USE OF AN ENTEROTOXIGENIC *ESCHERICHIA COLI* ($K88^+$) PIGLET MODEL TO ASSESS THE EFFECTS OF FEED SUPPLEMENTS

BY

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ABSTRACT

Post-weaning diarrhea (**PWD**) is associated with high mortality and reduced performance, and is a major cause of economic loss in the swine industry. Enterotoxigenic *Escherichia coli* (**ETEC**) plays an important role in neonatal and post-weaning diarrhea of piglets. Antibiotics are usually added to the feed of weaned pigs to prevent PWD. With the ban of antibiotics as feed additives in Europe, more and more research focuses on developing effective additives as alternatives to antibiotics. This research was conducted to evaluate the effects of an antibiotic (**PC**), a direct fed microbial (**DFM**), spray-dried porcine plasma (**PP**), **PP** + **DFM**, and **PP** + a combination of these non-antibiotic additives(specialized porcine powder, organic acids, a sweetener, and DFM) (**PP + Blend**) on pig performance as well as microbial, immunological and metabolic parameters in an ETEC ($K88^+$) piglet infectious model. No dietary effects on pig performance in *E. coli* $K88^+$ disease challenge model. The fecal score of pigs fed DFM diet was significantly lower ($P < 0.05$) than those fed the NC, PP and PP + DFM diet and had no difference with PC diet at 22 hour after infection. Villous height in the duodenum of pigs fed the PP was greater ($P < 0.05$) than that of pigs fed the NC diet. On day 14, a greater IFN-γ ($P < 0.05$) concentration was observed in pigs fed the PP + blend diet compared with those fed the PC and PP + DFM diet. In conclusion, the DFM has potential to reduce scouring in *E. coli* $K88^+$ disease challenge model for weaned pigs. However more research need to be done about the effect of this probiotic alone or combined with other additives on performance of post-weaned piglets. No dietary effects were observed on pig performance in *E. coli* $K88^+$ disease challenge model. SDPP

improved gut structure, especially villous height, in *E. coli* $K88^+$ disease challenge model for weaned pigs. Combination of SDPP and DFM decreased the inflammatory responses of weaned piglets to some extent in an *E. coli* $K88^+$ disease challenge model. However, more detailed studies need to be conducted in adaptive and cellular immunity of the digestive tract.

DEDICATION

This thesis is dedicated to my parents, Xiaotian Cui and Chunhai Bai. Thank you so much for your support and belief in me. I love you both.

我的论文奉献给我致爱的父母.是你们的信任和支持让我成功完成了在异国它乡的学习.

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LIST OF ABBREVIATIONS

AA	Amino acid
AB	Antibiotic
ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Alpha-1-acid glycoprotein
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CD	Crypt depth
cGMP	Cyclic guanosine monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
CP	Crude protein
DAEC	Diffuse-adhering *E. coli*
DE	Digestible energy
DFM	Direct fed microbial
DM	Dry matter
DMI	Dry matter intake
EAEC (EAggEC)	Enteroaggragative *E. coli*
EAST1	Enteroaggregative *E. coli* heat stable enterotoxin I
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*, that do not form exterotoxins and are not invasive
ETEC	Enterotoxigenic *E. coli*
GE	Gross energy

G:F ratio	Gain: Feed ratio
GIT	Gastrointestinal tract
IgG	Immunoglobulin G
IFN-γ	Interferon gamma
LPS	Lipopolysaccharide
LT	Heat labile enterotoxin
NSP	Non-starch polysaccarides
PUN	Plasma urea nitrogen
PWD	Post-weaning diarrhea
SDAP	Spray-dried animal plasma
SDBP	Spray-dried bovine plasma
SDPP	Spray-dried porcine plasma
SDSM	Spray-dried skim milk
SPP	Specialized porcine powder
STa	Heat stable enterotoxin 'a'
STb	Heat stable enterotoxin 'b'
TNF-α	Tumor necrosis factor-alpha
VFA	Volatile fatty acid
VH	Villous height

CHAPTER ONE

LITERATURE REVIEW

Post-weaning Diarrhea and Influence on Pig Performance

Diarrhea is one of the most common diseases of neonatal and weaned pigs worldwide. Post-weaning diarrhea usually develops 3 to 10 days after weaning (Hampson, 1994) and typically infects large numbers of pigs. It was first described as being associated with the proliferation of beta-hemolytic *E. coli* in the proximal small intestines of infected pigs in Canada (Richards and Fraser, 1961). Post weaning diarrhea (PWD) is characterized by yellowish or grey fluid-like feces, lasting for a week or more, and it causes progressive loss of weight (Richards and Fraser, 1961). Affected pigs appear depressed, lose appetite, and have a rough hair coat. Some become pot-bellied, suffer from tremors, and typically die within a few days if they do not go into remission. Group mortality rates can reach 25% in the absence of adequate medication (Richards and Fraser, 1961).

The 2000 annual report of the Canadian research network on bacterial pathogens of swine indicated that the Canadian swine industry produced 24.1 million pigs in 2000 (Jacques, 2001). It has been estimated that every year up to 80 million dollars in losses to the swine industry may be attributed to infectious diseases (Jacques, 2001). Based on these findings and the fact that antibiotics are being removed as the primary prophylactic treatment, alternative antimicrobial treatments are needed.

Major Causes of PWD

Although specific serotypes of *E. coli* play an important role in the etiology of PWD, the causations are highly complex and multifactorial. However, the exact mechanisms of pathogenicity leading to PWD remain unclear. It has been recognized that hemolytic *E. coli* is the primary cause of PWD (Nagy and Fekete, 1999). Other factors such as changes in diet, and environmental temperature, may also contribute to PWD (Shimizu and Terashima, 1982). Some feed ingredients, such as soybean, and high levels of protein in the diet are considered harmful to weaner pigs as this predisposes them to ETEC (Li et al., 1990; Li et al., 1991; Prohaszka and Baron, 1980). In addition, complex sources or a large number of sources of protein in the diet may increase the severity of diarrhea compared with a diet that contains only a few sources of protein (Etheridge et al., 1984). Management risk factors associated with PWD are chronic moderately cold temperatures (18 to 20 °C) in the nursery system (Le Dividich et al., 1980), continuously fluctuating temperature (23.5 ± 3 °C) (Le Dividich, 1981), inadequate hygiene of the nursery and lower feed intake during the first post-weaning week (Pluske et al., 1997). All of the factors mentioned above demonstrate how complex the etiology of PWD is.

Escherichia coli

Escherichia coli is a ubiquitous organism in the normal enteric flora of pigs (White, 2006). It can be classified by serotypes: O (somatic lipopolysaccharide), F (fimbrial), K (capsular), and H (flagellar) surface antigens (White, 2006). However, serotyping is expensive, lacks sensitivity and specificity and can be conducted reliably

only in a small number of reference laboratories (Nataro and Kaper, 1998). In order to differentiate diarrheagenic *E. coli* strains from non-pathogenic members of the normal flora, and since serotypic markers are not sufficient to identify a diarrheagenic strain, the detection of diarrheagenic *E. coli* focuses on the identification of characteristics which determine the virulence of these organisms (Nataro and Kaper, 1998). The requisite procedure of infection of pathogenic *E. coli* is: colonization of a mucosal site, evasion of host defenses, multiplication and host damage (Nataro and Kaper, 1998). Once colonization on the intestinal mucosal surface has been established, the pathogenic strategies of the diarrheagenic *E. coli* strains show remarkable variety (Nataro and Kaper, 1998). In this way, six groups of diarrheagenic *E. coli* strains are divided by pathogenic mechanisms. Enterotoxigenic *E. coli* (ETEC) is defined as *E. coli* strains that produce at least one member of two defined groups of enterotoxins: ST and LT. Five other distinct classes are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggragative *E. coli* (EAEC or EAggEC), enteroinvasive *E. coli* (EIEC), and diffuse-adhering *E. coli* (DAEC) (Nataro and Kaper, 1998). A summary of their characteristics is listed in **Table 1**.

Characteristic of E. coli Involved in Post-weaning Diarrhea.

Enterotoxigenic *E. coli* strains are one of the most important causes of diarrhea in both neonatal and weaned pigs (Nagy and Fekete, 1999). Enterotoxigenic *E. coli* adheres to the small intestinal microvilli without inducing morphological lesions and

Table 1 A summary of major virulence factors and pathogenic strategies of diarrheagenic *E. coli* strains, and animals typically infected [a,b]

Category	Virulence Factors	Pathogenic strategies	Typical infected animals
EPEC	EPEC adherence factor (EAF) plasmid eae gene bundle-forming pilus (BFP) enterotoxin:EAST1	1. localized adherence 2. signal transduction 3. intimate adherence	Weanling rabbits
EHEC	eae gene Shiga toxin (Stx) Plasmid	1. localized adherence 2. signal transduction 3. intimate adherence	Calves (hemorrhagic colitis)
EIEC	Actin-based motility: intercellular spread A (icsA)	1. epithelial cell penetration 2. lysis of the endocytic vacuole 3. intracellular multiplication 4. directional movement through the cytoplasm 5. extension into adjacent epithelial cells	Guinea-pig
EAEC	Plasmid	1. localized adherence	Rabbits and rats

	enterotoxin:EAST1	2. enhance mucus secretion from the mucosa	
		3. trap bacteria in a bacterium-mucus biofilm	
		4. elaborate exterotoxin	
ETEC	Adhesive fimbriae	1. colonize the surface of small bowel mucosa,	Piglets (PWD)
	enterotoxins: ST, LT	2. elaborate exterotoxin	
		3. increase a net secretory state	

[a] Adapted from Nataro and Kaper's review (1998).

[b] DAEC was not in the list, because little was known about DAEC. It has only been found in human beings (children) (Nataro and Kaper, 1998).

produces enterotoxins that act locally on enterocytes (Nagy and Fekete, 1999). The action results in hypersecretion and reduced absorption of water and electrolytes (Nagy and Fekete, 1999). During PWD, viable counts of hemolytic *E. coli* in the anterior or jejunum of affected pigs are 10^3 to 10^5 greater than they are in healthy weaning pigs (Svendsen et al., 1977). These bacteria are closely associated with the intestinal mucosa, and possess specific adhesive factors which prevent them from being dislodged. Adhesions and toxins are two prominent virulence factors of ETEC. Enterotoxigenic *E. coli* strains that cause PWD belong to a limited range of serotypes. The O serogroup is the most frequently involved with PWD; the O serogroup includes O8, O141, O138, O147, O149 and O157. The predominant serotype in most countries seems to be O149:$K88^+$ (Hampson, 1994). The fimbriae $K88^+$ has been renamed F4, and was found to be particularly associated with O149 (Tzipori, 1985). $K88^+$ ETEC may represent about 40 to 60% of the *E. coli* strains causing diarrhea in piglets (Hampson, 1994). These fimbriae can exist as antigenic variants, $K88^+$ab, $K88^+$ac and $K88^+$ad, all of which allow adhesion to villous enterocytes throughout the small intestine (Hampson, 1994). Other fimbriae (pili) on porcine *E. coli* include K99 (F5), 986P (F6) and F41. However, they are not commonly associated with PWD strains (Hampson, 1994).

Mechanism of Enterotoxigenic E. coli Associated with PWD.

Loss of protection from milk antibodies appears to contribute significantly to the susceptibility of weaned piglets to pathogenic *E. coli* (Deprez P et al., 1986). Pathogenic *E. coli* causing PWD enter animals by ingestion of feed, water or by other pathways.

Thereafter, ETEC proliferate in sufficient numbers, and colonize the small intestine by attaching to specific receptors on the small intestinal epithelium or in the mucus coating of the epithelium with their specific fimbrial adhesions (Fairbrother et al., 2005). $K88^+$ ($F4^+$) fimbriae mediate bacterial adherence to the intestinal epithelium throughout most of the small intestine, the whole length of the jejunum and ileum in pigs of all ages. Receptors specifically for $K88^+$ are fully expressed from birth to adult age (Nagy et al., 1992).

A significant symptom of PWD is a dramatic increase in excreted fluid volume in the bowel, due either to a failure of the bowel to reabsorb or absorb fluid or to a great increase in fluid secreted into the bowel (Fairbrother et al., 2005). The action of one or a combination of enterotoxins produced by the bacteria that have colonized the small intestine plays a major role in inducing PWD. As illustrated in **Figure 1**, LT binds to receptors on the surface of intestinal epithelial cells which then leads to an accumulation of cyclic adenosine monophosphate (cAMP) in the enterocyte (Fairbrother et al., 2005). However, cAMP-stimulated protein kinase A (**PKA**) phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR), thereby causing chloride secretion from the apical region of the enterocytes (Haan and Hirst, 2004; Thiagarajah and Verkman, 2003).

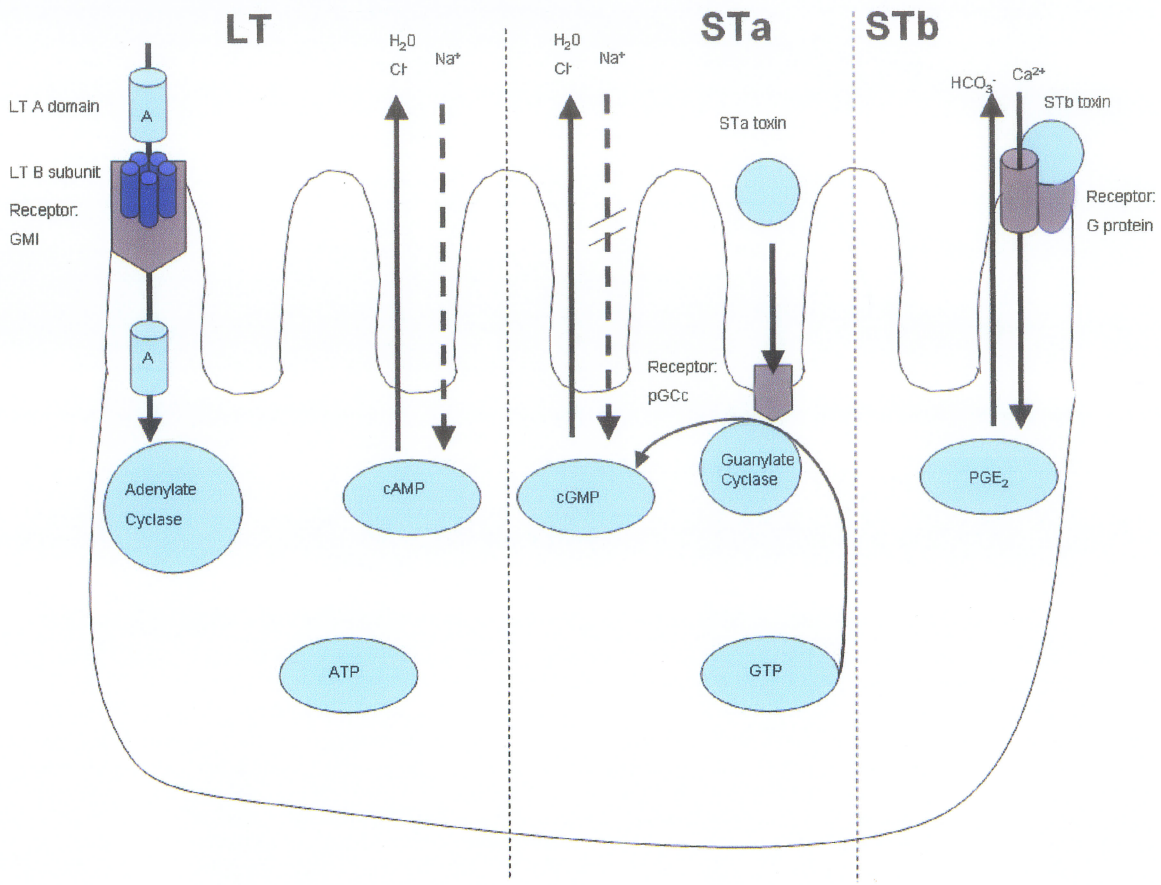

Figure 1 A schematic representation of mechanisms of action in heat-labile (LT) and heat-stable (STa and STb) enterotoxins of enterotoxigenic *Escherichia coli* (ETEC) of farm animals (Nagy and Fekete, 1999)

Heat stable exterotoxin 'a' (STa) toxin causes excessive levels of cGMP in enterocytes. The accumulation of cGMP leads to activation of CFTR and to increased secretion of Cl^- ions from crypt cells and inhibition of Na^+ and Cl^- absorption from cells at the tips of villi (Forte et al., 1992). Fluid accumulation in the lumen of the intestine in response to STa is also known to be under nitric oxide-dependent neuroendocrine influence. Mourad and Nassar (2000) inferred that nitric oxide and VIP (vasoactive intestinal peptide, which are known to have interactions) interact in promoting secretion by STa.

The mechanisms whereby the toxins STb and EAST1 induce diarrhea are not well understood. It is known that the mechanisms of STb toxins are not dependent on the elevation of cyclic nucleotides that characterizes the actions of LT and STa (Fairbrother et al., 2005). Enteroaggregative *E. coli* heat stable enterotoxin 1 (EAST1) has been shown to be widely distributed among *E. coli* strains isolated from piglets with PWD, especially among $K88^+$ ($F4^+$) ETEC of O149 (Osek, 2003). LT , $K88^+$ ($F4^+$) and EAST1 are a common combination in porcine ETEC (Osek, 2003; Yamamoto and Nakazawa, 1997). The *astA* gene encoding EAST1 has been detected to be widespread among porcine ETEC (Yamamoto and Nakazawa, 1997).

Challenge model of PWD

Due to the complexity of PWD, only a few serotypes of ETEC have been used in experimental replication of farm PWD incident in swine production. K88 is the major strain used in the experimental model of replicating PWD. A series of excellent PWD

reproducing experiments were done with SPF pigs by Madec and his colleagues (Madec et al., 2000). Madec's research group successfully induced short-term diarrhea in about 50% of 124 piglets that were challenged with ETEC (Madec et al., 2000). Diarrhea (average 1.7 days), low weight gain and depression were observed in inoculated groups (Madec et al., 2000). The greater dose, 10^{12} colony forming units (cfu)/mL, induced death in 10 of 16 piglets. A number of researchers successfully reproduced PWD by using 10^{10} cfu/mL of ETEC $K88^{+}$ (Bosi et al., 2004; Owusu-Asiedu et al., 2003). In some studies, clinical signs cannot be provoked by an oral challenge alone and stressors, such as cold stress, are also introduced into those experimental models (McDonald et al., 1999; Van Dijk et al., 2002). *Escherichia coli* shedding, weight loss and diarrhea are major symptoms of PWD. **Table 2** lists a few experiments involving PWD-reproducing models with ETEC K88. All the authors successfully replicated PWD despite differences in the weaning age of the piglets orally infected with *E. coli* K88 (10^{10} cfu/mL).

Prevention and Control of PWD

There are two major approaches to preventing PWD. One attempt is to minimize factors that predispose weaned pigs to PWD. Weaning age and weight, weaning diet, stocking rate, temperature in the nursery, and environmental contamination should be taken into account to reduce the incidence of the diarrhea disease (Hampson, 1994). For example, body weight at weaning is one of the critical factors determining the post-weaning performance (Lawlor et al., 2002). It is also positively correlated with birth weight (McConnell et al., 1987). A lag of 1.5 kg in pigs weaned at 21 days of age

Table 2 A summary of *E. coli* K88 challenge model in experiments testing feed additives to prevent PWD

E. coli strain	Dose (cfu/ ml)	Volume (ml)	Date of inoculum /weaning date(day)	Basic feed	Feed additives	Other stressor	Days diarrhea last(day)	Other syndromes	References
$K88^+$	10^{10}	N	22/21	corn	with/without oxytetracycline	cold (20 ± 2 °C)	3-4	acute, fulminating, and fatal diarrhea; shed *E. coli* K88	(Sarmiento et al., 1988)
K88ac	10^9	2	28/21	cereal	none	none	N/A	shed *E. coli* K88	(Jeyasingham et al., 1999)
K88; K87	$10^{8.5}$	50	25–30/23–28(2 days after weaning)(3 times at 24 hours	cooked white rice	with/without soluble NSP	none	2 to3	loss weight; shed hemolytic *E. coli*	(Van Dijk et al., 2002)

			intervals)						
K88	10^{10}	6	17/10 ± 1	wheat, whey, oat groats, soybean meal	SDPP, egg yolk antibody, ZnO, fumaric acid, antibiotics	none	3 to7	loss weight, shed *E. coli* K88	(Owusu-Asiedu et al., 2003)
K88	10^{10}	1	25/21	corn extruded, barely flaked, barley	SDP, fish protein	none	n/a	shed *E. coli* K88	(Bosi et al., 2004)

increased into a growth retardation of 8.6 days at slaughter (Wolter and Ellis, 2001). It is suggested that piglets with a birth weight above 0.9 to 0.95 kg have the ability to survive (Le Dividich et al., 2003). A stable ambient temperature of 26 to 28 °C was recommended for piglets penned on perforated floors during the first 10 to14 days after weaning (Madec et al., 2003).

The other approach is more specifically directed at the *E. coli*. This approach aims directly at reducing the attachment of large numbers of hemolytic *E. coli* in the intestine after weaning. Various additives, such as prophylactic antimicrobials, organic acids, probiotics, microencapsulated protease preparation, zinc oxide, cows' milk, and egg yolk containing specific antibodies against *E. coli* adhesions have all been employed by researchers to impede colonization of the intestine of the weaned pig by ETEC. To date, not a single strategy has proved to be totally effective. This suggests that the most successful approach will probably involve a combination of diet modification and other preventive measures.

Usage of Antibiotics and Problems

Antibiotics were used not only at therapeutic levels, but also at lower (sub-therapeutic) levels in feed for preventing diseases. Sub-therapeutic antibiotics improve growth rate and efficiency of feed utilization, reduce mortality and morbidity, and improve reproductive performance. So far, it is the most effective approach in preventing diseases in the swine industry. It is estimated that over the past 20 to 30 years, 70 to 80% of pig starters, 70 to 80% of grower feeds, 50 to 60% of finisher feeds, and 40 to 50% of

sow feeds contained antimicrobial agents (Cromwell, 2002). Cromwell's summary (2002) (**Table 3**) of more than a thousand experiments conducted in the United States between 1950 and 1985 on efficacy of antibiotics as growth promoters for pigs shows that antibiotics are more effective in improving growth and efficiency in young pigs than they are in growing–finishing period.

The small intestine is the principal site of nutrient and energy absorption. Cultured bacterial studies showed that micro-organisms in the small intestinal tend to compete with the host for energy and amino acids (Hedde and Lindsey, 1986). As much as 6% of the net energy in a pig's diet can be lost due to bacterial fermentation of feed (Hedde and Lindsey, 1986). One of the products of the fermentation is lactic acid in the small intestine which cannot be absorbed by host animals. Lactic acid reduces the availability of feed to host epithelia and increases the rate of nutrient transition through the intestine by enhancing peristalsis (Saunders and Sillery, 1982). The hypothesis of the mechanism of antibiotics is that it improves the efficiency of animal growth via the inhibition of the commensal microbiota, leading to increased nutrient utilization and a reduction in the maintenance costs of the GIT system (Gaskins et al., 2002).

Problems of resistance

Antibiotic substances as feed additives for promoting growth have been used in farm animal diets since the 1950s (Hardy, 2002). In the 1960s, antibiotic-resistant strains of *E. coli* were recovered from both healthy and diseased swine, poultry and cattle (Aden et al., 1969; Sojka and Carnaghan, 1961; Walton, 1966). One of the earliest reports,

Table 3 A summary of efficacy of antibiotics as growth promoters for pigs[a]

Stage	Control	Antibiotic	Improvement (%)
Starting phase, 7 to 25 kg			
Daily gain, kg	0.39	0.45	16.4
Feed : Gain ratio	2.28	2.13	6.9
Growing phase, 17 to 49 kg			
Daily gain, kg	0.59	0.66	10.6
Feed : Gain	2.91	2.78	4.5
Growing–finishing, 24 to 89 kg			
Daily gain, kg	0.69	0.72	4.2
Feed : Gain	3.30	3.23	2.2

[a] Source: Cromwell, 2002

published in the mid-1960s, found antibiotic-resistant *E. coli* in healthy pigs (8 to 20 weeks old) from farms in the northwest of England (Walton, 1966). Multiple-antibiotic-resistant strains of *E. coli* were obtained from 89 of the 105 swine fecal samples, with most being observed to be resistant to tetracycline, streptomycin, and sulfonamide (Walton, 1966). Numerous articles have been published over the past several decades describing antibiotic resistance among pathogenic and commensal swine *E. coli* isolates. In the late 1980s, an article reported the characteristics of antibiotic-resistant phenotypes among ETEC isolates of the O8 serogroup associated with young pig diarrhea in Quebec, Canada (Broes et al., 1988). Nearly 3 of the *E. coli* isolates demonstrated resistance to at least 4 antibiotics, and 11 strains were resistant to 6 or more antibiotics (Broes et al., 1988). Most of isolated *E. coli* were commonly resistant to streptomycin, tetracycline, and sulfonamides; however, all isolates were vulnerable to gentamicin and trimethoprim-sulfamethoxazole (Broes et al., 1988). A recent report documented the first gene-encoded resistance mechanism to the swine growth enhancer olaquindox among an *E. coli* isolate recovered from swine manure in Denmark (Hansen et al., 2004). The genetic elements (*oqx*AB) involved in resistance to olaquindox were subcloned and sequenced from a conjugative plasmid isolated from *E. coli*. The authors reported that plasmids which contained and expressed the *oqx*AB genes yielded high (>128 μg/ml) resistance to olaquindox in *E. coli*, as well as chloramphenicol (>64 μg/ml) (Hansen et al., 2004).

There has been great concern about antibiotic resistance in pathogenic bacteria since the publication of such findings. The major concern is that multi-antibiotic-resistant bacteria which are a result of the long-term use of growth promoting antibiotics as feed

additives might be transferred to human beings, resulting in serious health consequences. In the European Union (EU), the use of antibiotic substances in farm animals, especially as feed additives for growth promotion has caused much debate. Following the first ban on all growth-promoting antibiotics in Sweden in 1986, the EU banned avoparcin in 1997 and bacitracin, spiramycin, tylosin and virginiamycin in 1999, which has led to a diminution of resistance to avoparcin, macrolides and virginiamycin among Enterococci in animal feces, in Denmark and elsewhere (Casewell et al., 2003). However, experience in Sweden had shown that the bans might have adverse consequences on animal health and welfare and on human health, and might even result in economic loss for farmers (Acar et al., 2000; Casewell et al., 2003; Wierup, 2001).

Alternatives to antibiotics

As a result of political and economical pressure, the North American swine industry has planned to take action by looking for alternatives to antibiotics which can be used as growth promotants for pigs. The swine industry is a mainstay of Canadian agricultural exports and brings in approximately $1 billion per year (Reid and Friendship, 2002). If the same ban were imposed in the USA as has been imposed in Sweden, the production costs would increase by between $5.24 and $6.05 per head; net profit would decline by $0.79 per head. On the other hand, consumer costs would increase by 5 cents per pound (Hayes et al., 2001). However, if Canada's government does not have a policy limiting antibiotic usage, export of pork could ultimately decline.

There are several types of additives used or could be used as alternatives to

antibiotics in the swine industry. They are discussed below.

Probiotics

The widely accepted definition of a probiotic is "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance " (Fuller, 1989). Often-used probiotics are lactic-acid-producing bacteria species such as Bifidobacteria (e.g., *B. breve, B. infantis, B. liongum, B. bifidum* and *B. adolescensis*), Lactobacilli (e.g., *L. acidophilus, L. paracasei,* and *L. bulgaricus*), Enterococci (e.g., *E. faecium* and *E. faecalis*) and Saccharomyces(e.g., *S. boulardi* and *S. cerevisiae*) (Mosenthin and Zimmermann, 2000; Verstegen and Williams, 2002). The major selection criteria for probiotics are survival in the GIT with the ability of resistance to gastric acid and bile acids, and adherence to mucosal cells (Verstegen and Williams, 2002). To keep the use of probiotics safe, most of them were bacteria dominating in the host's GIT. The major bacteria species that have been cultivated or isolated from pig feces are *Streptococcus, Lactobacillus, Eubacterium, Bacteroides,* and *Peptostreptococcus* (Gaskins, 2001; Moore et al., 1987). Another study of microbial diversity in the mucosal layer of the pig colon demonstrated that streptococci and lactobacilli comprised the majority of isolates (54%) from the colon wall obtained by anaerobic culturing; however, this group accounted for only one-third of the sequence variation for the same sample obtained from the random cloning method (Pryde et al., 1999). *In vitro* studies have shown that two stains of lactobacilli inhibit the adhesion and growth of enteropathogens including *E. coli, Salmonella* and *Shigella* (Reid et al., 1993).

Another study also demonstrated that *Lactobacillus* strains produce proteinaceous substances that prevent attachment of *E. coli* to the mucosal surface in the ileum of pigs under *in-vitro* conditions (Blomberg et al., 1993). Ingestion of lactobacilli derived from the human intestine helped to reduce the duration of several types of diarrheal diseases caused by *E. coli* and reduced the activity of certain fecal enzymes of the intestinal flora, e.g., β-glucuronidase, urease, azo-reductase and nitro-reductase (Spanhaak et al., 1998; Verstegen and Williams, 2002).

One of the mechanisms of action of probiotics is bacterial antagonism. Probiotics produce inhibitory substances and by competitive exclusion prevent the attachment of pathogens to the gut wall (Kelly, 1998). They also modulate the immune system by improving systemic and mucosal immunity (Kelly, 1998; Mosenthin and Zimmermann, 2000). However, despite the beneficial effects that have been associated with probiotics for about a century, published studies show quite variable results in the swine industry. Studies of the performance response of sows and their litters to the probiotic strain *Bacillus cereus* var. *Bacillus toyoi* showed that BW of pigs in probiotic groups up to day 35 was greater than that of the control group and that both strains led to a reduction in the incidence of liquid feces and post-weaning diarrhea (Taras et al., 2005). Another experiment on the probiotic strain *Enterococcus faecium* NCIMB 10415 for piglets weaned at 28 days showed no overall differences in BW gain, feed intake, or feed efficiency between the probiotic experimental group and the control group (Taras et al., 2006). However, the percentage of post-weaning diarrhea infection in the probiotic group had reduced compared with that of the control group (Taras et al., 2006). Similarly, a

study of the same probiotic, *Enterococcus faecium* DSM 10663 NCIMB 10415 (EcF), showed a lower incidence of diarrhea in piglets fed this probiotic from birth to weaning compared with the control group (Zeyner and Boldt, 2006).The variable results may be due to different species of bacteria, dose levels, management of the animals, period administered and even feed processing (Fuller, 1999).

Prebiotics

A prebiotic is defined as 'a non-digestible dietary ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health' (Gibson and Roberfroid, 1995). Oligosaccharides, non-starch polysaccharides, sugar alcohols and fructo-oligosaccharides are all functional ingredients used as prebiotics (Crittenden, 1999; Verstegen and Williams, 2002). Bifidobacteria and lactobacilli are the dominant microflora in the colon which protect the host by preventing exogenous intestinal pathogens from establishing in the intestine (Crittenden, 1999). Prebiotics could promote rapid re-establishment of a normal microflora, thus decreasing the opportunity for a pathogenic organism to colonize in the intestine (Crittenden, 1999). A study showed that *Lactobacillus paracasei* combined with fructo-oligosaccharides as feed additives significantly increased the counts of lactobacilli, bifidobacteria, total anaerobes, and total aerobes compared with these counts in the control group (Nemcova et al., 1999). Fructo-oligosaccharides effectively accelerated the recovery of beneficial bacteria and potentially slowed down the recovery of pathogenic ones after acute secretory diarrhea in

cholera toxin challenged pigs (Oli et al., 1998). Further studies in animals are necessary before any accurate conclusions can be drawn.

Organic acids

Organic acids exist as constituents of normal animal and plant tissues in nature. Some organic acids, such as citric, formic, fumaric, lactic and propionic acids, and their salts, have been used as alternatives to antibiotics for a few years and have been reported to be beneficial in reducing the post-weaning lag period (Falkowski and Aherne, 1984; Tsiloyiannis et al., 2001).

The insufficiently developed digestive system of the early-weaned pig can barely handle the pressure of transition from milk to solid diets. The failure to maintain a low gastric pH by the hydrochloric acid secretion in the stomach has major implications for the poor performance of early-weaned pigs (Kidder and Manners, 1978). Lower pH, of 2 to 3.5, which is normal in mature pigs, optimizes the activities of pepsinogens. Conversely no pepsinogen activities were observed at pH > 6.0 (Taylor, 1959). Acid secretion in young pigs does not reach appreciable levels until 3 to 4 weeks post-weaning (Cranwell and Moughan, 1989). An elevated pH would, therefore, allow increased proliferation of *E. coli* which causes scour and mortality in post-weaned pigs (Smith and Jones, 1963). In addition, acid in the stomach also plays an important role in regulating gastric emptying by decreasing the digesta load in small intestine which also reduces the chance of supporting the proliferation of coliforms (Cornelius, 1988).

Based on the important value of gastric pH, organic acids have real potential to

be used as an alternative to feed-additive antibiotics. Hypotheses about the mechanisms of action of organic acids are mainly related to the reduction of gastric pH in pigs. Organic acids are expected to reduce gastric pH, scour and proliferation of coliforms and improve nutrient digestibility (Ravindran and Kornegay, 1993). In a recent study, it was demonstrated that the piglets whose diets were supplemented with different organic acids (1.0 - 1.6% of diet) had reduced incidence and severity of PWD and that they performed better than pigs that received no organic acids during the first 4 weeks after weaning (Tsiloyiannis et al., 2001).

Buffering capacity of the diets plays an important role in the response of weaned piglets to organic acids (Ravindran and Kornegay, 1993). Cereal or oilseed meal diets without milk products showed better response than those with milk products in weaned piglets (Ravindran and Kornegay, 1993). The age of piglets is another factor influencing response to organic acids. Several studies showed great response of piglets to organic acids added within the first 3 weeks post weaning, but little or no response is observed after 4 weeks (Risley et al., 1992; Tsiloyiannis et al., 2001). The age-dependent response to organic acid is due to the maturity of the GIT. At a very young age, especially immediately after weaning, pigs had limited capacity to secret gastric acids needed to maintain low enteric pH (Ravindran and Kornegay, 1993).

Spray-dried Plasma

Spray-dried animal plasma, mostly of porcine or bovine origin, is widely used as an additive in weanling pig diets to improve feed intake and reduce post-weaning

diarrhea. A study found that SDPP containing feed was more efficient in enhancing growth rate and feed intake in the conventional nursery than spray-dried skim milk for pigs 3 to 4 weeks after weaning (Coffey and Cromwell, 1995). This result indicated that SDPP can be an effective alternative to spray-dried skim milk (SDSM) for weanling pigs. Recent research demonstrated the positive effects of both SDPP and SDBP on pig performance, growth rate and feed intake, during the first week after weaning as a result of the Ig G fraction of plasma (Pierce et al., 2005). One possible mode of action of SDAP is the immunoglobulins and insulin-like growth factor-I content which prevent viral and bacterial infection by influencing intestinal mucosal function and gastrointestinal growth (Van Dijk et al., 2002). Coffey and Cromwell's research results supported this model (Coffey and Cromwell, 1995). Though a recent study showed that SDAP enhanced the growth of lactobacilli in the ileum and the cecum, SDAP had no effect on the integrity of the intestinal mucosa of pigs compared with colistin sulfate and control groups in an *E. coli* challenged model (Torrallardona et al., 2003). SDPP should be investigated as a means of preventing disease in pigs at weaning. Exciting results reported by Owusu-Asiedu et al. (2002) showed that a SDPP-based diet increased weight gains and lowered frequency of scours in early weaned pigs (Owusu-Asiedu et al., 2002). Another recent study also showed that, in addition to improve growth performance, a non-medicated SDPP diet protected F4-receptor-positive pigs against ETEC infection, inhibited excretion of ETEC, and reduced *E. coli*-induced inflammatory status in weaned pigs challenged by enterotoxigenic *E. coli* $K88^{+}$ (Bosi et al., 2004). However, SDPP is an expensive strategy for the controlling of PWD in pig production; the effective quantity

used in production was summarized as 6% SDAP in diet in a review (Van Dijk et al., 2001). Their continued study evaluated the effects of 8% SDPP diet in pigs in an *in-vivo* ETEC challenge model. The results show SDPP did not prevent infection in post-weaned, ETEC-challenged pigs, although substantially greater ADG and ADFI were observed in SDPP group (Van Dijk et al., 2002).

Summary of Literature Review

ETEC-induced PWD is an important cause of decreased growth rate, high mortality, morbidity and extra medication costs in weaned pigs. Recently, an increase in the incidence of outbreaks of severe *E. coli*-associated diarrhea has been observed worldwide. Traditionally, antibiotics are added to feed as growth-promoting additives and also to prevent diseases in the swine industry. With the ban of antibiotics as feed additives in Europe and the development of multiple bacterial resistances to a wide range of commonly used antibiotics, the search for effective alternatives to antibiotics is receiving more and more attention.

New approaches to controlling PWD include supplementation of the feed with egg yolk antibodies from chickens immunized with F4 or F18 adhesins, breeding of F4- and F18-resistant animals, supplementation with zinc oxide and /or spray-dried plasma, probiotics, prebiotics, or dietary acidification. Thus far, not a single strategy has proved to be totally effective. It is probable that the most successful approach will involve a combination of different diet modifications and other preventive measures.

Spray-dried porcine plasma, probiotics and acidifiers have been proposed as

effective means to promote the proliferation of lactobacilli or prevent the growth of *E. coli* in the mucosal layer of the pig's intestinal tract to control PWD in early-weaned pigs. They also modify the bacteria community in GIT in different ways. However, more research on their individual or combined efficacy compared with antibiotics is needed.

In this project, effectiveness of probiotic, SDPP, and organic acids or their combination will be evaluated in an *E. coli* challenge model as an alternative to antibiotic growth promoter in weaned pig diets.

CHAPTER TWO

USE OF AN ENTEROTOXIGENIC *ESCHERICHIA COLI* ($K88^+$) PIGLET MODEL TO ASSESS THE EFFECTS OF FEED SUPPLEMENTS

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ABSTRACT

Post-weaning diarrhea (PWD) induced by enterotoxigenic *E. coli* (ETEC) causes growth lag, high mortality, and economic loss in the swine industry. One hundred and eight 17-day-old weaned pigs were assigned to 6 dietary treatments in a completely randomized design to give 6 replicate pens per treatment with 3 pigs per pen. The diets were based on a corn-soybean meal and included a positive control with antibiotics (PC), or spray-dried porcine plasma (PP); or a direct fed microbial (DFM); or both (PP + DFM); or PP plus a combination of the non-antibiotic additives (DFM + organic acids + specialized porcine powder + a sweetener) (PP + Blend). Blood samples were taken on days 7 and 14 for determination of plasma urea N (PUN), ammonia nitrogen, IFN-γ and TNF-α. Alpha-1-acid glycoprotein (α-AGP) was only measured on day 14. All pigs were orally challenged with a 6 mL dose of 10^{10} colony forming units / mL of ETEC ($K88^{+}$) on day 8. Piglets were weighed on days 0, 7, and 14 of the experiment. On day 14, 6 pigs per treatment (one per replicate) were euthanized, and duodenum, jejunum and ileum samples were collected for histological assays, microbiological analysis, VFA, pH analysis. Average daily feed intake (ADFI), average daily gain and G : F ratio were similar ($P > 0.05$) for all treatments. Significant lower ($P < 0.05$) fecal scour was detected in pigs fed DFM diet compared with those fed NC, PP, or PP + DFM. Approximately 33% of pigs fed the NC diet died. The lactic acid bacteria count in the colon of PC fed pigs was significantly ($P < 0.05$) lower than in the colon of those fed the PP, PP + DFM, or DFM diets. Pigs fed PP had longer villi ($P < 0.05$) than those fed the NC diet. No diet effects were observed on VFA ($P > 0.05$) concentration and intestinal pH ($P > 0.05$)

value. On day 14, a greater IFN-γ ($P < 0.05$) concentration was observed in pigs fed the PP + Blend diet compared with those fed the PC and PP + DFM diet. In conclusion, no dietary effects on performance were observed in *E. coli* $K88^+$ disease challenge model for weaned pigs. DFM has potential to reduce post-weaning pigs scouring even though there were no improvements in performance. Spray dried porcine plasma improved gut health, especially villous height, of *E. coli* $K88^+$ disease challenge weaned pigs. Combination of SDPP and DFM decreased the inflammatory responses of weaned piglets to some extent in our *E. coli* $K88^+$ disease challenge model. However, more detailed studies need to be conducted in adaptive and cellular immunity of the digestive tract.

Key words: Probiotic, Spray-dried porcine plasma, Organic acids, Antibiotic, Post-weaning diarrhea, Pig, *Escherichia coli.*

INTRODUCTION

Post-weaning diarrhea (PWD) is a major cause of economic loss in the swine industry. It is recognized that enterotoxigenic *Escherichia coli* (ETEC) is the leading cause of diarrhea in early-weaned pigs (Nagy and Fekete, 1999). Subtherapeutic antibiotics are the most effective means of preventing PWD in young swine. With the emergence of antibiotic-resistant bacteria and the pressure to ban the use of growth-promoting antibiotics in Europe, more and more researchers are focusing on alternatives to antibiotics. Most recent research showed positive effects of both spray-dried porcine plasma (SDPP) and spray-dried bovine plasma (SDBP) on pig performance, growth rate and feed intake (Pierce et al., 2005). The SDPP reduced *E. coli* induced TNF-α in weaned piglets challenged by ETEC $K88^+$ but this depended on protein source (Bosi et al., 2004). Probiotic additives are reported to effectively reduce diarrhea in weaned pigs. Zeyner and Boldt (2006) demonstrated that, in piglets suffering from diarrhea, an oral dose of *Enterococcus faecium* given as a probiotic from birth to weaning reduced the rate of diarrhea. Mixed organic acids used in the study of Tsiloyiannis et al. (2001) improved all performance parameters.

Additives, probiotics, SDPP and organic acids mentioned above all may potentially be used as alternatives to antibiotics. As demonstrated by the highly variable results reported in different studies, none of these additives alone showed completely stable effect when competing with antibiotics. We were interested in testing additives SDPP, probiotics and organic acids in various combinations to see if they are effective in improving performance and preventing diarrhea in weaned pigs as alternative to

antibiotics. The objective of the current research was to evaluate the use of a soybean-meal basal diet supplemented with SDPP (PP), a direct feed microbial (DFM), SDPP + DFM (PP + DFM), and a combination of non-antibiotic additives [(SDPP + DFM + organic acids + SPP + a sweetener] (PP + Blend) on performance and gut microbial population and metabolites, gut morphological and immunological responses compared with antibiotics (PC) and the basal diet (NC) when the animals were challenged with ETEC $K88^{+}$.

MATERIAL AND METHODS

Animal Care, Housing, and Experimental Design

The use of pigs and experimental protocol were approved by the University of Manitoba Animal Care Committee and pigs were managed according to the guidelines of Canadian Council on Animal Care (CCAC, 1993). A total of 108 Cotswold piglets obtained from the University of Manitoba's Glenlea Swine Research Farm, weaned at 17 ± 1 days of age (5.91 ± 0.1 kg initial BW) and balanced for initial BW and litter of origin were used in a 14-day trial. Piglets were housed in plastic sides and plastic-covered expanded metal sheet flooring in a temperature maintained room (29 ± 1 °C) throughout the study. Due to the limitation of facilities, the experiment was conducted in 2 blocks (54 pigs per block). Animal care, housing, and experimental procedure were the same in the 2 blocks. In each block, piglets were randomly allotted to each of 6 dietary treatments in a completely randomized design. Three pens (3 pigs / pen) in each block were assigned to each treatment.

Diets, Feeding, and Experimental Procedure

Direct-fed microbials (DFM), antibiotics (Asp250), organic acids (PROMOTE ProAcid AD 201) and spray-dried porcine plasma (SDPP) were all obtained from Cargill Animal Nutrition (Minneapolis, Minnesota, USA). Asp250 is a commonly used antibiotic in swine industry. It widely kills both gram positive and negative bacteria. Corn and soybean meal provided the major energy and protein source in all 6 diets. One experimental diet was the basal diet (NC diet). The other 5 were: basal diet + antibiotic (PC); basal diet + bacillus direct fed microbial (DFM); basal diet + spray-dried porcine plasma (PP); basal diet + DFM + SDPP (PP + DFM); and basal diet + DFM, SDPP, SPP, organic acids and a sweetener (PP + Blend). The specialty ingredients were added at levels similar to those generally used in industry. All experimental diets were formulated to meet (NRC, 1998) nutrient requirements for piglets weighing 7.0 to 12.0 kg BW **(Table 4)**. Pigs had *ad-libitum* access to feed and water throughout the experiment. The ADG, ADFI, and feed conversion ratio (G : F) were determined weekly. On days 0, 7, and 14, blood samples (10 mL) were collected from all pigs via jugular vein puncture with sodium heparinized vacuum container tubes (Becton Dickinson, Rutherford, NJ), and immediately centrifuged at 2,000 xg for 10 minutes at 5 °C to recover plasma, which was stored at –20°C for further analysis. Likewise, on days 7 and 14, blood samples were collected into vacuum container tubes without additive and centrifuged at 3,000 xg for 20 minutes at 5°C to obtain serum. Serum samples were stored at –20 °C until α-1-acid glycoprotein (α-AGP), interferon gamma (IFN-γ), and tumor necrosis factor alpha

Table 4 Composition (g/kg, as fed basis) and nutrient analysis (g/kg, dry matter basis)[a] of experimental diets

Item	Diet[b]					
	NC	PC	PP	PP + DFM	DFM	PP + Blend
Corn	36.65	35.55	41.16	42.11	36.60	41.12
Soybean meal, 48% CP	28.00	28.00	22.00	28.00	28.00	22.50
Lactose	10.00	10.00	10.00	10.00	10.00	10.00
Dried whey	10.00	10.00	10.00	10.00	10.00	10.00
Menhaden fish meal	7.00	7.00	5.00	5.00	7.00	5.00
Oat groats	7.00	7.00	3.00	3.00	7.00	3.00
Biophos	0.35	0.35	0.80	0.80	0.35	0.80
Limestone	0.53	0.53	0.60	0.60	0.53	0.60
Iodized salt	0.25	0.25	0.25	0.25	0.25	0.25
Veg. oil	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[c]	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[d]	0.05	0.05	0.05	0.05	0.05	0.05
Lysine-HCl	0.07	0.07	0.01	0.07	0.07	0.01
SDPP	-	-	5.00	5.00	-	5.00
Methionine	-	-	0.03	0.03	-	0.03
Aureo S-P250G	-	0.10	-	-	-	-
Premix[e]						
DFM[f]	-	-	-	0.05	0.05	0.05

Luctarom[g]	-	-	-	-	-	0.04
Pro acid[h]	-	-	-	-	-	0.35
SPP[i]	-	-	-	-	-	0.10
DE, MJ/kg	15.0	15.0	14.9	14.9	15.0	14.8
CP,%	22.83	22.83	22.70	22.70	22.83	22.85
lysine, %	1.47	1.47	1.48	1.48	1.47	1.49

[a] Values are a mean of analyzed composition of the diets mixed for block 1 and 2.

[b] Diets: negative control (NC) = basal diet with no spray-dried porcine plasma (SDPP) or antibiotic; positive control (PC) = NC + antibiotics; DFM = negative control + direct fed microbial; PP = NC + SDPP; PP + DFM = PP + direct fed microbial; PP + Blend = PP + blend of direct fed microbial, organic acids and a sweetener.

[c] Provided per kilogram of complete diet: 18 mg copper, 110 mg zinc, 0.2 mg iodine, 50 mg iron, 50 mg manganese, 0.3 mg selenium.

[d] Provided per kilogram of complete diet:10 000 IU vitamin A, 1600 IU vitamin D, 120 000 IU vitamin E, 3.5 mg vitamin K, 0.035 mg B12, 0.1 mg biotin, 1.1 mg folic acid, 60 mg pantothenic acid, 2 mg pyridoxine B6, 8 mg riboflavin, 1.5 mg thiamin.

[e] Content: 110 g / MT Chlortetracycline, 55 g / MT Penicillin (as penicillin G Procaine), 110 g / MT Sulfamethazine.

[f] Direct fed microbial: calcium carbonate, *Bacillus subtilus* (124 billion cfu / kg), rice hulls.

[g] Luctarom sweet 500: Sodium saccharin.

[h] Pro acid 201: mixture of calcium propionate, fumaric acid, sodium benzoate, rice hulls,

mineral oil, and amorphous silicon dioxide.

[i] Specialized porcine powder: high protein meal with CP content of 70%

(TNF-α) were analyzed. Commercial test kits were used for AGP (Cardiotech Services, Inc., Louisville, KY), IFN-γ and TNF-α (Pierce Endogen, IL, USA) concentration measurement.

Bacteria Culture, Oral Challenge, and Health Status

The pure strain of ETEC expressing the $K88^+$ (F4) fimbria (P97-2554B, serotype O149:K91:F4 [$K88^+$]) used in the current study was obtained from Dr. Marquardt (Winnipeg, MB, Canada) which was the same strain used by Owusu-Asiedu et al. (2003). *E. coli* $K88^+$ challenge solution was prepared as described by Owusu-Asiedu (Owusu-Asiedu et al., 2003). Briefly, ETEC $K88^+$ was grown overnight in blood agar plate (Atlas Laboratories Co. Ltd. Winnipeg, MB, Canada) at 37° C using 1% inoculum from stock. Cells were washed twice with 2mL sterilized saline solution (0.9%, pH 7.2), and then the suspension (10^{10} cfu/mL) used for oral challenge. On d 8 of the experiment (25-d-old pigs), each pig received 6 mL of enterotoxigenic *E. coli* $K88^+$ culture in a syringe attached to polyethylene tube held in the oral cavity. Severity of diarrhea was characterized by using the fecal consistency score system described by Marquardt et al. (Marquardt et al., 1999). The health status of pigs was assessed using fecal consistency scoring (0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea). The assessment was performed by two trained personnel who had no prior knowledge of dietary treatment allocation. The total number of days that signs of scours were present in the pens was determined and is expressed as scouring days. No therapeutic antibiotics were given during the whole experiment.

Histological and Other Measurements

On day 15, 3 pigs per treatment, selected randomly from 3 of the 6 pens per treatment, were held under general anesthesia and euthanized by an intracardiac injection of sodium pentobarbital (50 mg/kg BW), and the weights of visceral organs and morphology of the gastrointestinal tract were determined.

Stomach, spleen, small intestine, and liver were removed, flushed with ice-cold phenylmethyl sulfonyl fluoride saline (2 L of 0.9% saline, pH 7.4 + 2 mL of 100 mM phenylmethyl sulfonyl fluoride), and 20 mL of digesta each from the stomach and the small intestine were obtained for pH measurement. Segments of intestine (duodenum, jejunum, ileum and colon) were taken for histology measurements. Ten centimeter segments of duodenum, jejunum and ileum was removed and stored in 10% neutral buffered formalin to fix the villi and the crypt for subsequent histological measurement. Six cross-sections were obtained from each formalin-fixed segment and processed for histological examination using the standard hematoxylin and eosin method. Villous height was measured from the tip to the base and crypt depth measured from the crypt-villous junction to the base on 10 well-oriented villi per specimen using a Zeiss photomicroscope equipped with a Sony 3-chip CCD color camera. The images were captured using Empix's Northern Eclipse Image Processing Software (Empix Imaging, Inc., Mississauga, ON, Canada) and measured by NIH image Software (Scion Corporation, Frederick, Maryland, USA).

Bacteria Count

Ten-centimeter colon and ileum section samples were collected in Petri dishes. The section was cleaned with sterilized peptone water (0.1%). The mucosa was then scraped with a blunt sterilized knife. A 1- to 2-g mucosa sample was diluted 10 times with sterilized peptone (0.1%). Ten millilitres of serial dilution (1 to 10 times) were cultured on chromogenic agar (Oxoid Ltd, England) and MRS agar (Marine BioProducts International Corporation, Canada) for *E. coli* and lactic acid bacteria counting at 30°C for 24 h and 48 h, respectively.

Chemical Analyses

All experimental diets were ground through a 1-mm screen (Cyclotec 1093 sample mill, Tecator, Hoganas, Sweden) prior to analysis. Samples were dried in a convectional oven at 105°C for 16 h for DM determination, whereas CP (N x 6.25) content was determined using Leco NS 2000 nitrogen analyzer (Leco Corp., St. Joseph, MI). A 100-mg sample was prepared for acid hydrolysis according to AOAC (1984) and analyzed for AA as modified by Mills et al. (1989). The method involved digestion in 4 mL of 6N HCl for 24 h at 110°C followed by neutralization with 4 mL (wt/vol) of NaOH and cooling to room temperature. The mixture was then made to 50 mL volume with sodium citrate buffer (pH 2.2). Methionine and cysteine were analyzed as methionine sulfone and cysteic acid, respectively, after oxidation with performic acid. Amino acids were then analyzed using a LK 4151 Alpha analyzer (LKB Biochrom, Cambridge, U.K.). Plasma urea N (PUN) was analyzed by a Nova Stat profile M blood gas and electrolyte

analyzer (Nova Biomedical Corporation, Waltham, MA). Ammonia N concentration in digesta samples was determined using the method described by Novozamsky et al. (1974). Volatile fatty acid in digesta samples were determined according to the gas chromatographic methods described by Erwin et al. (1961).

Calculations and Statistical Analysis

Villous height and crypt depths were determined by averaging the individual measurements in similarly treated pigs. Mean villi height and crypt depth were obtained by averaging the measurements from three pigs. Ten enteric villi and crypt were measured for the same section.

The ADG was calculated based on surviving pigs. The ADFI was calculated as follows: (total feed given – feed weighed back) / Σpidi where pi and di are individual pigs and the number of days in the pen, respectively.

Data were analyzed as a repeated measure design using the Proc Mix procedures of SAS (SAS Inst., Inc., Cary, NC). The Proc Mix model includes treatment (n = 6) and block (n = 2) effects as sources of variation. Pen was considered the experimental unit for all parameters measured. When a significant F-value ($P < 0.05$) for treatment means was observed in the ANOVA, treatments were compared using Tukey's multiple range-test, and the same was used to test IFN-γ and TNF-α, respectively, pre and post challenge. Mortality was compared using Chi-square test.

RESULTS

Animal Performance

The analyzed chemical composition of the experimental diets is shown in **Table 5**. The analyzed CP values of the diets were slightly lower than the formulated values. Treatment effects on ADG, ADFI, and G : F ratio during week 1 (pre-challenge, d 0 to 7), week 2 (post-challenge, d 8 to 14), and overall (d 0 to 14) are presented in **Table 6**. The ADG and ADFI for piglets fed PC diet compared with those of piglets fed other additive diets were not different ($P > 0.05$) during each week in the two-week experimental period. The overall ADG of piglets fed the PP + Blend diet tended to be greater ($P = 0.08$) than that of piglets fed DFM diet. The overall ADG of piglets fed the blend diet during the two weeks increased by 61.70 % compared with that of piglets fed the NC diet. The overall ADG of piglets fed the PP + Blend diet was numerically greater (7.80%) than that of piglets fed the PC diet. The ADFI of piglets fed the PP + Blend diet was numerically (15.56%) greater compared with that of the piglets fed the PC diet and greater (25.6%) than that of the NC diet group. The ADFI of the PP, PP + DFM and PP + Blend treatment groups increased by 4.89%, 12.44% and 15.6%, respectively, compared with PC diet group during the overall trial period. There was no dietary effect in G : F ratio ($P > 0.05$) during the second week and the overall trial period. A trend of greater G:F ratio ($P = 0.06$) was observed during the first week (pre-challenge) in pigs fed the PC diet compared with that of pigs fed DFM. Except for the pigs in the DFM diet group, pigs in every other treatment were numerically better in terms of ADG and ADFI than those in the NC group. Fecal consistency scores were shown in **Table 6**. Significantly lower fecal score was

Table 5 Analyzed DM, CP, ash and AA composition of feed used in formulating experimental diets, as-fed basis[a]

	Diet[b]					
Item, %	NC	PC	PP	PP + DFM	DFM	PP + Blend
DM	94.10	94.35	93.85	93.95	94.40	94.60
CP	21.75	21.75	21.75	21.375	21.69	22.19
Ash	6.65	6.70	6.30	6.40	6.40	6.50
Indispensable Amino Acid						
Arginine	1.40	1.40	1.31	1.31	1.40	1.33
Histidine	0.72	0.79	0.59	0.64	0.66	0.65
Isoluecine	1.18	1.22	1.19	1.12	0.95	0.90
Leucine	1.91	1.80	2.05	2.08	1.60	1.93
Lysine	1.72	1.62	1.87	1.54	1.88	1.73
Methionine	0.37	0.41	0.44	0.42	0.43	0.39
Phenylanine	0.88	1.12	1.09	1.05	0.96	0.88
Threonine	1.05	1.07	1.15	1.06	0.92	1.16
Valine	1.15	1.17	1.20	1.27	1.13	1.09
Dispensable Amino Acids						
Alanine	1.25	1.13	1.27	1.29	1.11	1.18
Aspartic acid	2.33	2.07	2.08	2.40	2.35	2.30
Cystine	0.34	0.41	0.44	0.43	0.38	0.42
Glutamic acid	4.19	4.44	4.59	4.30	4.14	4.03
Glycine	1.12	1.15	1.05	1.14	1.17	1.02
Proline	2.08	1.86	2.17	2.22	2.02	1.58

Serine	0.94	0.87	0.99	0.94	0.76	0.88
Tyrosine	0.70	0.82	0.76	0.59	0.67	0.74

[a] Values are as-fed basis.

[b] As in Table 4.

Table 6 Performance of post-weaned pigs fed different supplemented diets before and after being challenged with *E. coli* K88^{+} [a]

	Diet[b]							*P*-values
Item	NC	PC	PP	PP + DFM	DFM	PP + Blend	SEM[c]	Diet
Initial BW, kg	5.97	5.87	5.87	5.94	5.90	5.88	0.45	1.00
Final BW, kg	7.20	7.84	7.60	7.78	7.16	7.94	0.94	0.69
Day 0 to 7 (before-infection)								
ADG, g/d	65	106	93	120	52	114	24.85	0.21
ADFI, g/d	138	148	172	189	136	191	19.59	0.23
G:F ratio	0.44	0.70	0.57	0.65	0.32	0.59	0.12	0.06
Day 8 to 14 (after-infection)								
ADG, g/d	123	175	173	142	119	189	81.65	0.52
ADFI, g/d	275	303	301	316	274	329	57.47	0.44
G:F ratio	0.39	0.54	0.55	0.40	0.39	0.54	0.20	0.60
Day 0 to 14 (before and after-infection)								
ADG, g/d	94	141	133	131	86	152	32.90	0.05[d]

ADFI, g/d	207	225	236	253	205	260	25.21	0.06
G:F ratio	0.43	0.62	0.55	0.51	0.42	0.57	0.10	0.18
Fecal score[e] after infection, D 8								
22 hour	1.33^{y}	0.56^{xy}	0.94^{y}	0.94^{y}	0.22^{x}	0.78^{xy}	0.28	0.001
29 hour	1.31	0.50	1.28	1.33	0.44	0.94	0.43	0.27
46 hour	1.07	1.00	1.47	1.00	0.80	1.27	0.28	0.63
51 hour	0.78	0.61	1.22	1.11	0.50	1.22	0.22	0.21
Other items								
Mortality [f]	6	0	2	1	2	2	-	0.26

[a] Values are a mean of 6 replicates per diet.

[b] As in Table 4.

[c] Pooled SEM.

[d] DFM and PP + Blend are different, $P = 0.08$.

[e] Fecal score :0= normal, 1 = soft feces, and 2 = mild diarrhea and 3=severe diarrhea. Day 7 of experiment and after *E. coli* challenge

[f] Day 14 of experiment.

[x,y] Means with different letters are significantly different ($P < 0.05$).

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observed in DFM diet group compared with PP ($P < 0.05$) and PP + DFM ($P < 0.05$) diet group at 22 hours after infection. Six pigs in NC treatment died. No pig in the PC diet group died. The entire additive diets group had reduced mortality compared with the negative control (NC) treatment group.

Bacteria Population

One week after the challenge, scraped mucosa samples were cultured for *E. coli* and lactic acid bacteria counting. The bacteria count results are shown in **Table 7**. There were no differences among treatment groups in *E. coli* count numbers in ileum and colon. There were no differences among treatment groups in lactic acid bacteria count number in ileum; however, the lactic acid bacteria number in antibiotics (PC) group was significantly lower than in piglets fed PP ($P = 0.02$), DFM ($P = 0.03$), and PP + DFM ($P = 0.01$), and tended to be different ($P = 0.08$) from the count in the PP + Blend diet group.

Morphology

Visceral organ weights and intestinal morphology results are shown in **Table 8**. Dietary treatment had no effect ($P > 0.05$) on spleen weight. However, significantly greater ($P < 0.05$) liver weight was shown on piglets fed PP + Blend diet compared with those fed NC diet. Intestinal morphology in the ileum and jejunum was not influenced by diet ($P > 0.05$). Diet effect on villous height in the duodenum was statistically significant ($P < 0.05$). SDPP-fed pigs had greater villous height than those fed NC diet ($P < 0.05$) and DFM diet ($P < 0.05$). Crypt depth was not affected ($P > 0.10$) by diet in the duodenum, but the ratio of villous height to crypt depth of piglets fed PP diet tended to be greater than that of piglets fed NC diet ($P = 0.07$). These differences were mainly due to results obtained from piglets fed the diet with SDPP additive.

Table 7 Effect of diet on microbial population of ileum and colon mucins of post-weaned pigs after challenge on day 14 with *E. coli* K88^{+} [a]

	Diet[b]							*P*-value
Micro-organism, $\log_{10}$ cfu / mL	NC	PC	SDPP	PP + DFM	DFM	PP + Blend	SEM[c]	Diet
Escherichia coli								
Ileum	7.20	6.61	7.87	5.74	6.70	7.39	1.20	0.83
Colon	7.57	6.90	7.74	7.28	6.79	6.97	0.78	0.92
Lactic-acid bacteria								
Ileum	7.88	6.63	8.85	6.62	8.79	7.63	0.85	0.26
Colon	9.67^{xy}	7.87^{x}	10.24^{y}	10.35^{y}	10.02^{y}	9.76^{xy}	1.09	0.01

[a] Values are a mean of 6 replicates per diet.

[b] As in Table 4.

[c] Pooled SEM.

[x,y] Means with different letters are significantly different ($P < 0.05$).

Table 8 Effect of diet on small-intestine villous height, crypt depth, their ratio and organ weight after challenging on day 14 with *E. coli* K88^{+} [a]

	Diet[b]							*P*-values
Item	NC	PC	SDPP	PP + DFM	DFM	PP + Blend	SEM[c]	Diet
Intestinal morphology								
Duodenum								
Villous height, µm	280.55^{x}	362.48^{xy}	409.49^{y}	314.62^{xy}	284.31^{x}	350.60^{xy}	31.81	0.01
Crypt depth, µm	450.7	431.5	427.9	415.9	443.3	482.2	30.83	0.58
Villi: crypt ratio	0.65	0.89	0.99	0.80	0.68	0.75	0.12	0.06
Jejunum								
Villous height, µm	260.1	284.8	316.4	323.3	284.2	305.0	42.47	0.48
Crypt depth, µm	355.1	315.5	317.1	368.9	319.3	309.0	22.98	0.13
Villi: crypt ratio	0.76	0.93	1.13	0.89	0.94	1.07	0.13	0.33
Ileum								
Villous height, µm	290.5	315.5	305.2	292.1	283.1	258.2	21.49	0.54

Crypt depth, μm	285.8	261.0	289.9	296.5	295.5	321.6	26.05	0.51
Villi: crypt ratio	1.09	1.26	1.14	1.03	1.00	0.84	0.14	0.35
Organ weight								
Spleen, g	18.7	17.2	17.0	16.4	21.2	23.5	2.80	0.48
Liver, g	193.1^{x}	209.8^{xy}	231.0^{xy}	236.1^{xy}	213.8^{xy}	256.3^{y}	36.74	0.04

[a] Values are a mean of 10 slides from one pig, 6 pigs per diet.

[b] As in Table 4.

[c] Pooled SEM.

[x,y] Means with different letters are significantly different ($P < 0.05$).

Digesta pH, Ammonia N, and VFA Levels

Different diet additives had no effect ($P > 0.05$) on the pH of stomach, duodenal, jejunal, ileal and colonic digesta (**Table 9**). No dietary effect on the ammonia N concentration in intestinal digesta was observed ($P > 0.05$) after challenge. The plasma urea nitrogen value of piglets fed NC diet tended to be greater than that of piglets fed PP diet ($P = 0.08$) before infection. No dietary effect is shown after infection on plasma urea nitrogen ($P > 0.05$). In general, diet had no effect ($P > 0.05$) on the concentration of VFA in digesta obtained from the duodenum, jejunum and ileum (**Table 10**).

Immunology parameters

There was no dietary effect ($P > 0.05$) on concentration of IFN-γ (before infection), TNF-α (before and after challenge) and AGP (**Table 11**). However, IFN-γ concentration in pigs fed blend diet was significantly greater than in those fed PP + DFM ($P < 0.01$) and PC diet ($P = 0.02$).

DISCUSSION

There is a major concern in livestock production about the safety of animal products (Dagg et al., 2006 ; Schmid and Sinabell). This is not only a problem in the cattle, but also in swine and poultry production. For many years the swine industry has relied on sub-therapeutic levels of antibiotics as a means to reduce pathogen load in the digestive tract and as a growth promotant (Fairbrother et al., 2005; Ungemach et al., 2006). At the beginning of 2006, particularly in Europe, the use of antibiotics was banned because of a putative link between the use of antibiotics in animals and

Table 9 Effect of diet on intestinal pH, plasma urea nitrogen (**PUN**) and ammonia nitrogen in post-weaned pigs challenged with *E. coli* K88^{+a}

	Diet[b]							*P*-values
Item	NC	PC	PP	PP + DFM	DFM	PP + Blend	SEM[c]	Diet
Intestinal pH[d]								
Stomach	3.37	3.82	3.38	3.02	2.99	3.74	0.41	0.57
Duodenum	5.73	5.80	6.20	5.53	6.02	5.53	0.45	0.88
Jejunum	6.77	6.55	6.66	6.74	6.79	6.67	0.25	0.44
Ileum	6.81	6.47	6.92	6.64	6.85	6.72	0.30	0.83
Colon	6.39	6.46	6.20	6.11	6.51	6.23	0.24	0.38
Plasma Urea Nitrogen, mmol/L								
D 8, Before infection	6.23	4.15	3.65	4.10	4.82	3.80	0.08	0.07
D 14, After infection	5.53	4.20	3.93	3.95	5.05	4.12	0.10	0.59
Intestinal ammonia N[d], mg/L								
Jejunum	77.0	38.3	89.4	83.8	55.6	61.0	26.0	0.51
Ileum	108.2	70.0	54.7	60.7	88.5	75.6	32.2	0.84

Colon	347.3	363.9	340.5	316.0	410.7	395.6	66.4	0.92

[a] Values are a mean of 6 replicates per diet.

[b] As in Table 4.

[c] Pooled SEM.

[d] Day 14 of experiment.

Table 10 Effect of diet on intestinal VFA concentrations in jejunum, ileum and colon of post-weaned pigs after challenge on day 14 with *E. coli* K88[+a]

	Diet[b]							*P*-values
Item	NC	PC	PP	PP + DFM	DFM	PP + Blend	SEM[c]	Diet
Jejunum, mg/g								
Acetic	0.67	0.90	0.92	0.80	0.71	1.09	0.36	0.95
Propionic	0.05	0.08	0.60	0.13	0.02	0.02	0.09	0.24
Lactic acid	7.90	8.70	21.83	12.39	9.25	14.03	6.25	0.76
Ileum, mg/g								
Acetic	0.81	1.02	1.49	1.24	1.04	1.33	0.53	0.90
Propionic	0.06	0.06	0.22	0.21	0.05	0.21	0.09	0.65
Lactic acid	17.55	24.38	17.22	45.36	17.78	14.59	8.76	0.70
Colon, mg/g								
Acetic acid	17.70	22.42	23.08	20.90	21.84	16.58	3.89	0.79
Propionic acid	12.59	14.52	16.38	12.81	13.53	10.63	3.63	0.78

Iso-butyric acid	0.52	0.47	0.71	0.45	0.92	0.59	0.16	0.31
Butyric acid	5.35	6.16	7.30	5.10	6.83	4.63	1.80	0.76
Lactic acid	3.29	1.91	2.26	4.28	4.70	3.44	1.78	0.42

[a] Values are a mean of 6 replicates per diet.

[b] As in Table 4.

[c] Pooled SEM.

Table 11 Effect of diet on TNF-α, IFN-γ and AGP in post-weaned pigs before and after challenge with *E. coli* K88[+a]

	Diet[b]							*P*-values
Item	NC	PC	PP	PP + DFM	DFM	PP + Blend	SEM[c]	Diet
IFN-γ[d, e]								
Before infection[f], pg/mL	6.32	6.35	7.95	3.81	8.72	10.86	2.38	0.14
After infection[g], pg/mL	11.46^{xy}	9.28^{x}	10.67^{xy}	7.68^{x}	13.57^{xy}	19.15^{y}	5.11	0.01
TNF-α[e]								
Before infection[f], pg/mL	9.44	11.16	12.31	11.54	19.21	12.18	3.94	0.92
After infection[g], pg/mL	11.69	13.70	10.99	11.55	15.30	10.50	1.55	0.45
AGP[g], μg/mL	682.40	1031.29	836.67	828.33	645.26	868.13	296.95	0.76

[a] Values are a mean of 6 replicates per diet.

[b] As Table 4.

[c] Pooled SEM.

[d] Concentration in blend diet before and after challenge changes significantly ($P = 0.02$).

[e] No difference in concentration between pre and post challenge in all dietary treatment ($P > 0.05$).

[f] Day 7 of experiment.

[g] Day 14 of experiment.

[x,y] Diet means with different letters are considered to be significantly different($P < 0.05$).

antibiotic resistance in human medicine (Aarestrup and Wegener, 1999). One of the responses to this problem was to use cattle and swine rendered products, like spray dried porcine plasma (SDPP) and spray dried animal plasma (SDAP). Probiotics and acidifiers are also recognized as useful products (Fairbrother et al., 2005). In this research we have evaluated the ability of SDPP, probiotics and a combination of these products as alternatives to antibiotics.

It was anticipated that the non-antibiotic additives would improve pig performance and effectively reduce post-weaning diarrhea. However, in the current study, final BW and the overall ADG and ADFI of the pigs fed additive-supplemented diets were not significantly ($P > 0.05$) improved compared with those fed the NC and PC diets. Only numerical improvements in ADG and ADFI of piglets fed the PP + Blend diet were demonstrated in this experiment compared with the PC. The significance in liver weight of piglets fed PP + Blend compared with those fed NC diet may be due to individual body weight differences.

Other authors (Bosi et al., 2004; Yi et al., 2005) have noted that protein source has a major impact on animal performance in the *E. coli* $K88^+$ challenge model. Bosi et al., (2004) compared fish protein to SDAP in a challenge model with *E. coli* in medicated and non-medicated feed. They observed a significant effect for protein source in which SDAP was the superior ingredient (Bosi et al., 2004). However, no improvement in growth performance was obtained when antibiotics were added to the SDAP based diet (Bosi et al., 2004). This result was also true for the fish protein based diets but medication tended to have a beneficial effect (Bosi et al., 2004). Yi et al. (2005),

compared SDAP, glutamine, and a diet without either in an *E. coli* challenge model. They found no effects in growth performance (Yi et al., 2005). The positive effect they observed was in their positive control that was not challenged with *E. coli* $K88^+$ (Yi et al., 2005).

Even though we did not observe growth performance improvements we did see significant ($P < 0.05$) differences in fecal score at 22 hour after challenge (**Table 6**). The NC pigs scoured significantly ($P < 0.05$) more than pigs fed the DFM alone. In contrast, there was no significant difference in fecal scouring when SDPP or antibiotics were present in the diet when compared to the NC. This result would tend to be in agreement with Bosi et al. (2004) and Yi et al. (2005), who indicated that no differences in scouring could be attributable to SDPP in a challenge model, but other protein sources do.

Our results also indicated an improvement in villous height when SDPP was present (**Table 8**), which is an overall indicator of gut health. Owusu-Asiedu et al. also observed longer intestinal villi in pigs fed an SDPP containing diet compared with the villi in pigs not fed SDPP (Owusu-Asiedu et al., 2003a). The villous height, crypt depth, and their ratios found in the current research are in close agreement with results of the study by Hornich et al. (1973). The current result may be due to the protective function of SDPP glycoproteins which inhibit ETEC adhesion by competing for intestinal glycoprotein receptors in the intestine (Bosi et al., 2004). We conclude that SDPP has a protective effect on the gut, thus confirming its role as a feed supplement that helps protect weaned pigs from scouring.

Probiotics have been demonstrated to have benefit in nursery pig diets in

preventing post-weaning scours (Reid and Friendship, 2002). Species of bacteria used as probiotics may compete with pathogenic flora for nutrients, mask the surface of the GIT, occupy the adhesion site of pathogenic bacteria, and even stimulate bacterial enzyme activities (Pryde et al., 1999). While some studies showed that probiotics improved swine performance (Papaioannou et al., 2004; Taras et al., 2005), some showed no effects (Taras et al., 2006). In our studies the *Bacillus* probiotic we used had no beneficial effect on pig growth performance (**Table 6**). In fact, few if any studies have investigated *Bacillus* as a probiotic in a post-weaning diarrhea model with *E. coli* $K88^{+}$. Kyriakis et al., (1999) found a performance effect of a *Bacillus* probiotic in weaned swine but this did not include infection with a pathogenic bacterium to induce gastritis. Later studies by Alexpoulos et al., (2004) demonstrated performance benefits with *B. subtilis* and *B. licheniformis* spores as probiotics in sows and neonatal piglets, but once again no infectious diseases model was employed. However, *in vitro* studies do indicate that *Bacillus* can produce antimicrobial compounds to pathogenic *E. coli*, and even have beneficial effects on gut functions like mucin degradation and interconversion of bile salts (Guo et al., 2006).

The key experiments missing from the literature are those which experimentally infect pigs with *E. coli* pathogens and demonstrate reductions in disease when a *Bacillus* probiotic is present in the feed. Our experiment tested this hypothesis and under our conditions no growth performance benefit could be attributed to our *Bacillus* probiotic. However, we did see significant reductions in scouring (**Table 6**), with the *Bacillus* probiotic. This parameter is an important animal welfare indicator and as such the

Bacillus DFM shows potential as a feed supplement.

The *in vitro* results of Colliner et al., (2003), detected positive effects on gut health of a *Bacillus* probiotic, similar to our DFM, using mucus degradation among other gut health indicators. However, our results, if anything show a negative effect on villus architecture (**Table 8**), are in parallel with an increased tendency for inflammation (**Table 11**). Thus the effect on reduced scouring by the *Bacillus* probiotic appeared to be independent of affects on the enterocyte.

We found no differences in *E. coli* (**Table 7**), intestinal pH (**Table 9**), plasma urea nitrogen (**Table 9**), intestinal ammonia (**Table 9**), or VFA (**Table 10**). Intestinal pH, microbial populations, and by-products of microbial fermentation were measured to provide an indication of the effect of feed additives on intestinal health. The lack of response in the general *E. coli* but a response in the lactic acid population indicates that the effect of SDPP and DFM is not on the Gram-negative but the Gram-positive population. Both *Bacillus* and *Lactobacillus* utilize similar mechanism to transport nutrients across the cell wall and more effective competition for nutrients by *Bacillus* may explain the reduction in colonic lactic acid bacteria in our data (Djordjevic et al., 2001).

Interferon - γ was greater ($P < 0.05$) in the PP + Blend diet as compared to the PP + DFM, and the PC diets (**Table 11**). Interferon-γ is produced by both T-lymphocytes and natural killer (NK), cells which are part of the adaptive immune system that activate macrophages (Abbas and Lichtman, 2004). Interferon-γ is at the interface of the microbial interactions with macrophages (Abbas and Lichtman, 2004; Roitt and Delves,

2001). Thus, an increase in INF- γ indicates more inflammation as a result of increased microbial antigen reaching epithelial macrophages, probably via M-cells in the epithelial dome region (Abbas and Lichtman, 2004).

It is not surprising that the piglets fed PC diets had one of the lowest INF- γ levels because one would expect the antibiotics in the diet to reduce the adherence of many, but not all, microorganisms to the epithelium. The lower INF- γ level in PP +DFM diet group may be due to the protective function of SDPP glycoproteins which inhibit ETEC adhesion by competing for intestinal glycoprotein receptors in the intestine (Bosi et al., 2004). It is difficult to explain why the PP + Blend diet had such high INF- γ induction, but this appears to be the effect of the organic acids. Organic acids, SPP and a sweetener were the ingredients added to the PP + Blend diet that made it different from the PP + DFM diet.

IMPLICATION

In conclusion, SDPP showed some improvements in gut health of *E. coli* $K88^+$ challenge pigs. However, no growth performance improvement could be observed in our experiments. Spray-dried porcine plasma + DFM diet decreased inflammatory responses to some extent in our *E. coli* $K88^+$ disease challenge model. However, more detailed studies need to be conducted in adaptive and cellular immunity of the digestive tract. The probiotic, *Bacilli subtilis*, has potential to reduce scours even though there were no improvements in performance.

CHAPTER THREE

CONCLUSIONS

Based on the studies conducted in this thesis, it can be concluded that:

1. Significant effects in animal performance among dietary treatments were not detected. Animal growth and feed intake was calculated over a 7 day period, but if the infection was not severe, then it is possible that animals would have recovered within the 7 day period. Thus we may have missed a performance effect that occurred within the first 48 hours post inoculation. Given that we did observe diarrhea and some mortality, particularly in the negative control, we are fairly sure that infection did occur. However, animals did not exhibit illness for a prolonged period. Several authors (Van Dijk et al., 2002; Fairbrother et al., 2005) have noted that the *E. coli* $K88^{+}$ infectious diseases model can be variable. There are a number of possible reasons for this, but the primary one is that disease onset is not simply a function of the presence of the infectious bacterium, but the presence of other stressors, for example environmental temperature, that compromise the immune system allowing for induction of disease.

2. Fecal diarrhea was significantly lower in piglets fed the probiotic diet compared with other diets. As such, the probiotic, *Bacilli subtilis,* can potentially be included in the diet as an animal welfare measure. The presence of diarrhea reduces pig welfare outcomes because of increased odor, an increased level of microbial contamination,

and poor visual appearance of the pigs.

3. Spray-dried porcine plasma improved gut structure, especially villous height, in an *E. coli* $K88^+$ disease challenge model for weaned pigs. However, no growth performance improvement was observed in our experiment.

4. Significantly lower IFN – γ concentrations were observed in the blood of piglets fed PP + DFM and PC diets compared with those fed the PP + Blend diet. From our experiment, it is not possible to clearly conclude that there was a decreased inflammatory response. Because we would have expected that TNF-α and AGP respond in a similar manner to IFN – γ. It has been suggested that the glycoproteins in SDPP have a protective effect on the enterocyte (Van Dijk et al., 2002), but it can just as well be argued that it suppresses the immune response. However, given that the bulk of the literature indicates a beneficial effect of SDPP, it is much more likely that SDPP promotes enterocyte protection. To properly understand the effects of SDPP on gut immune function, more detailed studies need to be conducted in adaptive and cellular immunity of the digestive tract.

Recommendations

Studies should be conducted in the following areas:

1. One of the most important considerations is to develop an infectious diseases model with *E. coli* $K88^+$ that is less variable. For example, it would be helpful if more

severe diarrhea could be more consistently induced in animals. To improve the reliability of this model additional stressors, like lower environmental temperature, should be introduced as well as multiple strains of *E. coli* $K88^+$ with different suites of toxins.

2. Additional measurements should also be made to better characterize disease progression in pigs. For example, the first clinical indications of infection are reduced water and feed intake, and increased body temperature. Thus, animals should have their growth and feed intake monitored daily. Packed cell volume reductions would be an indicator of dehydration, and together with ear temperature measurements would be sensitive clinical measures of disease progression. By using these simple clinical measures a more reliable picture of the infectious model can be obtained.

3. More research must be conducted on the best dose, species, or combination of probiotics that could be used to reduce diarrhea. Since no performance improvements were detected in our experiment, more trials should be done on the effects of different probiotics on pig performance. However, if the infectious disease model could be improved resulting in less animal variation, statistical differences may be obtained. *Bacillus* species-based probiotics should be further investigated because their usage in swine diets is relatively uncommon. For example, *Bacillus cereus* and *Bacillus toyoi*, have been reported to improve BW of sows and piglets, but this was

not within the context of a challenge model (Taras et al., 2005); an infectious diseases model should be used. *Enterococcus faecium* was also reported to decrease fecal scouring in piglets between birth and weaning (Zeyner and Boldt, 2006). Thus additional *Bacillus* species and their combinations should be tested.

4. The effects of simple combinations of two additives, such as probiotics + organic acids, or SDPP + organic acids in weaned piglets' diet should be further investigated. Organic acids may be added alone as an experimental diet. Simple combinations of two supplements will give us a clearer idea of potential antagonistic or synergistic effects.

5. Since we did not find significant differences in AGP and TNF-α, a more complete analysis of immune mediators should be conducted. A full suite of pro-inflammatory (IFN-γ, TNF-α, and IL-12) and immunomodulatory (IL-10, TGF-γ) markers should be measured (Abbas and Lichtman, 2004; Roitt and Delves, 2001). Together these markers may be a better measure of the overall inflammatory process. In addition, measures of the effects on cell matrix structures (also part of the immune system), like gap-junctions should be conducted, because increased permeability is one of the phenotypes of infection with *E. coli*(Nagy and Fekete, 2005; Robins-Browne and Hartland, 2002).

6. Non-infected pigs should be added as a control in future experiments to provide a

comparison with infected pigs. Non-infected pigs should be kept in a separate room to prevent cross-contamination of feed, water and pens with *E. coli* $K88^+$ from infected pigs. All other conditions should be kept the same in each room, such as feed ingredients, room temperature, and management.

7. Due to differences in assay procedures and analytical approaches, the usefulness of VFA measurements as an index of the effect of dietary additives on enteric disease progression appears limited. Part of the problem is that VFA and ammonia are measures of recent fermentation events in the gut. If animals recover relatively quickly after infection then the gut sample may not be reflective of the fermentation at the time of infection. As mentioned in point two, if a clearer or more detailed analysis of disease progression can be obtained, a fecal sample that more accurately reflects the gut fermentation during infection can be taken.

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APPENDIX

AGP, IFN-gama and TNF-alpha were all measured by commercial Elisa kit. Detailed assay procedure of IFN- gama is listed below as an example:

1. Sample incubation:

Add 50 µl of standard diluent to each well of an anti-porcine IFN-gama precoated plate Then add 50 µl of standards or samples to each well in duplicate at room temperature (20-25 ℃) for 1 h.

2. Plate washing:

Wash plate THREE times with wash buffer.

3. Biotinylated antiobody reagent incubation

Add 100µl of Biotinylated antibody reagent to each well. Cover plate and incubate at room temperature for 1 h. Wash plate THREE times with wash buffer. Add 100µl of prepared Streptavidin-HRP solution (prepare immediately before use) to each well. Cover plate and incubate at room temperature for 30 minutes. Wash plate THREE times with wash buffer. Add 100µl of Premixed TMB substrate solution to each well. Develop the plate in the dark at room temperature for 30 minutes.

4. Substrate incubation and stop step

Stop reaction by adding 100µl of stop solution (0.18 M sulfuric acid) to each well.

5. Absorbance measurement

Measure absorbance on a plate reader at 450 nm-550 nm. Calculate results using curve-fitting statistical software by comparing with standard curve.

Fecal Score

Due to facility limitation, the experiment was separated into two periods. Fecal score measurement were late during the second period. The table below showed fecal scores at 6h after infection during the first period. Although they were not as high as 3, acute death, serious tremor and stress were started to be observed at 8-10 hours after infection.

	Diet[b]							*P*-value
Fecal score after infection	NC	PC	PP	PP + DFM	DFM	PP + Blend	SEM[c]	Diet
6h after infection	1.6	1.6	1	1	2	2	0.46	0.34

[a] Values are a mean of 3 replicates per diet.

[b] As in Table 4.

[c] Pooled SEM.

[e] Fecal score :0= normal, 1 = soft feces, and 2 = mild diarrhea and 3=severe diarrhea. Day 7 of experiment and after *E. coli* challenge