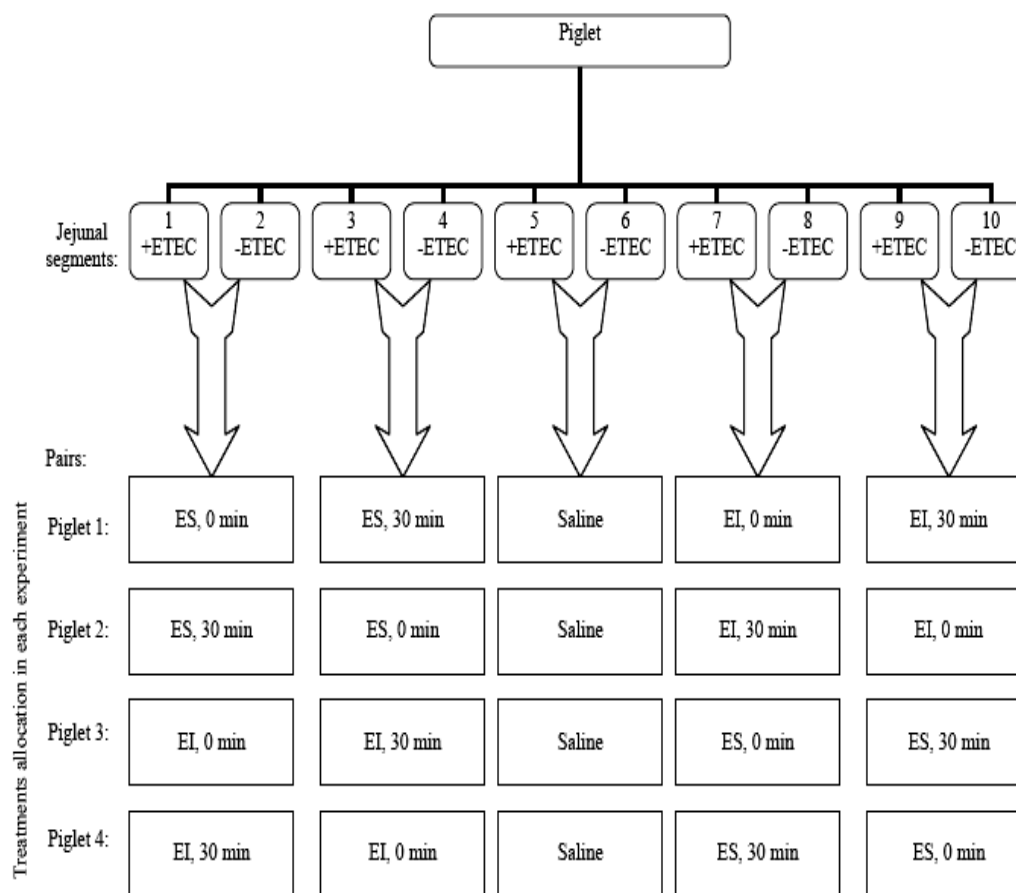
Figure 5.1. Assignment of jejunal segments to pairs in a piglet and treatment layout. Products were generated by incubating ethanol-extracted WM or FS with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI). Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min). Saline was always perfused in the middle pair.

At the end of the experiment the contents remaining in the segments were emptied into the drainage bottles (outflow). The piglets were killed by an intracardiac injection of sodium pentobarbital. The segments were dissected free of mesentery and the length measured. Tissue and fluid samples for ETEC counts were placed in sterile vials and transported (within 30 min) to the laboratory for processing.

Component sugars concentrations of NSP hydrolysis products

Carbohydrate levels were determined by GLC (component neutral sugars) and by colorimetry (uronic acids) using the procedure for NSP analysis described in Manuscript I.

Fluid, Electrolytes and Osmolality Measurements

Sodium and K^+ levels in the perfusion and outflow fluids were determined using Varian inductively coupled plasma mass spectrometer (Varian Inc., Palo Alto, CA, USA). Chloride was assayed using HPLC (Dionex Canada, Oakville, ON, Canada). Osmolality was determined using the Advanced™ Micro Osmometer (Model 3300, Advanced Instruments Inc., Norwood, MS).

Bacterial Enumeration

Bacterial enumeration was done only in the ETEC-infected segments (as in manuscript II) because in the pilot study (Manuscript II) growth was not observed when samples from the out-flow fluids and mucosal scrapings of the non-infected segments.

Calculations and Statistical Analysis

Net fluid, total solutes, Na^+, K^+ and Cl^- absorption were calculated as described in Manuscript I. Bacteria enumeration data was transformed as described in Manuscript I. Data were analyzed using the SAS statistical package (SAS 9.1, SAS Institute). Data

from the non-infected and ETEC-infected segments perfused with the same solution were compared using the Student's t test for equal and unequal variances. The effects of the product type, time of infection and interaction were analyzed as a Latin square design with piglet and segments within a pair as random effects (this analysis excluded saline perfused segments). Comparison between saline and test treatment combinations was performed using ANOVA and means separated by student-Newman-Keul's test (Steel and Torrie, 1980). For all the statistical analyses, $P < 0.05$ was considered significant.

5.4 RESULTS

Component sugars concentrations in NSP Hydrolysis Products and Characteristics of the Test Solutions

Component sugars, and total component sugar concentrations of the ES and EI products of WM and FS are shown in Table 5.1. The electrolyte and total solute profiles of perfusion solutions are shown in Table 5.2. Within feedstuff, electrolyte and solute contents as well as osmolality were similar.

Effect of WM Non-starch Polysaccharide Hydrolysis Products on Net Absorption

Enterotoxigenic *E. coli* infection reduced ($P < 0.05$) net fluid and Na^+ absorption upon perfusion with saline over the 7.5-h period compared to non-infected segments perfused with saline (Table 5.3). There was no interaction ($P > 0.05$) between product type and time of infection on net absorption. Non-infected and ETEC-infected segments perfused with ES had higher fluid absorption (617 vs. 472 $\mu L/cm^2$; $P = 0.043$ and 578 vs. 419 $\mu L/cm^2$; $P = 0.032$, respectively) than those perfused with EI.

Table 5.1. Component sugars concentrations (mg/g) of products derived from hydrolysis of wheat middlings and flaxseed non-starch polysaccharides by carbohydrase enzymes[1,2]

Item	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total
Wheat middlings								
Ethanol-solubles	nd[3]	51.5	135.3	5.6	4.5	259.2	92.4	548
Ethanol-insolubles	nd	56.3	72.9	2.3	11.1	32.5	74.0	249
Flaxseed								
Ethanol-solubles	16.1	18.1	107.4	nd	7.2	107.4	26.6	282
Ethanol-insolubles	47.3	27.5	88.6	nd	62.0	6.5	149.1	381

[1]Products were generated by incubating 50 g of ethanol-extracted wheat middlings or defatted flaxseed in distilled water medium with 0.5g of carbohydrase blend supplying pectinase, cellulase, mannanase, xylanase, glucanase, galactanase and other activities.
[2]Supernatant of enzyme degraded meals were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles and 80% ethanol-insoluble
[3]Not detected.

Table 5.2. Characteristics of perfusion solutions prepared from products derived from hydrolysis of wheat middlings and flaxseed non-starch polysaccharides by carbohydrase enzymes[1, 2, 3]

Item	Na^+ (mM/l)		Cl^- (mM/l)		K^+ (mM/l)		Osmolality (mOsmol/l)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Wheat middlings								
Ethanol-solubles	145	24	92	1.5	0.18	0.01	280	16
Ethanol-insolubles	155	18	103	3.0	0.47	0.02	286	6
Flaxseed								
Ethanol-solubles	103	11	160	2.1	0.38	0.03	268	16
Ethanol-insolubles	113	10	166	3.8	0.33	0.01	278	22

[1]Values are means and their SD.
[2]Products were generated by incubating ethanol-extracted wheat middlings or flaxseed with a blend of carbohydrase enzymes in distilled water medium. Supernatants were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles and 80% ethanol-insolubles.
[3]0.3 g of respective extract type were dissolved in 1-litre isotonic saline solution to constitute perfusion solution.

Table 5.3. Net absorption of fluid, Na^+, Cl^- and K^+ in non-infected and enterotoxigenic *E. coli* infected piglet jejunal segments after perfusion with saline or products derived from hydrolysis of wheat middlings non-starch polysaccharides by carbohydrase enzymes[1,2,3]

Treatment	Fluid ($\mu l/cm^2$)		Na^+ ($\mu M/cm^2$)		Cl^- ($\mu M/cm^2$)		K^+ ($\mu M/cm^2$)	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
Saline	529 ± 15^{ab}	$145 \pm 25^{**c}$	173 ± 13	$100 \pm 29^*$	125 ± 7.9	98 ± 26	-0.12 ± 0.10	-0.33 ± 0.15^a
ES-0 min	590 ± 33^{ab}	576 ± 46^a	121 ± 10	136 ± 25	129 ± 8.9	130 ± 13	-1.26 ± 0.51	-1.52 ± 0.46^b
ES-30 min	644 ± 94^a	579 ± 72^a	129 ± 16	124 ± 28	147 ± 14	133 ± 8.4	-1.73 ± 0.94	-1.19 ± 0.51^{ab}
EI-0 min	499 ± 60^{ab}	476 ± 63^{ab}	172 ± 19	155 ± 27	135 ± 15	120 ± 17	0.01 ± 0.16	-0.36 ± 0.42^a
EI-30 min	445 ± 54^b	362 ± 77^b	147 ± 39	124 ± 22	141 ± 16	113 ± 17	-0.63 ± 0.73	-0.45 ± 0.12^{ab}
Effects[4]								
HP	0.043	0.032	0.165	0.704	0.992	0.322	0.095	0.037
Time	0.997	0.417	0.714	0.427	0.419	0.908	0.418	0.775
HP x Time	0.416	0.391	0.493	0.722	0.672	0.745	0.900	0.613

[1]Values are means and their SE. Positive or negative values represent net absorption and secretion, respectively. Within response criteria asterisks indicate different from non-infected segments: $*P < 0.05$, $**P < 0.01$ analyzed using Student's *t* tests for equal and unequal variances. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline.
[2]Products were generated by incubating ethanol extracted-wheat middlings with a blend of carbohydrase enzymes in water. Supernatant were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI).
[3]Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).

However, in ETEC-infected segments, perfusion of EI resulted in lower K+ loss (-0.41 vs. -1.36 $\mu M/cm^2$; P = 0.037) compared to ES. Time of infection did not affect net absorption.

Effect of FS Non-starch Polysaccharide Hydrolysis Products on Net Absorption

Enterotoxigenic *E. coli* infection reduced ($P < 0.05$) net fluid and Na^+ absorption upon perfusion with saline over the 7.5 h period compared to non-infected segments perfused with saline (Table 5.4). There was no interaction ($P > 0.05$) between product type and time of infection on fluid and electrolytes absorption. Overall, there was no effect of product type and time of infection on net absorption in ETEC-infected segments.

Table 5.4. Net absorption of fluid, Na^+, Cl^- and K^+ in noninfected and enterotoxigenic *E. coli* infected piglet jejunal segments after perfusion with saline or products derived from hydrolysis of flaxseed non-starch polysaccharides by carbohydrase enzymes[1, 2, 3]

Item	Fluid ($\mu l/cm^2$)		Na^+($\mu M/cm^2$)		Cl^-($\mu M/cm^2$)		K^+($\mu M/cm^2$)	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
Saline	673 ± 76	232 ± 28**[c]	153 ± 25[a]	83 ± 11*	134 ± 12	111 ± 20	-0.63 ± 0.31[ab]	-1.49 ± 0.33*[b]
ES,0 min	539 ± 103	377 ± 70[bc]	81 ± 12[c]	62 ± 10	123 ± 17	136 ± 14	-0.86 ± 0.35[ab]	-0.89 ± 0.51[ab]
ES,30 min	645 ± 128	603 ± 76[a]	84 ± 10[c]	92 ± 22	169 ± 18	158 ± 20	-1.67 ± 0.39[b]	-0.95 ± 0.17[ab]
EI,0 min	692 ± 107	551 ± 58[a]	112 ± 15[b]	79 ± 18	137 ± 18	147 ± 17	-0.45 ± 0.36[ab]	-0.83 ± 0.32[ab]
EI,30 min	649 ± 74	544 ± 106[a]	121 ± 5.5[b]	94 ± 24	150 ± 10	142 ± 32	-0.18 ± 0.11[a]	-0.09 ± 0.07[a]
Effects[4]								
HP	0.470	0.480	0.036	0.636	0.893	0.908	0.013	0.167
Time	0.769	0.191	0.673	0.257	0.092	0.705	0.413	0.295
HP x Time	0.493	0.168	0.848	0.680	0.320	0.530	0.122	0.217

[1]Values are means and their SE. Positive or negative values represent net absorption and secretion, respectively. Within response criteria asterisks indicate different from non-infected segments: *$P < 0.05$, **$P < 0.01$ analyzed using Student's *t* tests for equal and unequal variances. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline.

[2]Products were generated by incubating ethanol extracted-flaxseed with a blend of carbohydrase enzymes in water. Supernatant were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI).

[3]Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).

Net Fluid Loss and Total Solutes Absorption

ETEC infection in saline perfused segments resulted in higher net fluid loss (384 and 440 μL/cm^2) for WM and FS experiments, respectively, compared to other test solutions (Figure 5.2). Product type and time of infection had no influence ($P > 0.05$) on net fluid loss in both experiments.

Compared with non-infected segments, ETEC-infected segments perfused with saline had the lowest total solute absorption compared with WM (Figure 5.3) and FS (Figure 5.4) extracts. There was no interaction between product type and time of infection on solute absorption in both experiments. In WM experiment, ETEC-infected segments perfused with ES had higher solute absorption (138 vs. 103 μOsmol/cm^2; $P =$ 0.046) than segments perfused with EI. In FS experiment, perfusing EI in non-infected and ETEC-infected segments resulted in higher solute absorption (205 vs. 157 μOsmol/cm^2; $P =$ 0.036 and 185 vs. 139 μOsmol/cm^2; $P =$ 0.036, respectively) than segments perfused with ES.

ETEC Count

The ETEC counts in outflow and mucosal scrapings of ETEC-infected segments are shown in Table 5.5. There was no effect of product type and time of infection on ETEC numbers in both experiments. The ETEC numbers in the mucosal scrapings of WM products were higher ($P < 0.05$) than those of segments perfused with saline. In the FS experiment, ETEC numbers in the outflow fluid and mucosal scrapings were similar ($P > 0.05$) among treatments.

(A)   (B)
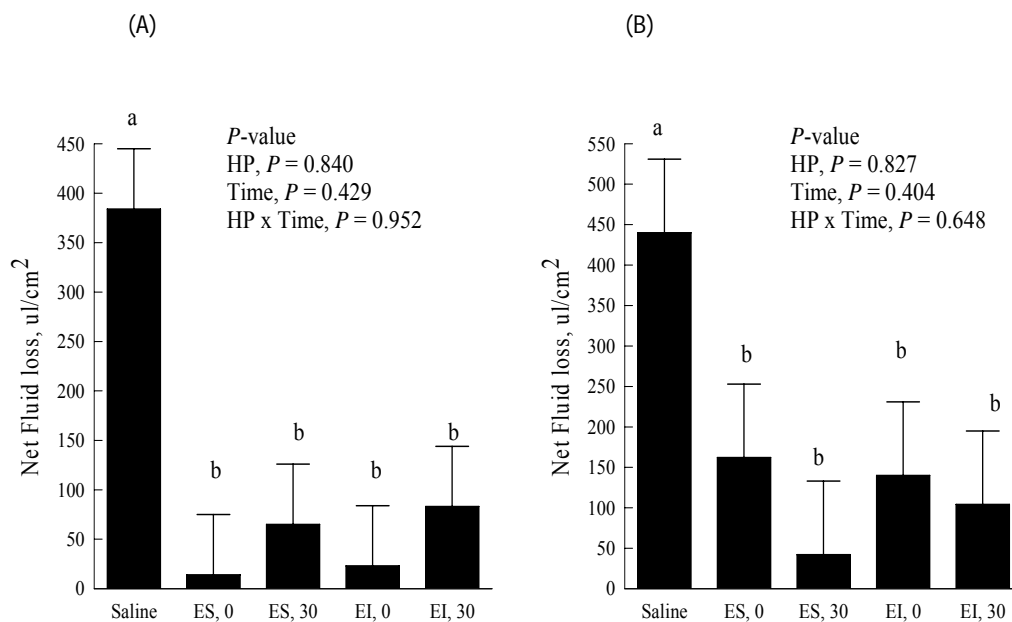
Figure 5.2. Fluid loss upon enterotoxigenic *E. coli* infection in piglet jejunal segments after perfusion with saline or products derived from hydrolysis of wheat middlings (Panel A) and flaxseed (Panel B) non-starch polysaccharides by carbohydrase enzymes. Values are means and their SE. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline. Products were generated by incubating ethanol extracted wheat middlings and flaxseed with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI). Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).

Figure 5.3. Net absorption of total solutes after perfusion with saline or products derived from hydrolysis of wheat middlings non-starch polysaccharides by carbohydrase enzymes in non-infected (■) and enterotoxigenic *E. coli*-infected (□) piglet jejunal segments. Values are means and their SE. Within response criteria asterisks indicate different from non-infected segments: **$P < 0.01$ analyzed using Student's *t* tests for equal and unequal variances. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline. Products were generated by incubating ethanol-extracted wheat middlings with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two extract types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI). Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).

Figure 5.4. Net absorption of total solutes after perfusion with saline or products derived from hydrolysis of flaxseed non-starch polysaccharides by carbohydrase enzymes in non-infected (■) and enterotoxigenic *E. coli*-infected (□) piglet jejunal segments. Values are means and their SE. Within a panel asterisks indicate different from non-infected segments: **$P < 0.01$ analyzed using Student's *t* tests for equal and unequal variances. Labeled means without a common letter differ ($P < 0.05$) and denotes comparison between saline and treatment combinations; [a, b, c] (for non-infected segments) and [x, y, z] (for infected segments). *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline. Products were generated by incubating ethanol-extracted flaxseed with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI). Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).

Table 5.5. Number of enterotoxigenic *E. coli* in the outflow fluid and mucosal scrapings of infected jejunal segments after perfusion with saline or products derived from hydrolysis of wheat middlings and flaxseed non-starch polysaccharides by carbohydrase enzymes[1,2,3]

Item	Wheat middlings		Flaxseed	
	Outflow fluid (Log$_{10}$CFU/mL)	Mucosal scrapings (Log$_{10}$CFU/g)	Outflow fluid (Log$_{10}$CFU/mL)	Mucosal scrapings (Log$_{10}$CFU/g)
Saline	6.7 ± 0.49	6.6 ± 0.72^b	6.9 ± 0.21	6.7 ± 0.50
ES, 0 min	6.3 ± 0.53	7.8 ± 0.32^a	7.2 ± 0.97	7.2 ± 0.35
ES, 30 min	6.4 ± 0.91	8.1 ± 0.19^a	6.9 ± 1.23	7.5 ± 0.84
EI, 0 min	6.9 ± 0.99	7.8 ± 0.31^a	7.1 ± 1.34	6.9 ± 0.92
EI,30 min	6.7 ± 0.54	7.7 ± 0.57^a	7.5 ± 0.75	7.3 ± 0.83
P-value				
HP	0.207	0.256	0.657	0.742
Time	0.472	0.844	0.451	0.913
HP x Time	0.291	0.525	0.899	0.585

[1]Values are means and their SE. Means in a column with superscripts without a common letter differ, *P* < 0.05 and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline.
[2]Products were generated by incubating ethanol-extracted wheat middlings and flaxseed with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI).
[3]Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).

5.5 DISCUSSION

Carbohydrase enzymes can partially hydrolyze NSP in feedstuffs (Castanon et al., 1997; Meng et al., 2005) releasing NSP hydrolysis products that the host animal cannot utilize. However, such products may influence gastrointestinal microbial activity (Chesson and Stewart, 2001) or expression of bacterial disease such as post weaning diarrhea (caused by enterotoxigenic *Escherichia coli,* K88) in piglets (Pluske et al., 2002). Indeed, feeding piglets diets containing CE influenced ileal digesta microbial activity in coincidence with increased NSP digestibility (Manuscript I). However, it is not known whether the NSP hydrolysis products as a result of CE application can influence piglet diarrhea, although there is evidence in pediatric research showing that hydrolyzed polysaccharides may be used to manage diarrhea (Alam et al., 2000). Thus, to test the hypothesis that products derived from hydrolysis of wheat middling and flaxseed NSP by CE could influence secretory diarrhea due to ETEC K88, an *in situ* model of secretory diarrhea (Nabuurs et al., 1993) was used.

It is prudent to expect that CE targeting NSP would release a wide array of NSP hydrolysis products differing in monomer content and molecular sizes (Chesson and Stewart, 2001). Thus, in the present study NSP hydrolysis products were fractionated on the basis of molecular size to ES and EI in an attempt to provide an insight of the molecular size likely to confer more protection as has been done previously (Kiers et al., 2007). Ethanol-soluble and EI differ in that the former are assumed to contain simple sugars, oligosaccharides and low molecular weight polysaccharides while the latter would represent high molecular weight polysaccharides (Slominski et al., 1993).

However, the types of component sugars of ES and EI from a particular source would be similar, albeit in different quantities as demonstrated in the present study.

The *in situ* model of secretory diarrhea used in the present study is considered suitable for quantitative assessment of the effect of enterotoxin-producing pathogens on net absorption in the small intestine (Nabuurs et al., 1993; Bruins et al., 2006). Post-weaning diarrhea is responsible for considerable economic loss due to mortality, decreased growth rate, and cost of medication (Fairbrother et al., 2005). The major causative agent for post-weaning diarrhea is ETEC which mostly carry K88 (F4) fimbrial adhesins and either secrete heat labile or heat stable enterotoxins or both (Fairbrother et al, 2005; Grange et al., 2006). Attachment of ETEC via fimbrial adhesins to small intestine epithelial cells is the initial step in colonization. After colonizing the small intestine, ETEC provokes hypersecretory diarrhea through release of enterotoxins that initiate metabolic cascades characterized by net fluid and electrolyte secretions into the intestinal lumen (Fairbrother et al., 2005).

Challenge of the jejunal segments with an ETEC strain resulted in reduced net fluid and total solutes absorption in segments perfused with saline in both experiments. This may be because of the inhibition of NaCl absorption by the villus cells as well as accelerated Cl^- secretion by the crypt cells (Hallback et al., 1982). It is noteworthy that segments showed net K^+ secretion in both experiments. This can be explained by the fact that K^+ transport in the small intestine is a passive process influenced by the luminal K^+ concentration (Turnberg, 1971). Perfusion solutions had lower K^+ ($< 1mM$) compared to ($> 4mM$) in the plasma of a 3-wk old piglet (Ullrey et al., 1967). That ES from WM showed higher solute absorption than EI whereas EI from FS showed high Na^+ and K^+

absorption than ES in non-infected segments. It is unclear as to what may have caused these effects.

It has previously been demonstrated that fermented soybean meal reduced ETEC-induced fluid and electrolyte losses in piglet small intestinal segments, whereas this was not the case for non-fermented soybeans (Kiers et al., 2006; 2007). Moreover, hydrolyzed guar gum (a water soluble polysaccharide) and wheat have been shown to enhance the efficacy of oral rehydration solution in controlling acute diarrhea in children (Alam et al., 2000). Perhaps, evidence for possible involvement of products derived from hydrolysis of WM and FS non-starch polysaccharides by CE in the protective effect described in the present study could be the presence of short-chain soluble polysaccharides.

Enterotoxigenic *E. coli* induced diarrhea involves a complex interplay between the host intestine and luminal bacteria, importantly ETEC must attach to the intestinal epithelium to initiate colonization (Fairbrother et al., 2005). We hypothesized that varying the time of infection relative to perfusion would give an indication of how test products would influence establishment of ETEC on the intestinal mucosa and subsequent infection as measured by fluid and electrolytes kinetics. However, the current data does not support this hypothesis as effect of time of infection did not influence any of the response criteria.

Various modes of action may be involved in protective effect of products derived from hydrolysis of WM and FS non-starch polysaccharides by CE. Firstly, compounds in the products may interfere with attachment of ETEC to the enterocytes, as has been shown for plant extracts on piglet brush borders *in vitro* (Kiers et al., 2002; Maiorano et al., 2007). However, since the number of ETEC attached to the mucosa scrapings were

similar for FS and higher for WM products compared to segments perfused with saline, probably no interference with the adhesion of ETEC occurred. The lack of differences in the number of ETEC attached to the mucosal scrapings of segments perfused with saline and products albeit evidence of infection in the former segments constitutes a known phenomenon associated with the present model of secretory diarrhea (Kiers et al., 2006; 2007). Bruins et al. (2006) suggested that the time frame used for the model may be too short to observe changes in mucosal ETEC count. Secondly, certain plant polysaccharides are now recognized as having a prebiotic effect (Cummings and Macfarlane, 2002). Prebiotics are defined as selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host well-being and health (Gibson et al., 2004). Thirdly, soluble fibers have been shown to enhance intestinal water and electrolyte absorption in normal and secreting rat small intestine (Alam et al., 2000; Turvill et al., 2000). Fourthly, the protective effect could have resulted from components in the products which could also interfere with the binding of enteroxin, as was shown elsewhere for certain toxin receptor analogues (Takeda et al., 1999) or interfere in the cascade of events leading to an increased Cl^- secretion and inhibited NaCl absorption, as was shown for polyphenolic compounds in plant extracts and boiled rice (Hor et al., 1995; Greenwood et al., 1999, Mathews et al., 1999). This could be mediated through stimulated Na^+–solute co-transport which is the basis for traditional World Health Organization oral rehydration therapy (Farthing, 1994).

In conclusion, irrespective of fractionation, soluble products derived from hydrolysis of WM and FS NSP by CE were beneficial in maintaining fluid balance during

ETEC infection. This significant finding may aid in designing enzyme systems tailored for maximizing dietary nutrient utilization by piglets as well as controlling enteric infections such as ETEC-secretory diarrhea. Further research is required to uncover the mode of action by which products derived from hydrolysis of WM and FS non-starch polysaccharides enhanced absorption under ETEC infection.

CHAPTER SIX

MANUSCRIPT IV

Non-starch polysaccharide hydrolysis products of soybean and canola meal protect against enterotoxigenic *Escherichia coli* in piglets

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6.1 ABSTRACT

Infectious diarrhea is a major problem in both children and piglets. Enterotoxigenic *Escherichia coli* infection results in fluid and electrolyte losses in the small intestine. The effects of NSP hydrolysis products of soybean (SBM) and canola meal (CM) on net absorption during ETEC infection were investigated. Products were generated by incubating SBM and CM with a blend of carbohydrase enzymes. Following incubation, slurries were centrifuged and the supernatants mixed with absolute ethanol to produce 2 product types: 80% ES and 80% EI. Products from SBM and CM were studied in 2 independent experiments in which 2 factors were investigated: product type (EI vs. ES) and time of ETEC infection (before vs. after perfusion). Pairs of small-intestine segments, one non-infected and the other ETEC-infected, were perfused simultaneously with different products during 7.5 h. Net absorption of fluid and solutes were determined. In both experiments, ETEC-infected segments perfused with saline control had the lowest ($P < 0.05$) net fluid and solutes absorption compared with SBM and CM products. Interaction ($P < 0.05$) between product type and time of infection on fluid absorption was only evident for SBM in which case perfusing ES products before infection resulted in high fluid (735±22 vs. 428±34 μL/cm^2) absorption compared with ETEC infection before perfusion. In conclusion, NSP hydrolysis products of SBM and CM, particularly ES from SBM, were beneficial in maintaining fluid balance during ETEC infection, suggesting potential in controlling ETEC induced diarrhea in piglets.

Key words: Carbohydrase, Early-weaned pig, NSP hydrolysis products, Post weaning diarrhea

6.2 INTRODUCTION

Enterotoxigenic *Escherichia coli* is a major cause of secretory diarrhea in piglets and in children from developing countries (Bhan et al., 2000, Fairbrother et al., 2005). Both pigs and children with *E. coli* induced gastroenteritis suffer from depressed growth, lack of apatite, and morbidity (Bhan et al., 2000, Fairbrother et al., 2005). Products such as water-soluble NSP (Go et al., 1994; Alam et al., 2000; Turvill et al., 2000), modified starch (Wingertzahn et al., 1999), rice gruel (Mathews et al., 1999), and fermented soybeans (Kiers et al., 2003; 2006; 2007) enhance fluid and electrolyte balance in models for intestinal secretory diarrhea. Previous work has demonstrated that high molecular weight polysaccharides derived from fermented soybeans enhance fluid and electrolytes balance in piglets infected with ETEC *in situ* (Kiers et al., 2007). Isolated soy polysaccharides have also been shown to reduce the duration of bacterial or viral induced diarrhea in children (Brown et al., 1993).

Carbohydrase enzymes are routinely used in piglet diets to improve nutrient utilization, putatively by depolymerizing complex cell-wall NSP in feedstuffs (Meng et al., 2005). In the process of depolymerizing NSP, supplemental CE may create NSP hydrolysis products *in situ* which might influence enteric bacterial infections in piglets (Chesson and Stewart, 2001; Pluske et al., 2002). Indeed, it was previously reported that addition of CE to piglet diets reduced frequency and severity of non-specific diarrhea (Inborr et al., 1988; Partiridge and Tucker, 2000). However, in no case was the cause of any episode of diarrhea determined. This is important since it is necessary to distinguish

diarrhea of dietary origin from that of bacterial etiology which might be influenced by the presence of NSP hydrolysis products (Chesson and Stewart, 2001).

A piglet small intestinal segment perfusion method was utilized to evaluate the efficacy of NSP hydrolysis products from SBM and CM in attenuating ETEC-induced fluid and electrolyte losses. Carbohydrase enzyme preparation was used to produce low (ethanol-soluble) and high molecular (ethanol-insoluble) weight hydrolysis products.

6.3 MATERIALS AND METHODS

Except for the hydrolysis products which were derived from the SBM and CM, the materials and methods were similar to those described in manuscript III.

6.4 RESULTS

Component sugars concentrations in NSP Hydrolysis Products and Characteristics of the Test Solutions

Total and component sugar contents of the ES and EI products derived from SBM and CM are shown in Table 6.1. Except for the EI products of CM, which had lower NSP component sugar content, other products had comparable carbohydrate content. The electrolyte and total solute concentrations of perfusion fluids are shown in Table 6.2. Within feedstuff products, electrolyte and solute contents were similar.

Table 6.1. Component sugars concentrations (mg/g) of products derived from hydrolysis of soybean and canola meal non-starch polysaccharides by carbohydrase enzymes[1,2]

Item	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total
Soybean meal							
Ethanol-solubles	14.7	nd[3]	5.2	95.6	29.9	7.1	153
Ethanol-insolubles	2.6	2.9	3.2	33.0	2.8	65.0	109
Canola meal							
Ethanol-solubles	65.8	36.3	nd	11.0	39.8	15.5	168
Ethanol-insolubles	15.0	3.1	nd	13.8	2.7	18.3	53.0

[1]Products were generated by incubating 50 g of ethanol-extracted wheat middlings or defatted flaxseed in distilled water medium with 0.5g of carbohydrase blend supplying pectinase, cellulase, mannanase, xylanase, glucanase, galactanase and other activities.

[2]Supernatant of enzyme degraded meals were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles and 80% ethanol-insoluble

[3]Not detected (minimum detection limit <1 mg/g).

Table 6.2. Characteristics of perfusion solutions prepared from products derived from hydrolysis of soybean and canola meals non-starch polysaccharides by carbohydrase enzymes[1,2,3]

Item	Na$^+$ (mmol/L)		Cl$^-$ (mmol/L)		K$^+$ (mmol/L)		Osmolality (mOsmol/L)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Soybean meal								
Ethanol solubles	158	2.4	133	1.5	0.86	0.01	311	4.5
Ethanol insolubles	141	3.1	120	3.0	0.98	0.02	307	5.6
Canola meal								
Ethanol solubles	144	4.5	161	2.1	0.43	0.03	296	6.3
Ethanol insolubles	154	5.6	175	3.8	0.41	0.01	308	4.8

[1]Values are means and their SD.

[2]Products were generated by incubating ethanol-extracted soybean and canola meals with a blend of carbohydrase enzymes in distilled water medium. Supernatants were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles and 80% ethanol-insolubles.

[3]0.3 g of respective product type were dissolved in 1-litre isotonic saline solution.

Effect of SBM NSP Hydrolysis Products on Net Absorption

Non-infected segments perfused with saline had the lowest ($P < 0.05$) net fluid and Na^+ absorption (Table 6.3). There was no interaction ($P > 0.05$) between product type and time of infection on the net absorption of fluid and electrolytes in non-infected segments.

Enterotoxigenic *E. coli* infection reduced ($P < 0.05$) net fluid and electrolytes (Na^+, Cl^- and K^+) absorption upon perfusion with saline over the 7.5 h period compared with non-infected segments perfused with saline. There was an interaction ($P < 0.05$) between product type and time of infection on net fluid and Cl^- absorption in infected segments such that infecting segments after perfusion of ES products resulted in higher net fluid and Cl^- absorption compared with segments infected before perfusion.

Effect of CM NSP hydrolysis products on net absorption

Except for K^+, non-infected segments perfused with saline had the highest ($P < 0.05$) net fluid and electrolytes absorption (Table 6.4). There was no interaction ($P > 0.05$) between product type and time of infection on net absorption of fluid and electrolytes in non-infected segments. Enterotoxigenic *E. coli* infection reduced ($P < 0.05$) net fluid and electrolytes absorption upon perfusion with saline over the 7.5 h period compared to non-infected segments perfused with saline. There was an interaction ($P < 0.05$) between product type and time of infection on net Na^+ and Cl^- absorption in infected segments such that infecting segments after perfusing with ES product resulted in lower net Na^+ and Cl^- absorption compared with segments infected before perfusion.

Table 6.3. Net absorption of fluid, Na^+, Cl^- and K^+ in non-infected and enterotoxigenic *E. coli* infected piglet jejunal segments after perfusion with saline or products derived from hydrolysis of soybean meal non-starch polysaccharides by carbohydrase enzymes[1, 2, 3]

	Fluid ($\mu l/cm^2$)		Na^+ (mM/cm^2)		Cl^- (mM/cm^2)		K^+ (mM/cm^2)	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
Saline	451 ± 50^c	$170 \pm 20^{**d}$	96 ± 7.29^b	$50 \pm 17.0^{*b}$	104 ± 8.75^a	$65 \pm 16.0^{*b}$	-0.86 ± 0.37^a	$-2.60 \pm 0.68^{*a}$
ES, 0 min	692 ± 58^a	$428 \pm 51^{*c}$	135 ± 12.3^a	137 ± 11.6^{ab}	87 ± 2.48^{ab}	74 ± 5.31^b	-0.08 ± 0.08^a	-1.23 ± 0.96^b
ES,30 min	665 ± 43^{ab}	735 ± 22^a	142 ± 12.0^a	$165 \pm 5.49^{*a}$	107 ± 15.0^a	127 ± 5.47^a	-0.51 ± 0.43^b	-0.34 ± 0.37^{ab}
EI, 0 min	640 ± 31^{ab}	$536 \pm 37^{*b}$	150 ± 8.50^a	$128 \pm 4.68^{*ab}$	72 ± 1.29^b	$59 \pm 5.17^{*b}$	-0.85 ± 0.37^a	-1.42 ± 0.56^{ab}
EI,30 min	578 ± 33^{bc}	546 ± 17^b	135 ± 8.37^a	131 ± 7.64^b	66 ± 5.48^b	70 ± 12.0^b	-0.14 ± 0.36^b	-0.36 ± 0.56^b
P-value								
HP	0.245	0.821	0.684	0.016	0.005	0.004	0.865	0.916
Time	0.481	0.023	0.701	0.069	0.408	0.041	0.058	0.932
HP x Time	0.203	0.010	0.315	0.141	0.137	0.016	0.434	0.325

[1]Values are means and their SE. Positive or negative values represent net absorption and secretion, respectively. Within response criteria asterisks indicate different from non-infected segments: *$P < 0.05$, **$P < 0.01$ analyzed using Student's *t* tests for equal and unequal variances. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline.

[2]Products were generated by incubating ethanol extracted-soybean meal with a blend of carbohydrase enzymes in water. Supernatant were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI).

[3]Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).

Table 6.4. Net absorption of fluid, Na^+, Cl^- and K^+ in noninfected and enterotoxigenic *E. coli* infected piglet jejunal segments after perfusion with saline or products derived from hydrolysis of canola meal non-starch polysaccharides by carbohydrase enzymes[1, 2, 3]

Item	Fluid (μl/cm^2)		Na^+ (mM/cm^2)		Cl^- (mM/cm^2)		K^+ (mM/cm^2)	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
Saline	517 ± 46^a	$265 \pm 15^{**b}$	108 ± 15^a	$59 \pm 7.4^{**a}$	101 ± 17^a	$54 \pm 16^{*bc}$	-1.24 ± 0.10	$-2.37 \pm 0.33^*$
ES, 0 min	378 ± 22^b	381 ± 31^a	68 ± 13^b	50 ± 16^a	79 ± 14^b	64 ± 5.3^{ab}	-1.22 ± 0.15	-1.96 ± 0.55
ES,30 min	378 ± 36^b	430 ± 40^a	63 ± 10^b	$7.7 \pm 19^{**b}$	87 ± 10^b	$40 \pm 4.2^{*c}$	-1.32 ± 0.52	-1.53 ± 0.37
EI, 0 min	341 ± 18^b	370 ± 11^a	63 ± 9.3^b	43 ± 15^{ab}	74 ± 16^b	61 ± 4.1^{ab}	-1.28 ± 0.24	-1.34 ± 0.53
EI,30 min	381 ± 26^b	389 ± 38^a	66 ± 9.2^b	52 ± 13^a	77 ± 80^b	81 ± 6.6^a	-0.97 ± 0.39	-1.02 ± 0.52
P-value								
HP	0.526	0.717	0.123	0.222	0.481	0.039	0.694	0.223
Time	0.468	0.839	0.120	0.271	0.603	0.872	0.773	0.408
HP x Time	0.459	0.553	0.204	0.025	0.800	0.030	0.576	0.896

[1]Values are means and their SE. Positive or negative values represent net absorption and secretion, respectively. Within response criteria asterisks indicate different from non-infected segments: $*P < 0.05$, $**P < 0.01$ analyzed using Student's *t* tests for equal and unequal variances. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline.
[2]Products were generated by incubating ethanol extracted-canola meal with a blend of carbohydrase enzymes in water. Supernatant were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI).
[3]Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).

Net fluid loss and total solutes absorption

ETEC infection in saline perfused segments resulted in higher net fluid loss (281 and 252 µL/cm^2) for SBM and CM experiments, respectively, compared to other test solutions except for ES from SBM infected before perfusion (Figure 6.1).

In the SBM experiment, there was an interaction effect ($P < 0.05$) of product type and time of infection on net fluid loss such that infecting segments after perfusion of ES protected against fluid loss compared to infection before perfusion. Net fluid loss in ETEC-infected segments was comparable upon perfusion with CM products and independent of the time of infection.

Infected segments perfused with saline had the lowest total solutes absorption compared with SBM (Figure 6.2) and CM (Figure 6.3) products. In the SBM experiment, there was an interaction ($P < 0.05$) between product type and time of infection on total solutes absorption such that perfusing ES before infection resulted in higher net solutes absorption compared with perfusion after infection.

ETEC count

The ETEC counts in outflow fluid and mucosal scrapings of ETEC-infected segments are shown in Table 6.5. There was no effect of product type and time of infection interaction on ETEC numbers in both experiments. The ETEC numbers in the outflow fluid and mucosal scrapings of SBM products were not different ($P < 0.05$) from those of saline segments. In the CM experiment, segments infected at time 0 and perfused with EI had higher ETEC count on mucosal scrapings than segments perfused with saline.
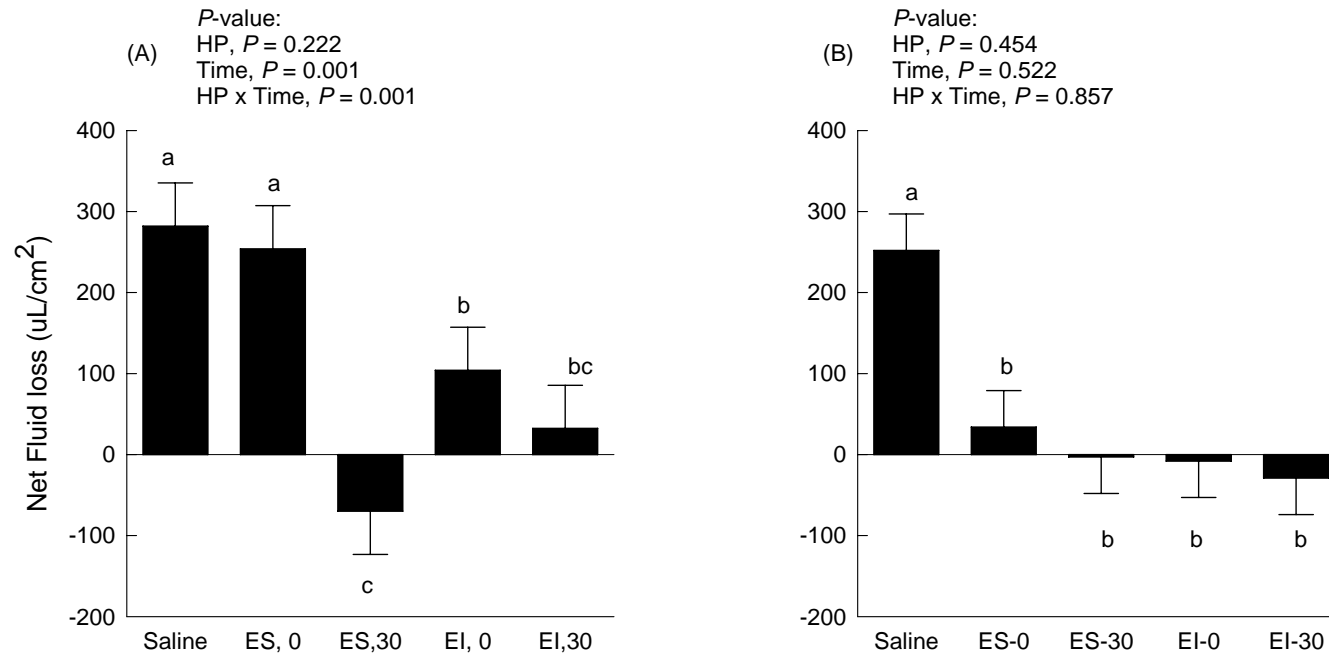
Figure 6.1. Fluid loss upon enterotoxigenic *E. coli* infection in piglet jejunal segments after perfusion with saline or products derived from hydrolysis of soybean meal (Panel A) and canola meal (Panel B) non-starch polysaccharides by carbohydrase enzymes. Values are means and their SE. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. P-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline. Products were generated by incubating ethanol extracted soybean meal and canola meal with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI). Segments infected before perfusion (ES,0 min) and (EI,0 min) or 30 min post perfusion (ES,30 min) and (EI,30 min).

Non-infected, *P*-value:
HP, *P* = 0.030
Time, *P* = 0.968
HP x Time, *P* = 0.362

Infected, *P*-value:
HP, *P* = 0.002
Time, *P* = 0.001
HP x Time, *P* = 0.04

Figure 6.2. Net absorption of total solutes after perfusion with saline or products derived from hydrolysis of soybean meal non-starch polysaccharides by carbohydrase enzymes in non-infected (■) and enterotoxigenic *E. coli*-infected (□) piglet jejunal segments. Values are means and their SE. Within response criteria asterisks indicate different from non-infected segments: *P < 0.05, **P < 0.01 analyzed using Student's *t* tests for equal and unequal variances. Means in a column with superscripts without a common letter differ, *P* < 0.05 and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline. Products were generated by incubating ethanol-extracted soybean meal with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two extract types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI).

Figure 6.3. Net absorption of total solutes after perfusion with saline or products derived from hydrolysis of canola meal non-starch polysaccharides by carbohydrase enzymes in non-infected (■) and enterotoxigenic *E. coli*-infected (□) piglet jejunal segments. Values are means and their SE. Within response criteria asterisks indicate different from non-infected segments: **$P < 0.01$ analyzed using Student's *t* tests for equal and unequal variances. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline. Products were generated by incubating ethanol-extracted canola meal with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two extract types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI).

Table 6.5. Number of enterotoxigenic *E. coli* in the outflow fluid and mucosal scrapings of infected jejunal segments after perfusion with saline or products derived from hydrolysis of soybean and canola meal non-starch polysaccharides by carbohydrase enzymes[1, 2, 3]

Item	Soybean meal		Canola meal	
	Outflow fluid	Mucosal scrapings	Outflow fluid	Mucosal scrapings
	$Log_{10}CFU/mL$	$Log_{10}CFU/g$	$Log_{10}CFU/mL$	$Log_{10}CFU/g$
Saline	7.0 ± 0.30	7.3 ± 0.24	5.7 ± 0.45^b	4.6 ± 0.45^b
ES,0 min	7.5 ± 0.29	7.0 ± 0.32	6.2 ± 0.35^{ab}	6.1 ± 0.47^a
ES,30 min	7.7 ± 0.23	7.1 ± 0.25	6.8 ± 0.48^a	6.2 ± 0.48^a
EI,0 min	7.4 ± 0.49	7.1 ± 0.33	7.3 ± 0.36^a	6.8 ± 0.30^a
EI,30 min	7.5 ± 0.39	7.3 ± 0.31	6.5 ± 0.39^{ab}	6.4 ± 0.42^a
P-value				
HP	0.616	0.710	0.431	0.277
Time	0.727	0.614	0.862	0.729
HP x Time	0.907	0.813	0.136	0.582

[1]Values are means and their SE. Means in a column with superscripts without a common letter differ, $P < 0.05$ and denotes comparison between saline and treatment combinations. *P*-value represents the effects of the product type (HP), time of infection (Time) and their interactions (HP x Time) and exclude segments perfused with saline.

[2]Products were generated by incubating ethanol-extracted soybean meal and canola meal with a blend of carbohydrase enzymes in water medium. Supernatants were mixed with absolute ethanol to yield two product types, 80% ethanol-solubles (ES) and 80% ethanol-insolubles (EI).

[3]Segments infected before perfusion (ES, 0 min) and (EI, 0 min) or 30 min post perfusion (ES, 30 min) and (EI, 30 min).
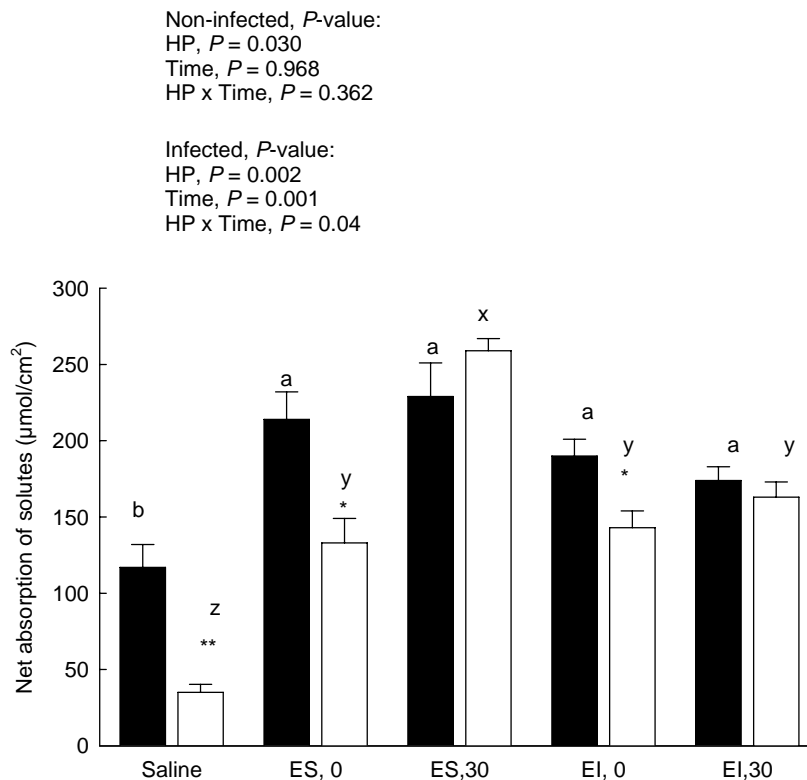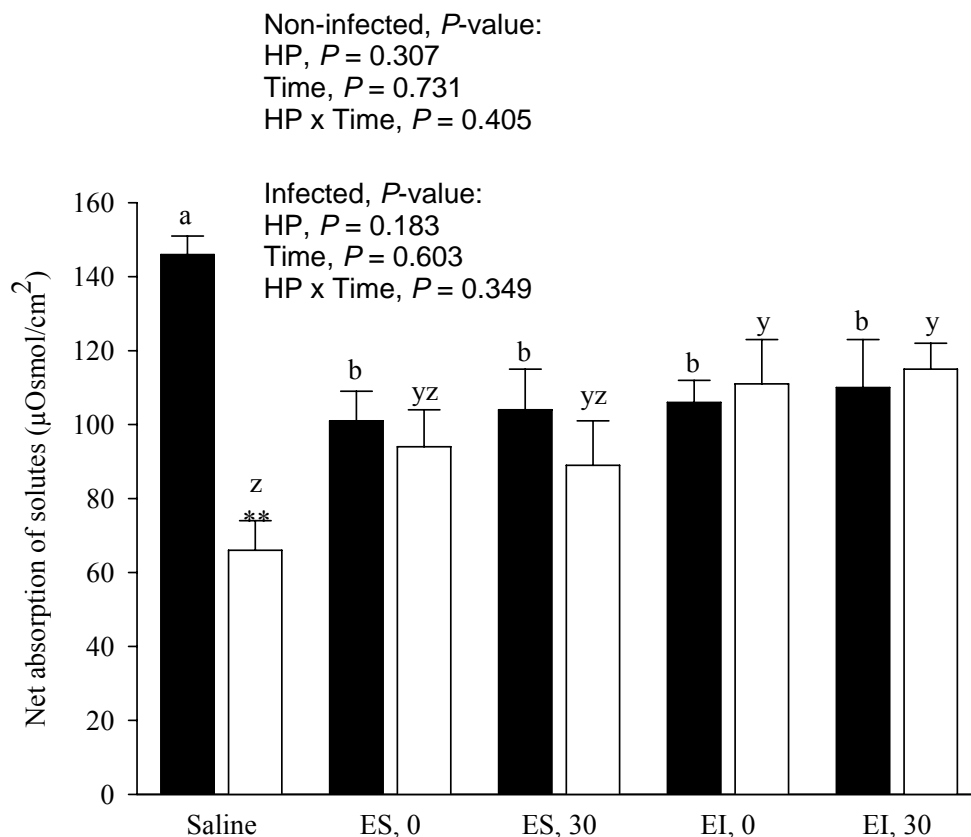
6.5 DISCUSSION

Enterotoxigenic *E. coli* is among the most common enteric pathogens causing acute secretory diarrhea in neonatal and weaned piglets (Fairbrother et al., 2005). Moreover, ETEC is among the most common enteric pathogens causing acute secretory diarrhea in young children in developing countries (Bhan et al., 2000) and in travelers to these regions. The pig gastrointestinal tract physiologically and anatomically resembles that of humans (Miller et al., 1987).

To test the hypothesis that products derived from hydrolysis of SBM and CM NSP by CE could influence secretory diarrhea caused by ETEC K88, we used an *in situ* model of secretory diarrhea. This model is considered a suitable model to quantitatively assess the effect of enterotoxin-producing pathogens on net absorption in the small intestine (Nabuurs et al., 1993). Ethanol-soluble and EI products differed in that the former were assumed to contain simple sugars, oligosaccharides and low molecular weight polysaccharides while the latter represented high molecular weight polysaccharides (Slominski et al., 1993).

Challenge of the jejunal segments with ETEC strain resulted in reduced net fluid and total solutes absorption in segments perfused with saline in both experiments. This may be because of the inhibition of NaCl absorption by the villus cells as well as accelerated Cl$^-$ secretion by the crypt cells (Hallback et al., 1982). It is noteworthy that segments showed net K$^+$ secretion in both experiments. This may be explained by the fact that K$^+$ transport in the small intestine is a passive process influenced by the luminal K$^+$ concentration (Turnberg et al., 1971). All perfusion solutions had lower K$^+$ (<1 m*M*) compared to (>4 m*M*) in the plasma of a 3-wk old piglet (Ullrey et al., 1967).

It has previously been demonstrated that products of fermented SBM reduced ETEC-induced fluid and electrolyte losses in piglet small intestinal segments, whereas this was not the case for non-fermented SBM (Kiers et al., 2006; 2007). Moreover, hydrolyzed guar gum has been shown to enhance net fluid absorption in models for secretory diarrhea (Alam et al., 2000). It has been suggested that the action of CE is mediated by degrading high molecular weight polysaccharides to simple sugars, oligosaccharides, and low molecular weight polysaccharides (Slominski et al., 1993; Meng et al., 2005). Evidence for possible involvement of products derived from hydrolysis of SBM and CM by CE in the protective effect described in the present study could be the presence of oligosaccharides and short chain polysaccharides.

ETEC-induced diarrhea involves a complex interplay between the host intestine and luminal bacteria, importantly ETEC must attach to the intestinal epithelium to initiate colonization (Fairbrother et al., 2005). We hypothesized that varying the time of infection relative to perfusion would give an indication of how products would influence establishment of ETEC on the intestinal mucosa and subsequent infection as measured by fluid and electrolytes kinetics. This hypothesis was only confirmed in segments perfused with ES products from SBM, which showed higher net fluid and solutes absorption when infected 30-min after perfusion compared to when infected before perfusion. However, as these segments had similar number of ETEC attached to the mucosa scrapings it is not clear as to what may have mediated this observation. In contrast, perfusing ES products from CM 30-min before infection resulted in lower Na^+ and Cl^- absorption compared to segments infected prior to perfusion. It is rather hard to explain this observation. Nevertheless, it is noteworthy that when compared with saline, CM products appeared to

suppress fluid absorption in non-infected segments through mechanisms we could not establish.

Various modes of action may be involved in the protective effect of NSP hydrolysis products in the present study. Firstly, compounds in the products may interfere with attachment of ETEC to the enterocytes, as has been shown for fermented SBM and piglet brush borders *in vitro* (Kiers et al., 2002). Secondly, certain plant polysaccharides are now recognized as having a prebiotic effect (Cummings et al., 2002). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selective stimulation of growth or activity of one or a limited number or activity of bacterial species especially lactic acid producing bacteria (Gibson and Roberfroid, 1995). In this context, lactic acid bacteria have been shown to have antibacterial effects on *E. coli* and *salmonella* species (Nout et al., 1989; Hillman et al., 1995; Korakli et al., 2002). However, since the number of ETEC attached to the mucosa scrapings were similar for SBM and higher for CM products compared with segments perfused with saline, probably no conclusive interference with the adhesion of ETEC occurred. The lack of differences in the number of ETEC attached to the mucosal scrapings of segments perfused with saline and NSP hydrolysis products albeit evidence of infection in the former segments constitutes a known phenomenon associated with the present model of secretory diarrhea (Bruins et al., 2006; Kiers et al., 2006). Bruin et al (2006) suggested that the time frame used for the model may be too short to observe changes in mucosal ETEC count. Thirdly, soluble fibers have been shown to enhance intestinal water and electrolyte absorption in normal and secreting rat small intestine (Turvill et al., 2000). Fourthly, the protective effect could have resulted from components in the products

which could also interfere with the (binding of) enteroxin, as was shown elsewhere for certain toxin receptor analogues (Takeda et al., 1999). Alternatively, such components may have interfered with the cascade of events leading to increased Cl^- secretion and inhibited NaCl absorption, as was shown for polyphenolic compounds in plant extracts and boiled rice (Hor et al., 1995; Greenwood et al., 1999; Mathews et al., 1999). This could be mediated through stimulated Na^+–solute co-transport which is the basis for traditional World Health Organization oral rehydration therapy (Farthing, 1994).

In conclusion, NSP hydrolysis products of SBM and CM, particularly ethanol-solubles from SBM administered before infection, were beneficial in maintaining fluid balance during ETEC infection. In relation to piglet nutrition, this finding may aid in designing enzyme systems tailored for maximizing dietary nutrient utilization as well as controlling enteric infections such as ETEC-secretory diarrhea. Further research is required to identify the component(s) in the products which are responsible for the protective effect and to evaluate the specific mechanism(s) underlying the improved net fluid balance.

CHAPTER SEVEN

MANUSCRIPT V

Response of piglets fed diets containing non-starch polysaccharide hydrolysis products and egg yolk antibodies upon challenge with *Escherichia coli* (K88)

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7.1 ABSTRACT

Response of piglets fed diets containing non-starch polysaccharide hydrolysis (HP) and egg yolk antibodies (EYA) upon oral challenge with ETEC were evaluated. Hydrolysis product was generated by incubating a mixture of ethanol-extracted wheat middlings, SBM, CM and flaxseed with a blend of CE. Following the incubation, the mixtures were centrifuged to recover supernatants which were then frozen, freeze dried and finely ground. Forty, 21-d old weaned pigs (7.2 ± 0.5 kg BW) were housed in pairs and allocated to one of the four diets: C (control), HP (5 g of HP/kg of C), EYA (5 g of EYA/kg of C) or EYA+HP (5 g of EYA + 5 g of HP/kg of C) in a completely randomized design to give 5 pens per diet. Piglets were fed the experimental diets for 9 d to evaluate pre-infection performance (ADFI, ADG and G:F). Between 0800 and 0945 of d 10 pigs were weighed, bled and rectal temperature recorded to establish baseline measurements (0 h). At 1000 on d 10 all piglets were orally challenged with ETEC. At 1600 of d 10 (6 h), 1000 of d 11 (24 h) and 1000 of d 12 (48 h) pigs were bled, rectal temperature recorded and the incidence and the severity of diarrhea was determined on pen basis using a fecal scoring system. At 24 and 48 h post-challenge, 1 pig/pen was sacrificed to collect digesta and intestinal tissue. Pigs fed the C diet ate less ($P = 0.04$) than pigs fed the diets containing HP within 48 h post challenge. Compared to pigs fed the C diet, pigs fed the diets containing additives showed low ileal adherent ETEC counts ($P = 0.06$) and digesta ammonia ($P = 0.002$) which coincided with less ($P = 0.01$) scours within 48 h post challenge. Feeding HP and EYA in combination resulted in higher ($P = 0.0001$) ileal lactic acid concentration than when fed singly. Pigs fed the diets containing EYA had shallow jejunal crypt depth ($P = 0.03$) and lower ileal pH ($P = 0.010$) whilst

pigs fed diets containing HP showed low gastric pH ($P = 0.03$) high ileal adherent lactobacilli counts ($P = 0.01$) than pigs fed the C diet. Interaction between diet and time post- challenge was observed for packed cell volume (PCV, $P = 0.0002$), plasma osmolality ($P = 0.04$), PUN ($P = 0.06$), glucose ($P = 0.04$), serum haptoglobin (Hp; $P = 0.05$) and interleukin-6 (IL-6; $P = 0.06$). Pigs fed the C diet showed lower PCV at 6 h, higher PUN at 6 and 48 h, higher glucose at 24 h, higher Hp and IL-6 at 6 and 24 h compared to pigs fed the diets containing additives. In conclusion, piglets fed the diet with additives exhibited less severe ETEC-enteritis within 24 h post infection diets.

Key Words: Egg yolk antibodies; Non-starch polysaccharides hydrolysis products; Piglet, Post weaning collibacillosis

7.2 INTRODUCTION

Enterotoxigenic strains of *Escherichia coli* are the most important cause of neonatal and post-weaning diarrhea in pigs (Fairbother et al., 2005). Colibacillosis induced by *E. coli* has become increasingly difficult to treat because of the growing antibiotic resistance by many strains (Fairbrother et al., 2005; Harvey et al., 2005). Subsequently, non-antibiotic intervention methods for these organisms are a high priority. One such solution would be the use of dietary components as reviewed by Pluske et al. (2002).

The present study was conducted to investigate the response of piglets fed diets containing HP and EYA upon oral challenge with ETEC.

7.3 MATERIALS AND METHODS

Hydrolysis Products

The HP was produced from wheat middlings (WM) , SBM, CM and flaxseed (FS). Prior to incubation with CE, each feedstuff was subjected to ethanol extraction as described in manuscript III. Non-starch polysaccharides HP was prepared by incubating a mixture of WM, SBM, CM and FS 4:4:1:1 wt/wt with 0.5 g of CE (same as in Manuscript III). The ratio of feedstuffs in the mixture represented typical inclusion rate in starter diets. Following incubation, the slurry was centrifuged at 1,838 x g for 20 min to recover the supernatant (i.e. HP) which was frozen at -80°C, freeze dried, finely ground and stored in sealed containers at 4°C until use.

Experimental Diets

The 4 experimental diets were: a control diet (C; Table 7.1) based on corn and the C plus HP, EYA or HP+EYA, each additive included at 5g/kg of complete diet. Corn was chosen because it contains low concentration of oligosaccharides and NSP (Houdijk et al., 2002). The C diet was formulated to meet the NRC (1998) nutrient specifications for pigs weighing 5 to 10 kg. The source, production and characteristic of the EYA was as described in Manuscript II.

Pigs, Housing, Feeding and Pre-Infection Performance Monitoring

The animal use protocol for this research was approved by the University of Manitoba Animal Care Committee (protocol # F06-025) and followed the principles established by the Canadian Council on Animal Care (CCAC, 1993). A total of 40 piglets 21 ± 1 d old and 7.2 ± 0.5 (mean ± SD) kg BW were used in the present study.

Table 7.1. Composition of control diet, as fed basis

Ingredient	%
Corn	37.0
Milk products[1]	37.2
Oat grouts	11.4
Select menhaden fish meal	9.0
Canola oil	3.0
Vitamin-mineral premix[2]	1.0
Salt	0.50
Biofos[3]	0.40
Limestone	0.30
L~Lysine-HCl	0.20
DL~Methionine	0.05
Calculated nutrient content	
DE, kcal/kg	3,537
CP, %	24.3
Total lysine, %	1.61
Ca, %	0.95
P available, %	0.62

[1]Dried skim milk (12%), casein (10%), dried whey (7.7%), and lactose (7.5%).
[2]Provided per kilogram of complete diet: vitamin A, 8,255 IU; vitamin D3, 1,000 IU; vitamin E, 10.9 IU; vitamin B12, 0.115 mg; vitamin K, 1.1 mg; Niacin, 36.8 mg; Choline chloride, 781.2 mg; Biotin, 0.25 mg, Folic acid, 0.75 mg, Mn (as MnO), 55 mg; Zn (as ZnO), 50 mg, Fe (as $FeSO_4.H_2O$), 80 mg, Cu (as CuO), 5 mg; Se (as $NaSeO_3$), 0.1 mg, I (as Ca $(IO_3)_2$), 0.28 mg. The premix did not contain any additional copper or zinc and was free of antibiotic.
[3]Ca, 21%; P, 17%.

Based on weaning BW, piglets were randomly assigned to 20 pens (n = 2 piglets per pen). Piglet breed and source were similar as in Manuscript II, III and IV. The pens were equipped with a feeder, a nipple type drinker, plastic-covered expanded metal floors and a wall partitioning between pens that allowed visual contact with pigs in adjacent pens. Room temperature was initially set at 29.5°C and gradually reduced by 1.5°C per wk. Dietary treatments were allotted in a completely randomized design to give 5 pens per treatment. Piglets had unlimited access to feed and water throughout the study. Piglets were fed experimental diets for a 9-d pre-infection period. At the end of the pre-infection period, the feed remaining in the feeder and individual BW were recorded to evaluate pre-infection performance. At 0800 of d 10 after weaning, all pigs were bled via jugular vein puncture for blood baseline (0 h) parameters; RT was also recorded for each piglet.

Bacterial Strain, Challenge, Fecal Score, Post-Infection Performance and Sample Collection

The ETEC strain and inocula preparations were as described in manuscripts II, III and IV. At 1000 of d 10, all pigs were orally challenged with 5 mL of Luria-Bertani broth (Fisher Scientific Company, Ottawa, ON, Canada) containing 10^9cfu of ETEC/mL using a syringe attached to a polyethylene tube. At 6, 24, and 48 h post challenge, all pigs were bled via jugular vein puncture and RT recorded. At each bleeding time, approximately 10 mL of blood from a piglet was collected in two tubes; one for plasma and the other for serum (Becton Dickinson & Co, Franklin Lakes, NJ). All blood tubes were placed on ice upon collection and transported to the laboratory for processing within 30 min. After determining the PCV using a hematocrit centrifuge, plasma was separated by

centrifugation (2,000 x *g* for 20 min) and immediately stored at -80°C until required for analysis. Samples for serum were allowed to clot for 20 min at 4°C, centrifuged (2,000 x *g* for 10 min at 5°C) and serum stored at -80°C until required for analysis. Fecal consistency scoring (1, hard feces; 2, Normal feces; 3, soft feces; 4, pasty diarrhea; 5 liquid diarrhoea) performed by two individually trained personnel with no prior knowledge of dietary treatment allocation was used to ascribe the diarrhea score of pigs. Fecal scores were measured at 0 (baseline, taken 1 h before challenge), 6, 24 and 48 h post-challenge.

To monitor post-infection piglet performance, feeders were emptied and BW was recorded at 0800 of d 10 after weaning to constitute acute phase baseline performance (0 h). Immediately after ETEC challenge feeders were refilled with respective diets. At 24 h post-challenge, BW and feed intake were recorded and one pig per pen euthanized as described in Manuscript I. The remaining one pig/pen was provided with fresh feed to record performance at 48 h post-challenge after which the piglet was euthanized.

On every occasion of euthanasia, the abdominal cavity was opened from the sternum to pubis to expose the whole GIT. The GIT was divided into four segments (stomach, small intestine, caecum and colon), which were clamped to minimize digesta movement. The small intestine was stripped free of its mesentery and further divided into three sections viz. ileum; from the ileal-cecal junction to 80-cm cranial to this junction, duodenum; from pylorus to 80-cm caudal to pylorus and the rest was designated jejunum (Adeola and King, 2006). The colon was further sectioned into proximal colon (from the cecal-colonic junction to apex of the spiral colon) and distal colon (from the apex of the spiral colon to rectum). Thereafter, all sections were weighed with and without digesta to

determine full and empty weights, respectively. The digesta pH was determined in all the sections using an electronic pH meter (Accumet Basic, Fisher Scientific, Fairlawn, NJ). Thereafter, digesta from the ileum and caecum was stored at -20°C until required for analysis.

A segment of the ileum (~5 cm) was placed in a sterile container and transferred to the laboratory (within 30 min of collection) for enumeration of bacteria adhered to the mucosal scrapings. Sections of mid-jejunum and ileum were removed and fixed by immersion in carnoy solution (4°C) for histomorphological analysis. Samples for the determination of dissacharidase activity were obtained from the mid-jejunum, snap frozen in liquid N and stored at -80°C until analysis.

Laboratory Analysis

Bacterial enumeration. Segments were aseptically opened longitudinally, and the mucosa was scraped from the luminal surface using a glass microscope slide. Under sterile conditions, 1 g of ileal mucosal scrapings was transferred to 15 mL tubes containing beads and 9 mL peptone water (Fisher Scientific, Fairlawn, NJ) and vortexed. Total coliforms, were determined using Eosin methylene blue agar (Difco, Detroit, MI) and ETEC were determined on Eosin methylene agar supplemented with 5μg ciprofloxacin/mL (Sigma, St. Louis, MO) as previously described by Setia (2007). Typical colonies were counted following incubation at 37°C for 24 h. Lactobacilli were enumerated using Rogosa agar (Oxoid, Ltd., Basingstoke, Hampshire, England) plates and incubated microaerophillically for 48 to 72 h at 37°C. All plating was performed in duplicate and the results expressed as cfu per g of mucosal scrapings.

Dissacharidase Activity. After thawing, mid-jejunal segments were opened longitudinally, and the mucosa was scraped from the luminal surface using a glass microscope slide. The mucosal scrapings were homogenized in aqueous Triton X-100 (1%, vol/vol; 6 mL/g of mucosa) containing protease inhibitors (0.001 *M* phenylmethylsulfonyl fluoride and 0.002 *M* iodoacetic acid) using a homogenizer (Ultra Turrax T 25 equipped with a S25N-8G probe, Janke & Kunkel GMBH & Co. KG, Staufen, Germany). Homogenates were centrifuged at 20,000 x *g* for 60 min at 4°C. The activities of lactase and sucrase were determined in the supernatants according to Dahlqvist (1968), using a glucose kit (Sigma, St. Louis, MO) to determine the amount of liberated glucose. To normalize measurements, total protein content in the supernatants was determined using a modified method of Lowry (Hartree, 1972) with bovine serum albumin as a standard. The unit of dissacharidase activity was defined as 1 µg of glucose released per min and 1 g of mucosal protein.

Intestinal Histomorphology. After 3 h in carnoy solution, samples were transferred into vials containing 80% ethanol and sent for processing in a commercial laboratory (Cancer Care Manitoba, Winnipeg, MB, Canada). Villus height (VH) from the tip of the villi to the villus-crypt junction and crypt depth (CD) from the villus-crypt junction to the base were measured at 10 x magnification using Axiostar plus microscope (Carl Zeiss, Oberkochen, Germany) equipped with a cannon camera and NIH image J software (U.S. NIH, Bethesda, MD) in at least 15 well-oriented villus and crypt columns. The VH to CD ratio (VCR) was calculated.

Digesta Characteristics. Ammonia and OA were analyzed as described in manuscript I whereas Na^+, K^+ and Cl^- were analyzed as described in Manuscripts II and III. Dry matter content of cecal digesta was determined using an oven incubator at 55°C.

Plasma Parameters. Plasma was assayed for PUN, Na^+, K^+, Cl^-, glucose and lactate using a Nova Stat profile M blood gas and electrolyte analyzer (Nova Biomedical Corporation, Waltham, MA). Osmolality was determined using the Advanced ^{TM} Micro Osmometer (Model 3300, Advanced Instruments Inc., Norwood, MS).

Serum Parameter. Serum concentration of Hp was determined using a commercially available Haptoglobin (porcine) enzyme immunoassay kit (ALPCO Diagnostic, Salem, NH). All samples were analyzed in duplicate within a single assay. Minimum detectability was 0.8 log10 ng/mL with an intra-assay CV of 4%. Serum concentrations of IL-1β, IL-6 and IL-10 were determined using commercially available Quantikine® kits (R&D Systems, Inc. Minneapolis, MN). All samples were analyzed in duplicate within a single assay. Minimum detection was 39 pg/mL, 10 pg/mL and 39 pg/mL with an intra-assay CV of 6.1%, 5.3%, and 4% for IL-1β, IL-6 and IL-10, respectively.

Component sugars concentrations of NSP hydrolysis products. Component sugars were determined by GLC (neutral sugars) and by colorimetry (uronic acids) using the procedure for non-cellulosic NSP analysis as described in Manuscript III.

Presentation of Results and Statistical Analyses

Data for bacterial enumeration and serum Hp were logarithmically transformed; GIT weights were expressed on BW basis before statistical analysis. For the performance and fecal scoring data the pen was the experimental unit, but for the other response

criteria, pig was the experimental unit. Data for performance and GIT measurements were subjected to the GLM procedures of SAS (SAS Inst., Inc., Cary, NC), however, diet (HP and EYA) was the fixed effect for the performance data while diet, day of sacrifice and their interations were fixed effects in the GIT measurements alaysis. Data for fecal scores, RT, plasma and serum parameters were analyzed with the Proc Mixed procedure of SAS with repeated measures over time (baseline, 6, 24 and 48 h post-infection). The model included terms for the fixed effects of diet (HP and EYA), time, and diet x time interaction, pigs were considered random effects. When F-value for the fixed effects was significant ($P < 0.05$) means were separated by PDIFF Adjust = Tukey option. Trends ($P < 0.10$) were also discussed.

7.4 RESULTS

Component sugars concentrations in HP

Component sugars concentrations of the HP are shown in Table 7.2. Overall, the total component sugars of the HP accounted for 356 mg/g.

Pre- and Post-Infection Growth Performance

Growth performance response during the 9 d pre-challenge period is shown in Table 7.3. Pigs fed diets containing HP tended to have greater ADG ($P = 0.08$) than pigs fed the C diet non-HP diets. Overall, treatments did not influence ($P > 0.10$) ADFI and G:F during the pre-challenge period. In post-challenge period, pigs fed the diets containing HP had higher ($P = 0.04$) feed intake than pigs fed non-HP diets.

Table 7.2. Component sugars concentrations of non-starch polysaccharides hydrolysis product[1]

Component sugars	mg/g
Arabinose	44.3
Xylose	81.8
Mannose	5.2
Galactose	26.3
Glucose	153.7
Uronic acids	44.8
Total	356.1

[1]Hydrolysis product generated by incubating ethanol-extracted wheat middlings, canola meal, soybean meal and flaxseed mixture with a blend of carbohydrase enzymes in a water medium.

Table 7.3. Effect of egg yolk antibodies and non-starch polysaccharides hydrolysis products on piglet performance during pre- and post-infection period[1]

Item	Dietary treatments[2]				SEM
	C	EYA	HP	EYA+HP	
Pre-infection					
ADG, g/d	199[b]	214[ab]	261[a]	223[ab]	16.82
ADFI, g/d	272	267	313	265	17.04
G:F	0.74	0.80	0.83	0.84	0.045
0-24h Post-infection					
Feed intake, g	316[b]	346[ab]	396[a]	374[ab]	18.7
Percent BW change	4.2	4.5	5.8	5.8	0.995
24-48h Post-infection					
Feed intake, g	329[b]	392[ab]	475[a]	461[a]	35.8
Percent BW change	1.7[b]	4.4[a]	3.0[ab]	5.0[a]	0.677

[1]n=5.
[2]C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in water medium, EYA+HP; combination of EYA and HP.
[ab]Means in a row that do not have a common superscript differ ($0.05 < P < 0.10$).

Organ Weights, Intestinal Histomorphology and Disaccharide Activity

Pigs fed the diets containing HP tended (P = 0.08) to have heavier empty intestines (Table 7.4). Irrespective of the dietary treatment, pigs sampled after 24 h post-challenge tended to have a lighter empty stomach (8.2 vs. 9.2 g/kg BW, P = 0.06) than those sampled 48 h post-challenge. Pigs fed the non-EYA diet had deeper (P = 0.09) jejunal crypts than pigs fed the diets containing EYA which resulted in lower VCR (Table 7.4). The day of sacrifice affected jejunal CD and VCR such that pigs sacrificed 24 h post challenge showed shorter CD (205 vs. 226 μm, P = 0.04) and higher VCR (3.03 vs. 2.68, P < 0.04) compared to pigs sacrificed 48 h post infection. At the ileal level, pigs fed the diets containing EYA had a higher VCR compared to pigs fed non-EYA diets. Pigs sacrificed at 24 h following infection had shorter CD (193 vs. 249 μm, P = 0.001) than pigs sacrificed 48 h post challenge. There was no diet effect (P < 0.10) on jejunal mucosal lactase and sucrase activities, however, time affected lactase activity such that pigs sacrificed 24 h post-challenge showed higher lactase activity (288 vs. 209 U/min•g protein, P = 0.02) compared to pigs sacrificed 48 h post-challenge (Table 7.4).

Table 7.4. Gastrointestinal measurements in piglets fed diets containing egg yolk antibodies and non-starch polysaccharides hydrolysis products upon *E. coli* challenge[1]

Item	Diet[2]:	24 h post-challenge				48 h post-challenge				SEM	P-value[4]		
		C	EYA	HP	EYA+HP	C	EYA	HP	EYA+HP		HPS	Diet	Diet*HPS
GIT sections weight, g/kg BW[3]													
Stomach		8.8	9.4	7.2	7.4	8.7	9.3	9.9	8.6	0.68	0.07	0.28	0.14
Small intestine		34.6	41.7	44.9	48.5	42.1	44.1	46.7	45.7	3.44	0.36	0.07	0.53
Large intestine		13.0	13.9	14.1	15.3	12.5	15.1	18.3	16.7	1.53	0.16	0.10	0.47
Jejunum													
Villi height, μm		596	593	601	638	548	607	608	614	33.6	0.61	0.50	0.79
Crypt depth, μm		231	193	189	208	246	209	246	206	14.48	0.05	0.09	0.24
Villi crypt ratio		2.6	3.3	3.2	3.1	2.3	2.9	2.5	3.1	0.25	0.04	0.05	0.58
Sucrase activity, U[5]		68	163	83	196	93	123	159	104	46.4	0.81	0.44	0.31
Lactase activity, U		269	294	275	312	190	219	212	215	45.9	0.02	0.89	0.99
Ileum													
Villi height, μm		406	391	489	459	450	501	515	489	39.8	0.03	0.14	0.54
Crypt depth, μm		215	140	227	190	244	219	259	273	20.1	0.001	0.04	0.40
Villi crypt ratio		1.9	3.2	2.3	2.5	1.9	2.3	2.0	1.8	0.26	0.04	0.03	0.43

[1]n=5

[2]C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in water medium, EYA+HP; combination of EYA and HP.

[3]Empty, organ without digesta.

4Effects: HPS, hours post-challenge.

[5]Values are μg of glucose liberated/ min·g of mucosal protein.

Gastrointestinal Microbial Activity

There was an interaction ($P = 0.024$) between diet and time post-challenge on ileal ammonia concentrations (Table 7.5). In this context, pigs fed the C diet had higher ileal ammonia concentration than pigs fed the other diets at 24 h but not at 48 h post-challenge. Pigs fed the EYA+HP diet had higher ($P < 0.0001$) ileal lactic and total OA concentrations compared with pigs fed the other diets. There tended to be an interaction ($P = 0.09$) between diet and time after challenge on cecal VFA (Table 7.5) such that the diet effect was only evident for pigs sacrificed at 24 but not at 48 h post-challenge. In this context, pigs fed diets containing HP had higher cecal VFA (84 vs. 43 mmol/l) than pigs fed the non-HP. Stomach pH differed between pigs fed the HP and non-HP diets in which case pigs in the HP group showed lower gastric pH (Table 7.5). Pigs fed the diets containing EYA had lower ileal pH than pigs fed non-EYA diets.

Total coliforms, ETEC and lactobacilli counts in the mucosal scrapings of terminal ileum are shown in Table 7.6. Mucosal scrapings of pigs fed the C diet had higher ($P = 0.06$) ETEC counts than pigs fed the other dietary treatments, which in turn had similar ETEC count. Feeding diets containing HP increased ileal mucosa lactobacilli count resulting to higher lactobacilli to ETEC ratio relative to pigs fed non-HP. Dietary treatments did not influence ($P > 0.10$) ileal total coliform counts; however, pigs fed diets containing HP showed higher ($P = 0.01$) lactobacilli to coliform ratio than pigs fed fed non-HP diets.

Table 7.5. Digesta fermentative characteristics in pigs fed diets containing egg yolk antibodies and non-starch polysaccharides hydrolysis products upon challenge with *E. coli*[1]

Item	Diet[2]:	24 h post-challenge				48 h post-challenge				SEM	*P*-value[3]		
		C	EYA	HP	EYA+HP	C	EYA	HP	EYA+HP		HPS	Diet	Diet*HPS
Ileum													
VFA, mmol/l[4]		5.55	9.54	7.53	10.0	7.72	6.34	7.88	11.2	1.749	0.92	0.16	0.45
BCVFA, mmol/l[5]		0.20	0.37	0.16	0.34	0.22	0.31	0.20	0.16	0.079	0.42	0.24	0.54
Lactic acid, mmol/l		44	90	88	137	54	51	57	123	13.76	0.07	<0.001	0.31
Total OA, mmol/l[6]		50	100	96	147	62	58	65	135	14.41	0.08	<0.001	0.28
Ammonia, mg/l		154	102	104	107	112	100	113	98	8.232	0.07	0.002	0.72
Caecum													
VFA, mmol/l		43	62	84	63	70	76	51	74	12.29	0.57	0.69	0.10
BCVFA, mmol/l		15	17	16	16	23	14	9	14	5.001	0.80	0.68	0.52
Lactic acid, mmol/l		31	39	27	31	51	39	32	43	9.147	0.18	0.62	0.73
Total OA, mmol/l		89	118	127	111	143	130	92	131	24.13	0.45	0.94	0.34
Ammonia, mg/l		433	356	295	378	375	302	304	348	33.25	0.17	0.02	0.72
pH													
Stomach		3.9	3.2	2.6	2.9	3.2	3.0	2.4	2.5	0.33	0.13	0.03	0.85
Jejunum		5.9	6.4	6.2	6.1	6.2	6.3	5.9	5.9	0.199	0.70	0.38	0.41
Ileum		7.3	6.3	6.7	6.1	6.7	6.7	6.6	6.4	0.213	0.85	0.01	0.07
Caecum		5.5	5.6	5.6	5.7	5.6	5.6	5.9	5.7	0.188	0.58	0.80	0.77
Proximal colon		6.0	6.3	6.0	6.2	6.0	6.1	5.9	6.1	0.136	0.52	0.25	0.77
Distal colon		6.4	6.5	6.3	6.5	6.4	6.4	6.4	6.7	0.101	0.79	0.10	0.64

[1]n=5

[2] C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in water medium, EYA+HP; combination of EYA and HP.

[3]Effects: HPS, hours post-challenge

[4]Volatile fatty acids; sum of acetate, propionate and butyrate.

[5]Branched chain volatile fatty acids; sum of isobutyrate, isovalerate and valerate.

[6]Total organic acids; sum of VFA, lactate and BCVFA.

Table 7.6. Adherent microbial populations in ileum of piglets fed diets containing egg yolk antibodies and non-starch polysaccharides hydrolysis products upon challenge with ETEC[1,2]

Item	Diet[3]:	24 h post-challenge				48 h post-challenge				SEM	*P*-value[4]		
		CON	EYA	HP	EYA+HP	CON	EYA	HP	EYA+HP		HPS	Diet	Diet*HPS
Total coliform[5]		6.7	7.1	5.8	5.7	6.3	6.3	5.3	6.6	0.618	0.69	0.27	0.52
ETEC, K88[6]		5.3	4.8	4.8	4.4	5.7	4.7	4.6	5.0	0.340	0.49	0.06	0.55
Lactobacilli		6.9	7.6	9.3	7.8	5.9	5.9	8.1	8.6	0.702	0.11	0.01	0.32
Lactobacilli to coliform ratio		1.1	1.1	1.7	1.5	0.9	1.0	1.5	1.3	0.181	0.20	0.003	0.99
Lactobacilli to ETEC ratio		1.3	1.6	2.1	1.8	1.0	1.3	1.8	1.8	0.212	0.16	0.004	0.84

[1]n=5

[2]Log_{10} cfu per gram of ileal mucosal scrapings, wet basis.

[3]C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in water medium, EYA+HP; combination of EYA and HP.

[4]Effects: HPS, hours post-challenge.

[5]Plated on eosin methylene blue agar.

[6]Plated on Luria-Bertani agar containing 0.5 µg ciprofloxacin/mL (Setia, 2007).

Digesta Electrolytes and Fecal Scores

There tended to be an interaction between the time after ETEC challenge and diet for ileal Cl^- concentration ($P = 0.07$) content (Table 7.7). In this context, digesta of pigs fed diets containing HP had higher Cl^- concentration than pigs fed non-HP diets at 24 h but not at 48 h post-challenge. Irrespective of dietary treatment, time post-challenge effect on ileal digesta electrolyte content was such that, pigs sacrificed 24 h post-challenge had lower Na^+ (3.06 vs. 3.69 mM, $P = 0.02$), K^+ (0.39 vs. 0.46 mM, $P = 0.03$) and Cl^- (5.3 vs. 6.6 mM, $P = 0.07$) compared to pigs sacrificed at 48 h post-challenge. At the cecal level, pigs fed the HP+EYA diet had higher Na^+ ($P = 0.06$) and K^+ ($P = 0.06$) concentration. Pigs fed the diets with additives had more ($P = 0.009$) cecal digesta DM than pigs on the C diet.

There tended to be a treatment x time interaction ($P = 0.08$) for fecal scores (Figure 7.1). At 48 h post-challenge, pigs fed diet containing combination of HP and EYA showed low incidence of diarrhea. Generally, pigs fed diets containing EYA showed less fecal score at all times.

Table 7.7. Digesta electrolytes content in piglets fed diets containing egg yolk antibodies and non-starch polysaccharides hydrolysis products upon challenge with *E. coli*[1]

Item Diet[2]:	24 h post-challenge				48 h post-challenge				SEM	*P*-value[3]		
	C	EYA	HP	EYA+HP	C	EYA	HP	EYA+HP		HPS	Diet	Diet*HPS
Ileum												
Na$^+$, mmol/l	3.41	2.91	3.21	2.71	3.48	3.98	3.93	3.36	0.361	0.02	0.49	0.58
Cl$^-$, mmol/l	5.07	5.20	5.19	5.67	5.48	4.83	10.0	6.22	1.017	0.07	0.07	0.06
K$^+$, mmol/l	0.38	0.42	0.34	0.40	0.39	0.46	0.48	0.51	0.046	0.03	0.48	0.48
Caecum												
Na$^+$, mmol/l	2.68	2.38	2.17	2.15	1.93	3.12	1.77	3.61	0.441	0.41	0.17	0.06
Cl$^-$, mmol/l	6.82	4.71	4.70	2.69	4.44	5.40	3.59	4.76	1.320	0.85	0.47	0.36
K$^+$, mmol/l	0.83	0.67	0.66	0.80	0.59	0.64	0.46	0.91	0.107	0.22	0.06	0.36
DM, g/kg	183	243	245	247	192	253	239	243	19.1	0.86	0.01	0.96

[1]n=5.
[2] C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in water medium, EYA+HP; combination of EYA and HP.
[3]Effects: HPS, hours post-challenge.

Figure 7.1. Effect of egg yolk antibodies and NSP hydrolysis products on fecal scores during 48 h following oral challenge with E. coli K88 (+) *Escherichia coli* challenge. Each mean represents five pens. C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in water medium, EYA+HP; combination of EYA and HP. Scores:1; well formed feces but fir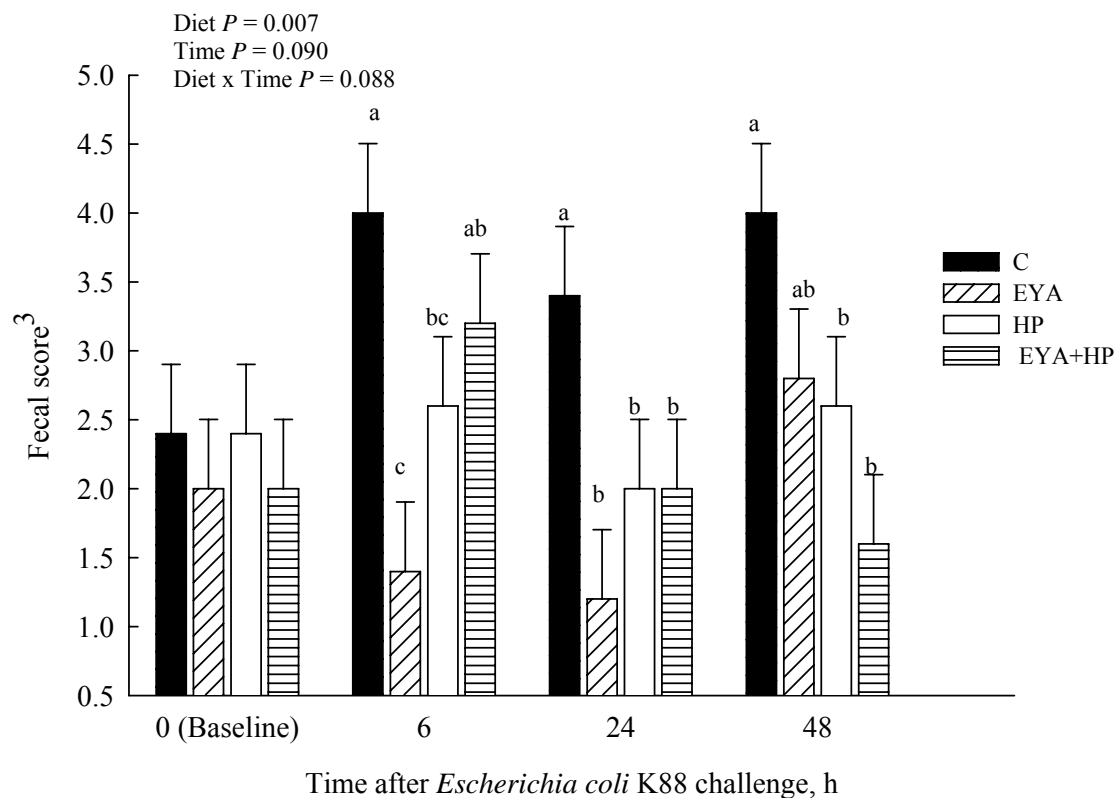m to cut, 2; well formed feces but soft to cut, 3; pasty diarrhea, 4; pasty diarrhea, 5: liquid diarrhoea. [abc]Within time point after challenge, bars without common letters differ, 0.05< *P* < 0.10.

Packed Cell Volume and Rectal Temperature

There was a diet x time interaction ($P = 0.0002$) on PCV within 48 h following ETEC challenge (Figure 7.2). Pigs fed diets with additives had greater ($P = 0.0002$) PCV than did pigs in the C group at 6 h. By 24 h, PCV was lowest for all pigs relative to baseline levels and there was no diet effect ($P > 0.10$) at this time point. There was an upsurge of PCV for all groups by 48 h post-challenge but this did not reach baseline levels. There was no diet x time interaction ($P = 0.28$) or diet effect ($P = 0.74$) on RT. However, a time effect ($P = 0.002$) was observed in which pigs across dietary treatments had the highest RT at 6 h post challenge (39.5 vs. 39.1°C; SEM $= 0.10$) relative to baseline levels (data not shown).

Plasma Biochemical Parameters

At 6 h post-challenge, plasma osmolarity for all groups was lowest ($P < 0.0001$) but similar ($P = 0.41$) among treatment groups (Figure 7.3). However, by 24 h after inoculation, plasma osmolarity in pigs fed diets containing EYA was restored to baseline levels and was higher ($P = 0.04$) than in pigs fed non-EYA diets. At 48 h post challenge plasma osmolarity was similar ($P > 0.10$) among dietary treatments and commensurate to baseline levels. There was an interaction ($P = 0.06$) between diet and time on PUN (Figure 7.4; panel A). Relative to pigs fed diets with additives, pigs fed the C diet had elevated PUN at 6 and 24 h post ETEC challenge, which declined to baseline levels by 48 h. In contrast, pigs fed diets with additives showed a relatively constant circulating PUN at all time points.
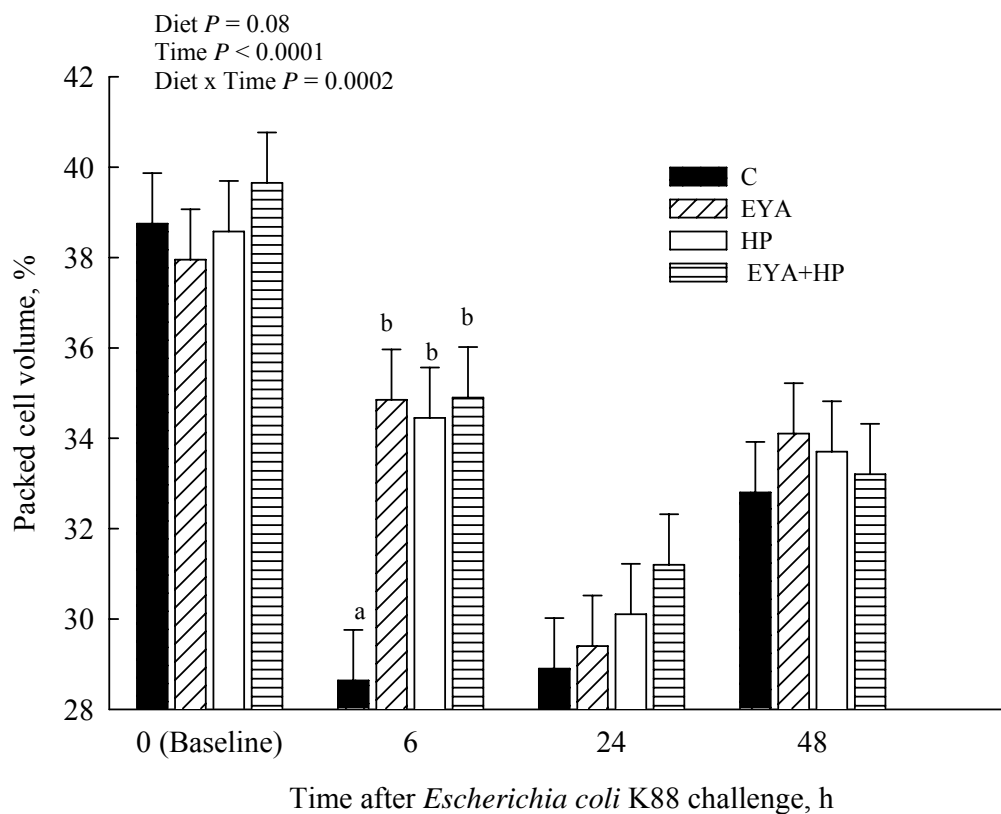
Figure 7.2. Effect of egg yolk antibodies and non-starch polysaccharides hydrolysis products on packed cell volume during 48 h following an oral challenge with *E. coli* K88. C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in a water medium, EYA+HP; combination of EYA and HP. [ab]Within a time point, bars without common letters differ, $0.05 < P < 0.10$.
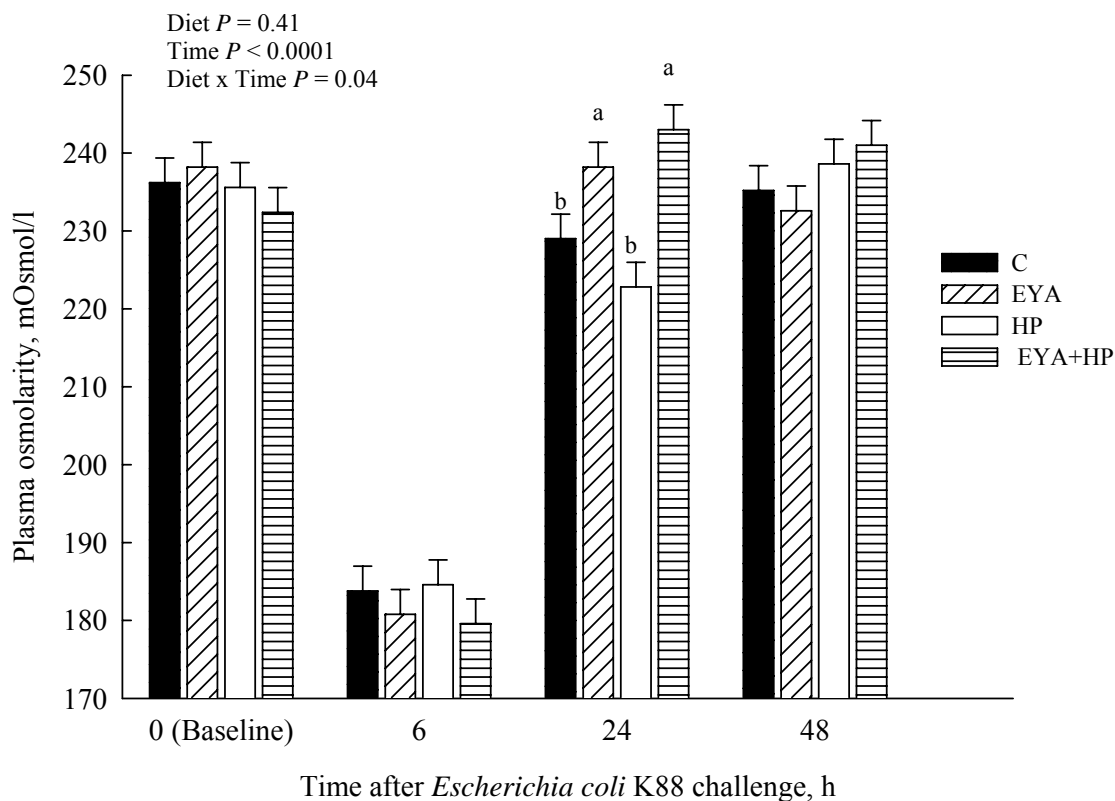
Figure 7.3. Effect of egg yolk antibodies and non-starch polysaccharides hydrolysis products on plasma osmolarity during 48 h following an oral challenge with *E. coli* K88. C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in a water medium, EYA+HP; combination of EYA and HP. [ab]Within a time point, bars without common letters differ, $0.05 < P < 0.10$.
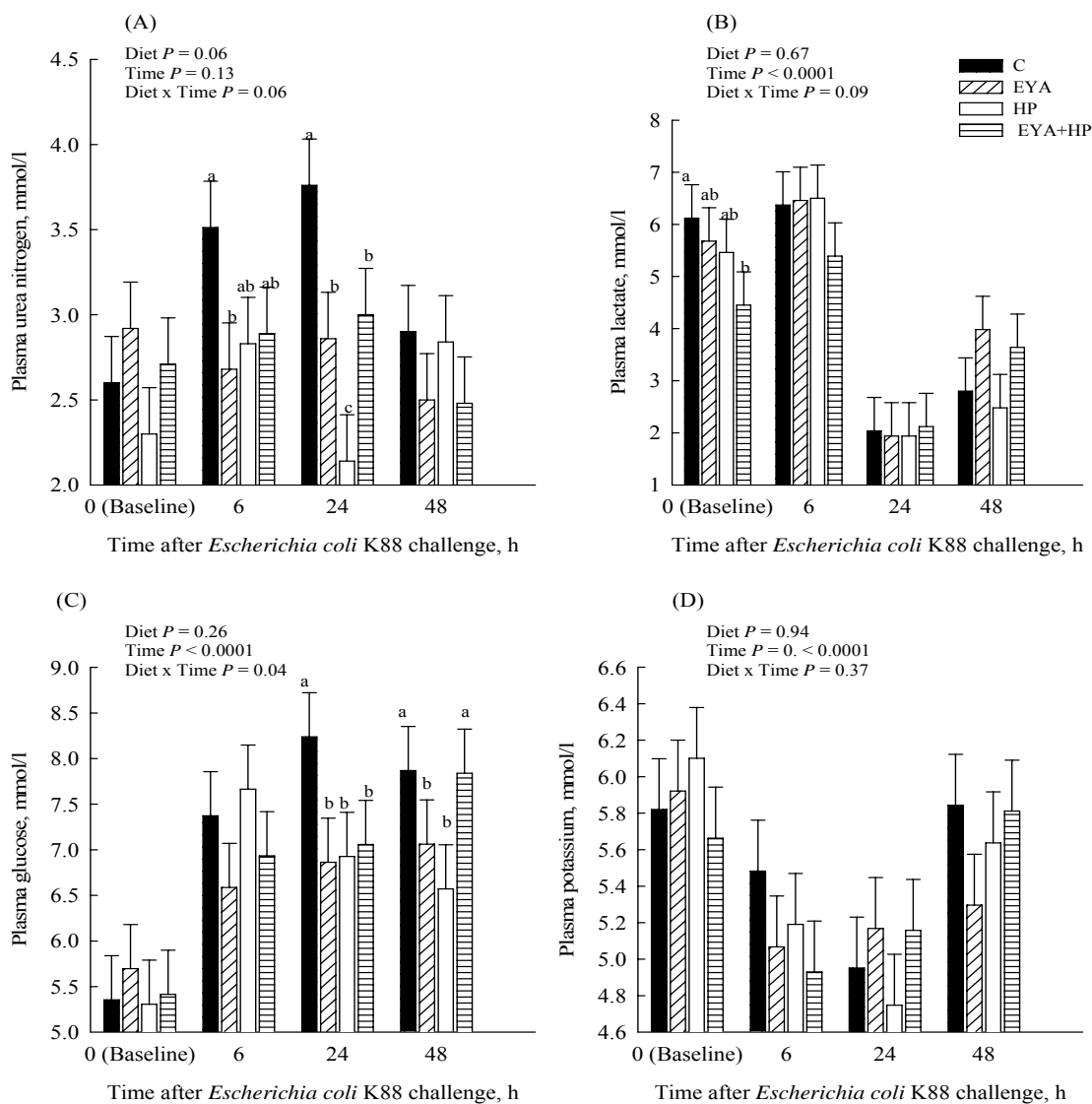
Figure 7.4. Effect of egg yolk antibodies and non-starch polysaccharides hydrolysis products on plasma urea nitrogen (panel A), lactate (panel B), glucose (panel C) and potassium (panel D) levels during 48 h following an oral challenge with *E. coli* K88. C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis product was generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in a water medium, EYA+HP; combination of EYA and HP. [ab]Within a time point, bars without common letters differ, $0.05 < P < 0.10$.

There tended to be a diet x time interaction ($P = 0.09$) on plasma lactate concentration such that at baseline, EYA+HP group had lower circulating lactate compared to C group (Figure 7.4; panel B). Overall, there was a time effect ($P < 0.0001$) on plasma lactate such that by 24 h pigs across all treatments showed a sharp decline in circulating lactate relative to baseline levels. Data for circulating plasma glucose within 48 h post-challenge is shown in Figure 7.4 (panel C). Diet and time after challenge affected plasma glucose levels interactively ($P = 0.04$). By 24 h post-challenge piglets in the C group had higher plasma glucose concentration than other groups which were in turn similar. At 48 h, the EYA+HP. Overall, a time effect on plasma glucose levels was such that there was a sharp increase by 6 h post-challenge and levels remained high relative to baseline thereafter. Plasma Na^+ and Cl^- contents were not affected by diet and time after ETEC challenge or their interactions ($P > 0.10$; data not shown). Although there was no diet x time interaction ($P = 0.37$) or diet effect ($P = 0.94$), a time effect was observed ($P < 0.0001$) in which pigs across dietary treatments had the lowest plasma K^+ at 24 h post challenge (5.00 vs. 5.89, mmol/l; SEM = 0.28) relative to baseline levels (Figure 7.4; panel D). There was a resurgence of increasing plasma K^+ for all groups by 48 h post-challenge.

Serum Haptoglobin and Cytokines

There was a diet x time interaction for serum Hp ($P = 0.05$) within 48 h following ETEC challenge (Figure 7.5; panel A). At 6 h post-challenge, pigs in the C group had greater ($P = 0.01$) circulating Hp than those fed diets with additives which in turn had similar Hp concentrations.
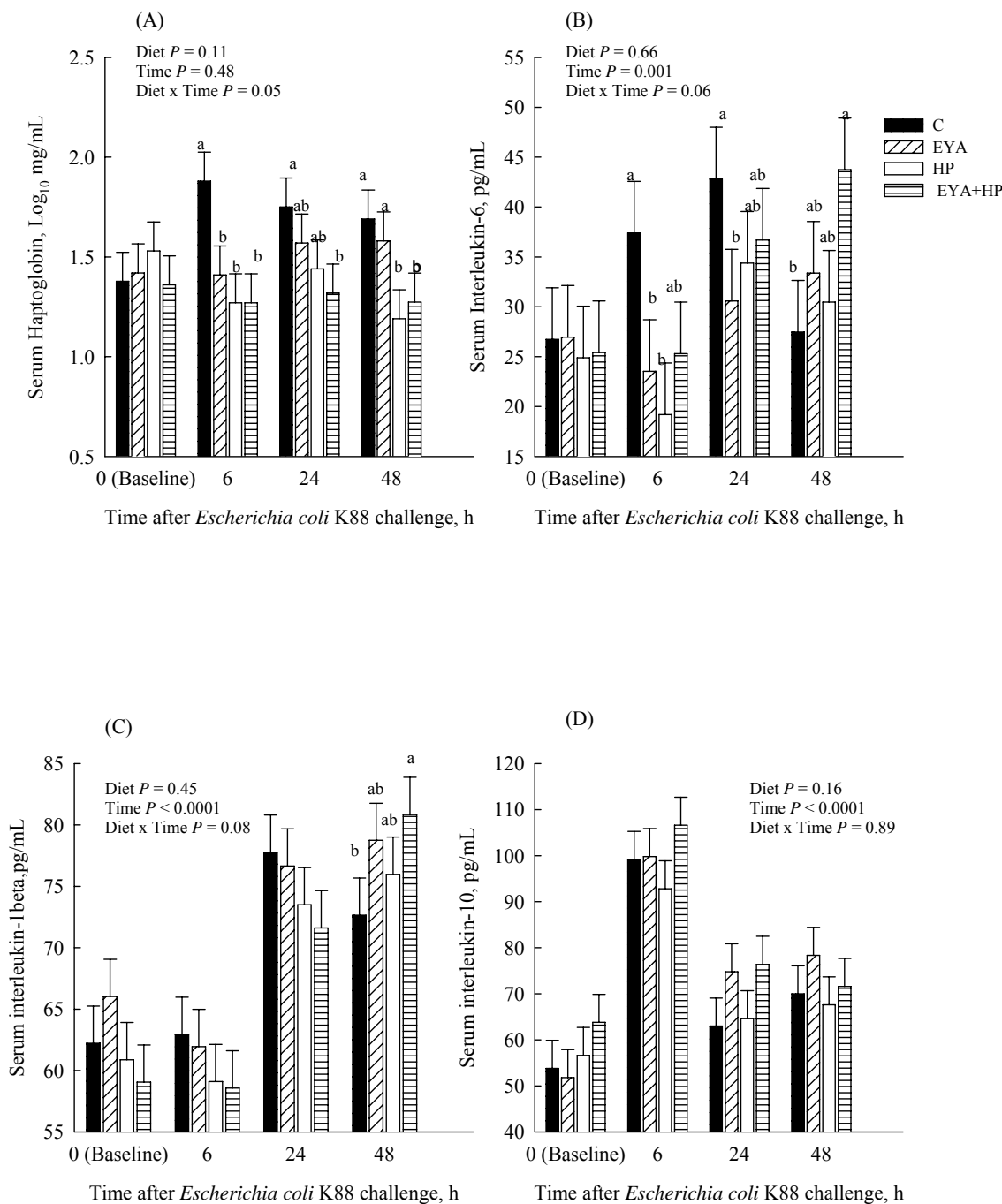
Figure 7.5. Effect of egg yolk antibodies and non-starch polysaccharide hydrolysis products on serum haptoglobin (panel A), Interleukin-6 (panel B), Interleukin-1β (panel C) and Interleukin-10 (panel D) levels during 48 h following an oral challenge with *E. coli* K88. C, control diet with no additives, EYA; egg yolk antibody against ETEC K88 fimbriae, HP; hydrolysis products generated by incubating ethanol-extracted wheat middlings, canola meal, soybean and flax mixture with a blend of carbohydrase enzymes in a water medium, EYA+HP; combination of EYA and HP. [ab]Within a time point, bars without common letters differ, $0.05 < P < 0.10$.

Twenty four hours after the ETEC challenge pigs in the C group still showed higher Hp concentrations than pigs fed the EYA+HP diet. By 48 h, serum Hp in EYA group was similar to the C group but greater than for pigs fed the EYA + HP diet. Overall, circulating serum Hp in pigs fed the HP diet remained relatively constant at all time points.

Diet and time after ETEC challenge affected serum IL-6 interactively (P = 0.06; Figure 7.5; panel B). At 6 h post challenge, pigs in the C group had greater serum IL-6 than pigs fed additives. By 24 h, serum IL-6 in the C group was greater than in the EYA group whilst pigs fed diets containing HP were intermediate. By 48 h, circulating serum IL-6 for the EYA+HP group was higher (P = 0.06) than for the C group whilst the EYA and HP groups were intermediate at this time point.

Diet and time after ETEC challenge affected serum IL-1β interactively (P = 0.08; Figure 7.5; panel C). At 48 h post challenge, pigs in the EYA+HP group had greater serum IL-1β. Overall, there was a time effect (P < 0.0001) on IL-1β such that pigs across dietary treatments had the highest circulating concentrations of IL-1β at 24 h (75 vs. 62 pg/mL; SEM = 3.02) relative to baseline concentrations. Irrespective of dietary treatments, circulating IL-1β remained greater than baseline concentrations at 48 h post-challenge.

Data for circulating serum IL-10 is shown in (Figure 7.5; panel D). Although there was no diet x time interaction or diet effect (P > 0.10) for IL-10, a time effect was observed (P < 0.0001). Pigs across dietary treatments had the highest circulating concentrations of IL-10 at 6 h (100 vs. 57 pg/mL; SEM = 6.08) relative to baseline

concentrations. Circulating IL-10 was, however, restored to baseline concentrations by 24 h and remained unchanged at 48 h post-challenge.

7.5 DISCUSSION

The challenge of formulating control diet to evaluate additives such as HP which naturally occurs in feedstuffs has been highlighted before (Houdijk et al., 2002). In the present study, we used corn not only because it contains low concentrations of oligosaccharides and NSP but to ensure that the basal diet was devoid of feedstuffs used to generate the HP studied. Theoretically, NSP hydrolysis products may protect piglets from ETEC by interfering with attachment to the enterocytes as has been shown for plant extracts (Kiers et al., 2002; Maiorano et al., 2007). Alternatively, NSP hydrolysis products may promote proliferation of beneficial/commensal bacteria which are known to have anti-pathogenic capabilities (Nout et al., 1989; Hillman et al., 1995). In relation to EYA, it has been shown that they block the binding of ETEC to immobilized piglet mucus (Jin et al., 1998). In the present study, characteristics describing gastrointestinal ecology of a piglet when fed a basal diet containing HP and EYA singly or in combination upon challenge with ETEC were investigated. Furthermore, it was hypothesized that pigs fed a combination of HP and EYA might have synergistic containment of ETEC induced gut inflammatory response such that they might have less pronounced acute phase responses.

The high ADG in pigs fed diets containing HP may have be due to the numerically higher ADFI observed for this group. Surprisingly, the effect of HP was not evident when fed in combination with EYA as pigs fed the EYA+HP diet performed commensurate to the other groups. This observation suggests antagonism between EYA

and HP on piglet performance. Generally, egg products have been shown to support poorer piglet performance (Schmidt et al., 2003). Anorexia and attendant shift in the partitioning of dietary nutrients away from skeletal muscle accretion toward metabolic responses that support the immune system is the hallmark of sub-clinical and clinical infections in animals (Johnson, 1997). In the present study, within each time point post-challenge pigs fed the C diet showed less feed intake compared to pigs fed diets containing HP whereas pigs fed diets containing EYA added more weight at 48 h. Overall, this observation suggest that HP and EYA afforded protection against ETEC and that there is merit in feeding these additives in combination to support adequate piglet performance during ETEC infection.

High intestinal weight is often not a desirable attribute in swine growth as viscerals are known to metabolize considerable amount of nutrients (Nyachoti et al., 2000). However, with respect to piglet, high intestinal weight is relevant as they preferentially develop their gastrointestinal tract to cope with the new weaning diet before muscle accretion begins in earnest (Cranwell and Moughan, 1989). In agreement with Owusu-Asiendu et al. (2003b), pigs fed EYA diets had shallower intestinal crypts which resulted in higher VCR. Crypt hyperplasia is a consequence of increased apoptosis in response to enteropathogens (Willing and Van Kessel, 2007). Previously, Jin et al. (1998) showed that EYA blocked the binding of ETEC to immobilized piglet mucus. In this context, piglets fed EYA diets maintained intestinal integrity due to less mucosal and ETEC interaction. However, the benefits noted for pigs fed EYA diets on mucosal integrity were not reflected in less severe enteritis-associated suppression in dissacharidase activity. It is noteworthy; however, that pigs fed additives had almost a 2-

fold higher sucrase activity compared to the C group although due to high variability this did not approach statistical significance.

Low ammonia concentration was observed in the ileal digesta of pigs fed diets containing additives. Enterotoxic products of bacterial metabolism such as ammonia contributes to increased enterocytes replacement rate as they negatively affect growth and differentiation of intestinal epithelial cells (Gaskins, 2001; Suzuki et al., 2002). Subsequently, it is no coincidence that pigs in the C group showed higher ammonia content and pronounced crypt hyperplasia. Furthermore, a reduction in intestinal ammonia is a primary mechanism for the growth response induced by antibiotics in piglets (Visek et al., 1978). At cecal level, piglets fed diets containing HP alone also resulted in lower ammonia which might have maintained cecal mucosal integrity although not measured in the present study. Feeding piglets a combination of EYA and HP elicited higher ileal lactic acid and OA production than when fed alone which suggested synergism between the two additives. Organic acids are products of bacterial fermentation of carbohydrates, and the quantity of OA produced depends on the amount and composition of the substrate and on the type of microbes present in the GIT (van Beers-Schreurs et al., 1998a). At the present time it is rather difficult to explain why a combination of EYA and HP would result in greater OA production as EYA is mainly of nitrogenous composition. In agreement with Manuscript I, dietary differences in OA in the terminal ileum were not reproduced at the cecal level perhaps due to extensive fermentation owing to a large reservoir of bacteria in this organ.

Gastric acidity is the first line of defense against bacterial invasion in neonatal pigs (Cranwell et al., 1976). However, at weaning, natural acidification is attenuated,

predisposing piglets to orally derived pathogens (Pluske et al., 2002). In the present study, pigs fed diets containing HP showed lower gastric pH, an effect that may have been meditated by enhanced microbial fermentation in the stomach using HP as substrates. This observation confirms the finding in Manuscript I that up to 12% of NSP were fermented before the terminal ileum. Maintaining low gastric pH is important in the control of enteric pathogens such as ETEC which are transmitted within the herd via oral-fecal cycling (Pluske et al., 2002). Additives, particularly EYA lowered ileal pH which correlated well with lower ammonia and higher OA concentrations observed in pigs fed these diets. The lower distal colonic pH for the pigs in the HP group relative to pigs fed non-HP diet suggest nitrogen fermentation in the latter group.

Pigs fed diets containing additives showed lower ETEC numbers in the ileal mucosa scrapings which coincided with less severity of the ETEC-infection associated symptoms as shown by lower visual assessment of fecal consistency within 48 h post-challenge. That HP supported higher number of lactobacilli which resulted in higher lactobacilli to ETEC ratio in the mucosa scrapings suggests competitive exclusion as the mode of action of these products (Harvey et al., 2005). In relation to EYA, it appears not to support lactobacilli.

After colonizing the small intestine, ETEC provokes hypersecretory diarrhea through release of enterotoxins that initiate metabolic cascades characterized by net fluid and electrolyte secretions into the intestinal lumen (Fairbrother et al., 2005). Results of the present study showed that pigs fed the HP diet had higher Cl^- content relative to pigs fed non-HP diets. Whether the pathogenesis of ETEC leads to diarrhea depends, on the amount of fluid entering the large intestine and on its capacity to compensate for the loss

from the small intestine by enhanced absorption (Argenzio et al. 1984; van Beers-Schreurs et al. 1998b). It has been suggested that the absorption of electrolytes and water by the large intestine are facilitated by the action of OA (Argenzio and Whipp, 1979), and that these compounds may help to increase the percentage of DM and lower electrolytes in the contents of the large intestine. Subsequently, we observed higher cecal digesta DM for pigs fed the diets with additives which, however, did not coincide with elevated cecal OA concentration. Nevertheless, pigs fed the HP diet showed lower cecal electrolyte contents which was coincidental to the higher cecal VFA observed for pigs fed this diet.

Thus, piglets exhibited reduced PCV 6 h post-challenge with the lowest level being achieved at 24 h and signs of recovery at 48 h. Similarly, Vellenga et al. (1988) and Cox et al. (1989) reported a decline in PCV in piglets infected with ETEC. In secretory diarrhea, such as the one caused by ETEC, PCV is expected to increase due to hypovolaemia (i.e. loss of extra cellular fluids) (Mitchell, 2004). In this context, it would appear that piglets in the aforementioned studies and the present study did not experience ETEC induced hypovolaemic shock albeit evidence of profuse diarrhea. However, transient reduction of PCV is plausible in hypovolaemia. Firstly, one of the prominent aspects of acute phase responses is the modification to vascular tone which can lead to edema and in some instances red-cell extravasation and thus reducing PCV (Baumann and Gauldie, 1994). Secondly, reduced osmolality, hyponatraemia, and hypokalaemia are the usual transient outcome of infectious diarrhea and reflect renal water retention, under the influence of antidiuretic hormone, in response to hypovolaemia (Lord, 1999; Mitchell, 2004). Subsequently, the time dependent reduction in PCV (lowest at 24 h), plasma osmolality (lowest at 6 h) and K^+ (lowest at 6 and 24 h) as observed in the present

study is a consequence of acute phase responses to ETEC-enteritis. Nevertheless, a diet and time post-challenge interaction was observed for PCV such that pigs fed diets with additives sustained a numerically higher PCV at 6 h and 24 h compared to the C group. This observation suggested that piglets fed additives were able to contain to some extent the severity of ETEC inflammatory response. Protective effect of EYA against ETEC insults were further demonstrated when pigs fed diets containing EYA showed quick plasma osmolarity recovery relative to pigs fed diets without EYA by 24 h post-challenge.

Alteration of liver metabolism is a primary physiological response associated with acute inflammation (Johnson, 1997; Carroll, 2007). Plasma urea N has been used as an indicator of AA breakdown as a result of less than optimal systemic AA supply for protein synthesis (Coma et al., 1996) and of protein catabolism to release AA for synthesizing acute phase proteins in APR (Johnson, 1997). In the present study, pigs fed the C diet showed higher PUN and serum Hp at all times post-challenge which correlated well with anorexia observed for this group. Overall, pigs fed diets with additives maintained much lower PUN and Hp within 48 h post-challenge which reflected the superior performance observed for these groups. Hyperglycemia is often an outcome of acute phase responses following secretion of glucocorticoids as part of a negative feedback loop that regulates the immune system to prevent it from overreacting (McCallum et al., 1990; Johnson et al., 1997). Subsequently, a time dependent elevation of plasma glucose observed in the present study is a result of ETEC-enteritis. However, diet effects were such that the C-fed pigs showed higher plasma glucose at 24 and 48 h which suggested severe ETEC infection. A time dependent decline in plasma lactate was

noted in coincidence with hyperglycemia suggesting gluconeogenesis from lactate (Hatenyi et al., 1988).

Changes in plasma acute phase proteins are driven by changes in circulating cytokines, notably IL-1β and IL-6 (Baumann and Gauldie, 1994). The cytokine IL-6 acts directly on hepatocytes to stimulate AA uptake and synthesis of a broad array of acute-phase proteins (Carroll, 2007). In pigs, increased Hp levels have been associated with reduced growth rate that is consistent with repartitioning of AA toward acute phase protein synthesis and away from growth (Eurell et al., 1992; Johnson, 1997). Therefore, the finding that pigs fed the C diet had elevated IL-6, Hp and PUN correlated well with reduced growth observed for this group at 48 h. At 48 h pigs fed diets with the additives and in particular in combination showed high pro-inflammatory cytokines (IL-1β and IL-6) concentrations compared to pigs in the C group, which is difficult to explain as pigs fed diets with additives had a lower incidence of diarrhea at 48 h post-challenge. Interleukin-10 is an anti-inflammatory cytokine known to down regulate pro-inflammatory cytokines (e.g. IL-1β and IL-6) (Moore et al., 1993). In this context, IL-10 is produced in response to pro-inflammatory cytokines as has been shown in human monocytes following lipopolysaccharides challenge (Foey et al., 1998). The present study shows that IL-10 peaked at 6 h post challenge and declined to baseline levels by 24 h and remained so upto 48 h. At the peak of IL-10, pro-inflammatory cytokines remained at baseline level and peaked when IL-10 plummeted. It is likely that the initial peak of IL-10 observed in the present study was in response to local intestinal pro-inflammatory cytokines upon ETEC challenge. On the other hand the upsurge of pro-inflammatory

cytokines as observed at 24 h is the result of the secondary wave of cytokines which characterize the complex process of the APR (Baumann and Gauldie, 1994).

Responses reported in the present study are a consequence of gastrointestinal tract already conditioned with additives (additives fed for 9 d before challenge) and therefore 'mimic' prophylactic use of antimicrobials. At 48 h pigs showed deeper CD, less lactase activity and higher ileal electrolyte than pigs sampled at 24 h, which suggested that that irrespective of dietary treatment ETEC enteritis became more severe at 48 h post challenge. Serial slaughter negate an opportunity of observing an animal over time, however, it is clear from the present study that diets were associated with an altered piglet intestinal microenvironment over time. The present data contribute to the understanding of the mechanisms through which HP and EYA prevents ETEC-induced secretory diarrhea in piglets.

In conclusion, piglets fed diets containing the additives singly or in combination exhibited less severe ETEC-enteritis. Because hen eggs are such a rich source of immunoglobulin that is easily purified, these results provide further implications for prevention and perhaps treatment of common infectious diarrhea in piglets. In respect to HP, the present data may aid in designing enzyme systems tailored for maximizing dietary nutrient utilization by piglets as well as controlling enteric infections such as ETEC-secretory diarrhea.

CHAPTER EIGHT

GENERAL DISCUSSION

The newly weaned pig is simultaneously subjected to nutritional, psychological and environmental stressors which in concert with immature digestive and immune systems predispose the piglet to GIT disturbances (Lallès et al., 2004; Main et al., 2004). A major challenge for the pig industry is to formulate economically viable growth promoting diets to ease the transition from sow milk to nursery diets without recourse to AGP (Whitemoore and Green, 2001). Further to satisfying the nutritional requirements of weaned pigs, such diets should also improve the piglet digestive capacity, modulate microbial succession, stabilize the commensal microbiota, improve immune function and enhance disease resistance (Kelly and King, 2001).

The present research evaluated the efficacy of CE targeting NSP in enhancing the gut health and function in weaned pigs. Carbohydrase enzymes targeting feedstuff NSP constitute a major grouping of supplemental exogenous enzymes that are routinely added to piglet diets to improve nutrient utilization (Partridge, 2001). To achieve this end, CE breakdown the NSP to release encapsulated nutrients; in the process a variety of HP are released (Slominski, 2000; Chesson and Stewart, 2001; Meng et al., 2005). It has been speculated that such HP could modulate gastrointestinal microbial activity (Chesson and Stewart, 2001) or influence expression of enteric diseases such as PWC (Pluske et al., 2002). However, whether the HP would influence GIT microbial activity in the context of post weaned piglet GIT disturbances remains to be determined.

In Manuscript I, the effects of supplementing baby pig diets with a multi-CE supplement targeting NSP in wheat, barley, CM, SBM, peas and FS on growth performance, GIT bacteria activity and nutrient digestibility were studied. Pigs fed CE supplemented diets had higher ileal lactobacilli count, lactate and total OA concentrations as well as high ileal mannose, arabinose, and xylose digestibility. This suggested that HP containing arabinose, xylose and mannose sugar residues may have support lactic acid bacteria activity in the ileum. Furthermore, reduction of ileal digesta ammonia concentration in pigs fed diets with CE was commensurate to putative mode of action of AGP (Francois, 1962; Visek, 1978; Anderson et al., 1999).

Whereas the effect of CE on ileal microbial activity observed in Manuscript I suggested a healthier gut, they were not translated into better growth performance. Presumably because the study was conducted in a research facility in which piglet were not under GIT health challenges. The study of a specific enteric pathogen, particularly if it causes economic loss, offers a means of assessing the usefulness of nutritional interventions/strategies on the survival of that particular pathogen in the GIT to cause disease (Madec et al., 1998; 2000; Jones et al., 2001; Pluske et al., 2002). Consequently, the effectiveness of CE targeting NSP on piglet intestinal microflora was further evaluated using ETEC challenge model to evaluate the impact on the gut health and function. Enterotoxigenic *E. coli* was chosen because it is among the most prevalent bacterial pathogens in the GIT of piglet upon weaning and has been implicated in the pathogenesis of PWC (Pluske et al., 2002; Fairbrother et al., 2005). Post weaning collibacillosis is a disease of the small intestine and has been estimated to be responsible for as much as 50% of the economic losses seen in the production of weaned pigs

(Fairbrother et al., 2005; Cutler et al., 2007). In herds with PWC, up to 2% mortality is recorded, but of greater economical significance is the morbidity and reduction in growth performance (Cutler et al., 2007).

Using the ETEC disease challenge two approaches were adopted; an *in situ* model (Manuscripts III and IV) and an *in vivo* model (Manuscript V). The *in vivo* model of ETEC challenge has been widely used at the University of Manitoba, Department of Animal Science (e.g. Owusu-Asiedu et al 2003a, b). However, the *in situ* model has not been used at the University of Manitoba. Thus, before the planned studies could be implemented, a pilot study (Manuscript II) was conducted to establish this model in our laboratory. In the pilot study, conventional anti-diarrhea agents FA, ZnO, EYA and AB were used as treatments with saline as control. The results showed that these agents' attenuated fluid losses in ETEC infected jejunal segments. These observations agreed with studies in the literature showing efficacy of these agents in controlling ETEC-induced diarrhea in piglets (e.g. Tsiloyiannis et al., 2001; Savoini et al., 2002; Owusu-Asiedu et al., 2003a; Roselli et al., 2001).

Following the establishment of the *in situ* model, a series of four experiments were conducted to study the effects HP from CM, SBM, WM and FS on ETEC-induced secretory diarrhea. These ingredients were used in the diets fed to piglets in the study presented in Manuscript I. The HP were produced following the procedures developed at the University of Manitoba, Department of Animal Science and variously reported (e.g. Slominski et al., 1993; Slominski et al., 1994; Meng et al., 2005; Slominski et al., 2006). In all experiments saline was used as an internal control for maximum response to infection (Kiers et al., 2006). The results of the four experiments were reported in

Manuscript III (WM and FS experiments) and Manuscript IV (CM and SBM experiments). Overall, the results demonstrated that HP from various feedstuffs attenuated ETEC induced fluid and electrolyte losses. However, as the numbers of the ETEC count on the mucosal scrapings of the jejunal segments did not differ with saline perfused segments the data did not provide conclusive evidence on the mechanism in which HP protected against ETEC infection.

In Manuscript V, the HP singly or in combination with EYA were fed to piglets for 9 d after which they were orally challenged with ETEC and observed for 48 h during which time several response criteria associated with ETEC-enteritis were evaluated. In the generation of the HP, the ratio of the WM, SBM, CM and FS in the mixture represented a typical composition in piglet diet. The EYA were chosen because their protective effect against ETEC-induced diarrhea was previously demonstrated at the University of Manitoba, Department of Animal Science (e.g. Owusu-Asiedu et al., 2002; 2003a, b). The perceived advantage of AGP is that they decrease the energetic costs associated with constitutive low level inflammation caused by enteropathogens (Anderson et al., 2000). Responses reported in Manuscript V are a consequence of GIT already conditioned with additives (additives fed for 9 d before challenge) and therefore 'mimic' prophylactic use of antimicrobials.

Results presented in Manuscript V showed that pigs fed diets containing HP had lower gastric. In Manuscript I, pigs fed diets supplemented with multi-CE had higher pre-cecal NSP digestibility (14 vs. 4%) than pigs fed unsupplemented diets. Such effects may have been meditated by enhanced microbial fermentation, predominantly lactic acid bacteria in the stomach (Ewing and Cole, 1994). Overall, these results suggest that CE

targeting feedstuff NSP could avail substrates for the acid fermentors in the fore gut. Maintaining low gastric pH is important in the control of acid sensitive enteric pathogens such as ETEC which are spread between animals primarily by fecal-oral route (Berschinger, 1999). Furthermore, at low gastric pH, digestion of proteins and populations of beneficial bacteria (lactobacilli) are maximized (Cranwell et al., 1995; Doyles, 2001; Lallés et al., 2007).

The diversity of GIT bacterial populations is directly related to the number and composition of limiting substrates, since each limiting substrate will support the one bacterial species or strain that is most efficient in utilizing it (Gaskins, 2001). Moreover, the stability of bacterial populations will also be influenced by the inhibitory actions of a number of microbial metabolites such as OA and ammonia (Anderson et al., 2000; Gaskins, 2001; Williams et al., 2001). It is likely, therefore, that certain substrates and their associated physico-chemical effects play a major role in maintaining the balance of the microbiota in the GIT, and subsequently in determining whether a pathogenic bacterium proliferates (or not) to cause disease (Pluske et al., 2002).Since ETEC-induced diarrhea is a disease of the small intestine, it follows that the piglet response in different treatments groups to ETEC challenge as presented in Manuscripts III, IV and V include a sequela of ETEC interactions with GIT ecology.

After colonizing the small intestine, ETEC provokes hypersecretory diarrhea through release of enterotoxins that initiate metabolic cascades characterized by net fluid and electrolyte secretions into the intestinal lumen (Fairbrother et al., 2005). Whether the pathogenesis of ETEC leads to overt diarrhea depends, on the amount of fluid entering the large intestine and on its capacity to compensate for the loss from the small intestine

by enhanced absorption (Argenzio et al., 1984; van Beers-Schreurs et al., 1998b). In this context, net fluid absorption results presented in Manuscript III and IV, cecal DM and fecal score results presented in Manuscript V converge to suggest that HP were effective in reducing ETEC induced diarrhea.

In Manuscript V, pigs fed diets containing additives showed low ETEC numbers in the ileal mucosa scrapings which coincided with less severity of the ETEC-infection associated symptoms. It has been demonstrated that EYA block the binding of ETEC to immobilized piglet mucus (Jin et al., 1998). Since feeding the HP alone or in combination with EYA supported similar numbers of ETEC attached to the mucosa, it can be deduced that HP excluded ETEC on the piglet mucosa. However, these observations on the ETEC numbers did not concur with the *in situ* model studies reported in Manuscripts III and IV. Indeed, the microbial data for the study presented in Manuscript II showed that ETEC numbers attached to the mucosal scraping of segments perfused with EYA were similar to those of segments perfused with saline. With respect to the *in situ* model it appears that the duration of perfusion may be too short to detect differences in ETEC numbers as was suggested by Bruins et al. (2006). Alternatively, as speculated in Manuscripts III and IV, perhaps the protective mechanisms of HP when applied directly to the intestinal lumen may be mediated by other mechanisms which may require future determination. For instance, except for the ES from SBM, time of infection relative to perfusion did not appear to influence net absorption. Furthermore, although segments perfused with ES from SBM 30 min before ETEC challenge showed higher net absorption than segments perfused with the same product but challenged before perfusion, still the number of ETEC attached to the mucosal scrapings were similar.

Digesta characteristics data presented in Manuscript V shows that additives, particularly EYA, lowered ileal pH which correlated well with lower ammonia and higher OA concentrations observed in pigs fed these diets. The overall effect is less number of viable ETEC in the intestinal lumen and subsequently less number of ETEC on the mucosa as discussed earlier. Indeed, HP supported higher lactobacilli numbers in the intestinal mucosa with a consequence of higher lactobacilli: ETEC ratio (Manuscript V). Furthermore, enterotoxic products of bacterial metabolism such as ammonia contributes to increased enterocytes replacement rate as they negatively affect growth and differentiation of intestinal epithelial cells (Gaskins, 2001; Suzuki et al., 2002). Moreover, additives resulted to almost 2-fold high (non-significant) sucrase activity compared to the C group. Overall, reduction of intestinal pathogens or toxic bacterial metabolites contributes to the GIT health and function a consequence of which is improved piglet performance in the presence of pathogens as presented in Manuscript V. It should be noted, however, that limitations of the *in vivo* experiment design negate determination of whether pigs fed additives without ETEC challenge might have similar effects on the reported response criteria.

The multi-enzyme supplement used in the present research was previously shown to be effective in the hydrolysis of NSP in various feedstuffs (Meng, 2005). In those studies the reductions in NSP recoveries following incubation of feedstuff with CE were 34, 36, 26 and 34 % for wheat, CM, SBM and FS, respectively. In the present research (Manuscripts III, IV and V), the water soluble fraction of partially hydrolyzed NSP were isolated and either perfused or fed to piglets. Whereas, the results were discussed in the context of carbohydrate content of HP, a variety of water soluble phyto-chemical

constituents released following partial hydrolysis may also be attributable to results presented. Nevertheless, since the meals were subjected to extensive ethanol extraction before incubation, the HP studied in the present research demonstrate that CE targeting NSP may be beneficial in controlling ETEC-induced diarrhea in pigs. These novel results will expand the scope of enzyme technology in animal nutrition in the new paradigm of dietary approaches to gut health and function.

CHAPTER NINE

CONCLUSIONS AND FUTURE STUDIES

CONCLUSIONS

The following conclusions can be drawn from the present research:

1. Supplementing starter diets containing wheat, barley, soybean meal, canola meal peas and flaxseed with a multi-carbohydrase enzyme containing pectinase, cellulase, mannanase, xylanase, glucanase and galactanase activities increased pre-cecal total NSP digestibility (14% vs. 4%) compared to the unsupplemented diets.

2. Ileum digesta of piglets fed diets containing multi-carbohydrase supplement had higher lactobacilli count, higher organic acid concentration and lower ammonia concentration.

3. A short intestinal segment perfusion *in situ* model for quantitative assessment of ETEC effect on net absorption in the small intestine was established and validated using ZnO, FA, carbadox and EYA as conventional anti-diarrhea agents.

4. Non-starch polysaccharides hydrolysis products produced from wheat, flaxseed, soybean meal and canola meal under *in vitro* conditions and fractionated into oligosaccharides and low molecular weight polysaccharides (80% ethanol solubles) and high molecular weight polysaccharides (80% ethanol insolubles) enhanced net absorption in ETEC infected jejunal segments. However, the number of ETEC attached to the mucosal scrapping of segments after perfusion were generally similar between the test products and the saline control.

5. Supplementing starter diets with HP alone or in combination with EYA attenuated ETEC-enteritis symptoms such that piglets fed additives showed less pronounced acute phase responses and superior performance in the presence of a pathogen compared to control.

6. Feeding piglets HP and EYA led to lower gastric pH, less number of ETEC attached to the mucosa, more organic acid in the ileal digesta and a lower incidence of diarrhea compared to control.

FUTURE STUDIES

Application of enzyme technology in animal nutrition has consolidated around their use in improving nutrient utilization. In this respect, their apparent value to the feed industry has extended little from that established in the earliest days of use when bulk enzymes first became available in volumes and at prices which allowed their use in animal feeds. The present research demonstrates that carbohydrase enzymes targeting NSP might offer a means of manipulating the gastrointestinal microbial activity to the benefit of the host. However, future studies are required to:

1. Identify the component(s) in the NSP hydrolysis products which are responsible for the protective effect to allow maximization of those components with greater effects.

2. Evaluate the specific mechanism(s) underlying the protective effect of HP.

3. Expand scope of enteric disease models afflicting swine GIT such as swine dysentery, salmonellosis, porcine intestinal spirochaetosis, non-specific colitis, porcine proliferative enteropathies and gastric ulcers among others.

4. Design carbohydrase enzymes systems based on the data generated above and evaluate their protective effects in the *in vivo* trials through dietary supplementation.

5. Further evaluate synergism between enzymes and other nutritional strategies/intervention in enhancing the GIT health and function.

CHAPTER TEN

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