Gut microbiome analysis in piglet models infected with *Escherchia coli* K88: The role of charcoal and dietary crude protein supplemented with probiotic *Escherchia coli* strains UM2 and UM7.

by

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ABSTRACT

Entrotoxigenic *Escherichia coli* (ETEC) K88 is a causative agent of post-weaning diarrhea (PWD) in early-weaned pigs. This study investigated the efficacy of two alternative diets, charcoal (0.1, 0.5, 1, and 2%) and a low crude protein (CP) diet (17%) supplemented with probiotic *E. coli* strains (UM2 and UM7), against PWD infection in ETEC K88 challenged piglets. The present study found that charcoal had no effect on the challenged piglets' performance, ileal and colonic microbiota or their fermentation end products. There was, however, a correlation between charcoal dosage and fecal consistency score. Charcoal reduced the ileal mucosal attached ETEC K88. Feeding a low-CP diet resulted in a lower ileal ammonia concentration. The low-CP diet reduced the *E. coli* populations in the ileal digesta as well as lowered mRNA expression of the IL-1ß. We concluded that the use of both 1-2% charcoal diet and a low-CP diet supplemented with probiotic *E. coli* strains were effective in reducing the incidence and severity of PWD infection.

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DEDICATION

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FOREWORD

This thesis has been written in chapter format and includes an abstract, a general introduction, a review of literature, material and methods followed by a discussion of the first experiment and a discussion of the second experiment and conclusions. The research along this topic started in 2009 by Dr. Denis Krause in the Animal Science Laboratory at the University of Manitoba. The purpose of this research was to study the effect of alternatives to in-feed antibiotics, such as charcoal and differing levels of crude protein supplemented with probiotic *E. coli* strains UM2 and UM7, on GIT microbiota of piglets. This thesis has been formatted in Microbiology style.

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LIST OF ABBREVIATIONS

AB	Antibiotics
ACE	Abundance-based coverage estimation
ADFI	Average daily feed intake
ADG	Average daily gain
BCFA	Branched-chain fatty acids
B. adolescentis	*Bifidobacterium adolescentis*
B. lactis	*Bifidobacterium lactis*
B. licheniformis	*Bifidobacterium licheniformis*
B. subtilis	*Bifidobacterium subtilis*
B. thermofilum	*Bifidobacterium thermofilum*
BW	Body weight
C	Charcoal
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
CP	Crude protein
CCA	Canonical correspondence analysis
C. parvum	*Cryptosporidium parvum*
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E. rectale	*Eubacterium rectal*

EAST1	Entroaggregative heat-stable toxin-1
E. faecium	*Enterococcus faecium*
EMB	Eosin Methylene Blue
ETEC	Enterotoxigenic *Escherichia coli*
E. rectale	*Eubacterium rectal*
FCS	Fecal consistency score
G/F	Gain to feed ratio
cGMP	Cyclic guanosine monophosphate
G_S	GTP-binding protein
GIT	Gastrointestinal tract
HPAB	High crude protein + antibiotics
HPNA	High crude protein + no additives
HPPRO	High crude protein + probiotics
IEC	Intestinal epithelium cell
IL	Interleukin
LPAB	Low crude protein + antibiotics
LPNA	Low crude protein + no additives
LPPRO	Low crude protein + probiotics
LT	Heat-labile toxin
LT-I	Heat-labile toxin-I
LT-II	Heat-labile toxin-II
LTh-I	human Heat-labile toxin-I
LTp-I	porcine Heat-labile toxin-I

LB	Luria-Bertani
LPS	Lipopolysaccharide
LGG	*Lactobacillus rhamnosus* GG
L. acidophilus	*Lactobacillus acidophilus*
MCP-1	monocyte chemoattractant protein-1
mRNA	Messenger ribonucleic acid
NA	No additives
NS	Non-significant
OA	Organic acids
OTU	Operational taxonomic units
PC	Principal components
PCA	Principal component analysis
PCR	Polymerase chain reaction
PWD	Post-weaning diarrhea
PRO	Probiotics
Q-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
S. enterica	*Salmonella enteric*
S. tryphimurium	*Salmonella tryphimurium*
S. aerus	*Staphylococcus aerus*
S. dysentariae	*Shigella dysentariae*
SCFA	Short chain fatty acids
SDPP	Spray-dried porcine plasma

SEM	Standard error of the mean
ST	Heat-stable toxin
STh-I	human Heat-stable toxin
STp-I	porcine Heat-stable toxin
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
T1SS	Type I secretion system
T2SS	Type II secretion system
VFA	Volatile fatty acids
16S rRNA	small subunit ribosomal ribonucleic acid
16S rDNA	small subunit ribosomal deoxyribonucleic acid

GENERAL INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) K88 is a major cause of the frequent infection, *Escherichia coli* (*E. coli*) post-weaning diarrhea (PWD) or post-weaning colibacillosis in early-weaned pigs. This condition leads to extreme economical losses throughout the swine industry due to morbidity, mortality, and decreased growth performance of infected piglets (Fairbrother *et al*., 2005). Traditionally, in-feed antibiotics such as chlortetracycline-sulphamethazine-penicillin, tylosin-furazolidone, apramycin, and neomycin have been used as a prophylaxis and treatment for PWD infection. With the rise of antibiotic resistance bacteria (commensal and pathogenic) and well founded adverse effects of in-feed antibiotics on human health, this practice is no longer considered acceptable (Casewell *et al*., 2003, McEwen & Fedorka-Cray., 2002, Vondruskova *et al*., 2010). In January 2006, the European Union completely banned the use of in-feed antibiotics in livestock diets (Vondruskova *et al*., 2010, Wellock *et al*., 2008). Alongside this decision, other efforts are being made to reduce the usage of antibiotics in livestock diets in other parts of the world (Vondruskova *et al*., 2010). Therefore, efforts for developing effective alternatives to antibiotics such as charcoal, probiotics, or different nutritional strategies (such as using a low crude protein diet) are at the forefront of most research objectives.

The charcoal has been found to be an effective absorbent for the removal of bacteria and their toxins both in *vitro* and in *vivo* (Busscher *et al*., 2008, Drucker *et al*., 1977, Naka *et al*., 2001, Watarai & Tana., 2005). The ability of charcoal to bind to bacteria and a variety of other molecules is due to numerous pores that significantly increases its sorption surface (Chandy & Sharma., 1998). Charcoal is a nonselective absorbent and its removal activity and effectiveness

depends on the pore size of charcoal; smaller pores are needed for removal of small material while larger pores are needed to remove larger material (Naka *et al*., 2001). Little research has been done to investigate the effect of charcoal in reducing PWD in early-weaned pigs. Therefore it seems worthwhile to evaluate the effect of charcoal during the onset of PWD and potential changes in the GIT microbiota in early-weaned pigs experimentally infected with ETEC K88 (Watarai & Tana., 2005).

Probiotics have recently received increased attention due to their potential role in maintaining or restoring the host's natural microbial flora, in addition to out-competing undesired pathogens, often by means of antimicrobial substances (Paul., 2008). In particular, one antibacterial substance, called colicin, is also produced by certain *E. coli* strains (Gillor *et al*., 2008). Recent in *vitro* studies have shown that colicin-producing *E. coli* strains are effective against ETEC K88 (Setia *et al*., 2009). In addition, manipulation of the level of dietary CP is another strategy to reduce the incidence and severity of PWD in early-weaned pigs. It has been shown that a decrease in dietary CP results in decreased protein availability for the proliferation of pathogenic bacteria such as ETEC in the GIT (Prohászka & Baron., 1980, Wellock *et al*., 2006). Indeed, feeding a low-CP diet is associated with a decrease in the undigested protein in the distal small intestine and colon and is less favorable for nitrogen utilizing bacteria (Stein & Kil., 2006). A low-CP diet is also associated with decreased production of toxic compounds such as ammonia, amines, indoles, phenols, and branched-chain fatty acids (BCFA), all indicators of GIT health (de Lange *et al*., 2010, Gaskins., 2001, Stein & Kil., 2006). Moreover, feeding a low-CP diet has demonstrated beneficial effects on the GIT microbiome and its associated immune response (Lynch *et al*., 2007, Opapeju *et al*., 2010, Wellock *et al*., 2006). It is therefore worth evaluating any synergistic effects of varying levels of dietary CP supplemented with probiotic *E.*

coli strains during the onset of PWD, GIT microbiota, and immune response of experimentally ETEC K88 infected piglets.

LITERATURE REVIEW

1. *Escherichia coli* diarrhea in early-weaned pigs

Escherichia coli is a gram-negative, rod-shaped, facultative anaerobic bacterium belonging to the Enterobacteriaceae family of Gammaproteobacteria. It is a common inhabitant of the GIT of animals and humans. Some strains of *E. coli* are harmless while some are pathogenic. Based on virulence mechanisms, pathogenic strains of *E. coli* responsible for causing diarrhea are classified into six groups; entrotoxigenic *E. coli* (ETEC), entrohemorragic *E. coli* (EHEC), entroinvasive *E. coli* (EIEC), entrophatogenic *E. coli* (EPEC), entroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC).

ETEC are commonly found to be the cause of diarrhea among humans in developing countries and weaned animals, particularly early-weaned pigs and calves (Nagy & Fekete., 1999, Stephen., 2001). Various research studies are investigating strategies to inhibit the ETEC infection in farm animals, as there is always a risk of human infection.

ETEC K88 (F4) is an etiologic agent in outbreaks of PWD, which is a major challenge to the swine industry resulting in low performance rates, high morbidity, and mortalities (Cutler *et al*., 2007, Fairbrother *et al*., 2005). Besides ETEC, members of the genera *Clostridium, Lawsonia,* and *Brachyspira* as well as viruses such as rotaviruses, coronaviruses, and transmissive gastroenteritis viruses are frequently reported as etiological agents of PWD infections (Morin *et al*., 1983, Vondruskova *et al*., 2010).

In the wild, weaning of piglets is a gradual and long process occurring between 8 to 20 weeks of age. This is contrary to industry practices where piglets are immediately weaned between the age of 21 to 35 days (Lalles *et al*., 2007, Mavromichalis., 2006). This early-weaning process induces stress in immature piglets making them more susceptible to enteric diseases, particularly PWD (Fairbrother *et al*., 2005, Lalles *et al*., 2007, Vondruskova *et al*., 2010). Such non-infectious stress factors include receiving infrequent, low digestible feed compared to frequent, highly digestible milk, lack of maternal antibodies in solid feed, separation from their mother, exposure to a new environment and mixing with other litters (Lalles *et al*., 2007, Vondruskova *et al*., 2010).

1.1. Pathogenicity of entrotoxigenic *Escherichia coli* (ETEC)

1.1.1. Mechanism of virulence

The pathophysiological events of ETEC infection are as follows: ingestion of bacteria, attachment to intestinal epithelium, colonization, proliferation, and local entrotoxin secretion, leading to a net secretory state and ETEC diarrhea (Candy., 1980). Therefore, the attachment of ETEC to the intestinal epithelium is a prerequisite step for development of ETEC infection. This attachment is mediated by an array of different fimbriae such as K88 (F4), K99 (F5), 987P (F6), and type 1 (F1) that mostly target sugar moieties of glycoproteins and glycolipids of porcine epithelial cells (DuPont *et al*., 2009, Klemm *et al*., 2010). Fimbriae and their adhesins play a major role in the development of an ETEC infection as their loss results in avirulent strains and even probiotics (Klemm *et al*., 2010). Parallel studies in humans also demonstrated that fimbirae,

designated as colonization factor antigens (CFA), are essential for ETEC attachment and diarrhea (Candy., 1980).

The fimbriae, K88 and 987P, is responsible for ETEC infections in weaned piglets and newborn piglets, respectively. The K99 fimbria is responsible for ETEC infections in calves, lambs, as well as newborn piglets (DuPont *et al*., 2009, Fairbrother., 2006). Type 1 (FimH) fimbria is the most common type of fimbriae in *E. coli* strains, although its role in attachment of ETEC strains to the small intestinal mucosa is not yet known (Jayappa *et al*., 1985, To *et al*., 1984). Type 1 fimbriae have been found to mediate the small intestinal attachment (Fairbrother., 2006, Fleckenstein *et al*., 2010, Sokurenko *et al*., 1998). K88 fimbriae are long appendages with a length of 0.1-1 µm and a diameter of 2.1 nm (Snoeck *et al*., 2004). K88 is the most prevalent fimbriae causing PWD in piglets and possessing the different serological antigenic variants K88ab, K88ac, and K88ad. Porcine small intestine receptors for these variants include: intestinal mucin-type glycoproteins (IMTGP) for K88ab and K88ac, transferrin GP74) for K88ab, and intestinal glycosphingolipid (IGLad) for K88ad (Erickson *et al*., 1994, Jin *et al*., 2000, Koh., 2007, Snoeck *et al*., 2004).

Binding of ETEC strains, to their specific receptors in the small intestine, triggers the secretion of one or a combination of enterotoxins. These toxins include heat-labile toxin (LT), heat-stable toxin (ST), and entroaggregative heat-stable toxin-1 (EAST1), which result in a flux of water and electrolytes into the intestinal lumen. This can lead to a watery diarrhea if malabsorption of excess fluid occurs in the large intestine (Fairbrother., 2006).

The LT is a large toxin complex (~ 88 kDa) which functionally resembles the *Vibrio cholera* toxin at 77% identity at the nucleotide level (Fairbrother *et al*., 2005, Fairbrother., 2006, Nagy & Fekete., 1999, Nataro & Kaper., 1998). This toxin can be impaired at 60 ºC for 15

minutes (Fairbrother., 2006). LT consists of five B subunits in an aromatic-like arrangement that are responsible for binding to the gangliosides on the host cell with one A subunit and two A1 and A2 domains joined by a disulphide bond. The A1 domain is the biologically active toxin molecule with adenosine diphosphate ribosylase activity and the A2 domain mediates the interaction to the B subunits (Fleckenstein *et al*., 2010, Turner *et al*., 2006). Based on their B subunits, LT exists in two distinct serogroups, LT-I, and LT-II. LT-I is pathogenic and is divided into two groups, LTh-I and LTp-I that are found primarily in human and porcine isolates, respectively. LT-II (LT-IIa and LT-IIb) is nonpathogenic and found mostly in other animal isolates (Fairbrother *et al*., 2005, Nagy & Fekete., 1999). Upon binding of bacteria to epithelial cells, B subunits bind with a high affinity to GM1 gangliosideson the surface of the host's cell followed by a translocation of the A1 toxin molecule to the target cell through type II secretion system (T2SS). The A1 domain transfers an ADP-ribosyl moiety from nicotinamide adenine dinucleotide (NAD) to the alpha subunit of GTP-binding protein (G_S), which regulates adenylate cyclase activity. This ribosylation of the alpha $G_{S,}$ results in constitutive activity of adenylate cyclase, leading to elevation of cyclic adenosine monophosphate (cAMP). Increased levels of cAMP activate the chloride channels (cystic fibrosis transmembrane regulator-CFTR) located in the apical epithelial cell membrane. This in turn promotes excessive secretion of chloride ions and water into the intestinal lumen resulting in a watery diarrhea (Fairbrother *et al*., 2005, Fleckenstein *et al*., 2010, Nataro & Kaper., 1998, Turner *et al*., 2006).

The LT may have a potential role in promoting the attachment and colonization in the small intestine by ETEC strains (Allen *et al*., 2006, Johnson *et al*., 2009). It has been reported that LT knockout isolates resulted in decreased incidence of diarrhea and intestinal bacterial colonization in gnotobiotic pigs (Berberov *et al*., 2004). This could be a result of the association

of LT with lipopolysaccharide (LPS) and the bacterial cell surface components, acting as an adhesin (Horstman *et al*., 2004).

The ST is a small toxin that resists heating up to 100 ºC for 15 minutes and divided into two structurally and functionally distinct groups with different mechanisms of action, ST-I (STa) (~ 2 kDa) and ST-II (STb) (~5 kDa) (Peterson & Whipp., 1995, Turner *et al*., 2006). The ST-I can further be classified into two subgroups, STh-I and STp-I, identified from human and infected pig strains, respectively. STp-I was also isolated from human and bovine strains. Upon attachment of ETEC to epithelial cells, ST-I is released through the type 1 secretory system (T1SS) and binds to guanylate cyclase C reseptor (GC-C), which in turn activates the intracellular catalytic domain of guanylyl cyclase, resulting in enhanced levels of cyclic guanosine monophosphate (cGMP). Elevated intracellular cGMP activates cGMP-dependent protein kinase II that in turn activates chloride ion channels (CFTR). The consequent secretion of chloride ions and inhibition of sodium ions (Na^+) absorption subsequently drives water into the intestinal lumen through osmosis (Fleckenstein *et al*., 2010, Turner *et al*., 2006). ST-II is primarily associated with swine isolates and functions independently of cyclic nucleotide secretion. Once binding of ETEC to epithelial cells occurs, ST-II is released through T1SS and binds to a glycosphingolipid, so called sulphatide, which is widely located on the intestinal epithelium. After internalization, ST-II activates a pertussis toxin-sensitive GTP-binding regulatory protein resulting in secretion of calcium through a receptor-dependent ligand-gated calcium channel. This in turn activates calmodulin-dependent protein kinase II leading to activation of intestinal ion channels. This toxin can also phosphorylate protein kinase C resulting in activation of CFTR. The elevated calcium levels mediate phospholipases A2 and C releasing arachidonic acid from membrane phospholipids resulting in the formation of prostaglandins E2

(PGE2) and 5-hydroxytryptamine (5-HT), which regulate water and electrolyte transport out of the intestinal cells (Dubreuil., 2010, Fairbrother., 2006, Turner *et al*., 2006).

Entroaggregative heat-stable toxin-1 is a small toxin and was first isolated in EAEC. EAST1 shares structural and functional similarity to ST-I and, like ST-I, binds to GC-C as a receptor and activates production of intracellular cGMP. EAST1 has been isolated from human and animals, particularly swine and cattle; however, its role in development of diarrhea has not been found (Fairbrother., 2006, Turner *et al*., 2006, Veilleux & Dubreuil., 2006).

1.1.2. **Host factors**

Age can be a critical factor in piglet susceptibility to ETEC infection. This is the result of changes to host mucosal and/or intestinal epithelial cell receptors for each putative adhesin (Fairbrother., 2006, Jin *et al*., 2000). As mentioned earlier, ETEC K88 is most commonly associated with early-weaned pigs aged from 21 to35 days, but not in older piglets (~47days old). Reduction in the number of K88 receptors in mucus accounts for the age resistance of older piglets. This is also the case for ETEC 987P strains, however the age resistance to infection by ETEC K99 strains in older pigs is due to the decline in intestinal epithelium receptors of K99 fimbriae (Fairbrother., 2006, Jin *et al*., 2000).

2. Microbial composition of GIT in early-weaned pigs

2.1. Normal GIT flora in pigs

Complex populations of microorganisms densely inhabit the GIT of mammals. In fact, presence of food (carbon and mineral sources), moisture, and appropriate temperature make the GIT a favorable habitat for a diverse array of microorganisms to grow and proliferate. Certain bacteria, aerobic and facultative anaerobic bacteria such as streptococci, lactobacilli, clostridia, and *E. coli*, populate the germ-free GIT during and shortly after birth (Gaskins., 2001, Macpherson *et al*., 2005, Richards *et al*., 2005, Sorum & Sunde., 2001). Indeed, host-microorganisms and microorganisms-microorganisms interactions determine the microbial composition of the GIT (Vondruskova *et al*., 2010).

The normal bacteria of the GIT have been long appreciated for their various beneficial effects to the host. They out-compete non-indigenous pathogenic bacteria via competition for binding sites (colonization resistance) and resources, volatile fatty acids (VFA) production, and production of antimicrobial compounds, such as bacteriocins (Busscher *et al*., 2008, Gaskins., 2001, Vondruskova *et al*., 2010). Likewise, they are involved in production of essential nutrients including, short chain fatty acids (SCFA), amino acids, and vitamins including B1, B2, B3, B6, B7, B12, C, and K (Gaskins., 2001, Sorum & Sunde., 2001). The GIT microbiota also has a significant impact on intestinal structure by stimulating rates of epithelial cell turn over and villus size leading to an increase of absorptive surface area and mucus secretion (Richards *et al*., 2005). In addition, the normal GIT flora contributes to the development of a balanced and regulated host immune system. It has been widely known that germ-free pigs have an impaired

immune system in comparison to their conventional counterparts populated with commensal bacteria, conferring a dynamic interaction between the gut bacteria and the host immune system. Pigs under germ-free conditions are unable to produce serum antibodies to T-dependent and type 2 T-independent antigens (Butler *et al*., 2002). Additionally, lack of dendritic cells and T cells for germ-free pigs in the diffused lymphoid tissue of villi and crypts of the jejunum has been reported (Haverson *et al*., 2007). It has also been shown that germ-free mice have hypoplastic peyer's patches with few germinal centers, in addition to reduced IgA-producing plasma cells and lamina propia $CD4^+$ T cells (Macpherson & Harris., 2004, Round & Mazmanian., 2009). In parallel, germ-free chickens resulted in a lower IgG serum when compared to conventional chickens (Haverson *et al*., 2007, Richards *et al*., 2005).

The stomach and proximal small intestine are acidic and are generally populated by Gram-positive bacteria such as lactobacilli and streptococci. However, the distal small intestine (ileum) is less acidic and is mainly colonized by *Enterobacteriaceae* spp., *Bacteroides* spp., lactobacilli, and enterococci (Hopwood & Hampson., 2003, Sorum & Sunde., 2001). In the large intestine due to higher pH, lower digesta passage rate, and optimal temperature, this favours increased numbers of bacteria, mainly obligate anaerobic bacteria such as *Bacteroides* spp., *Fusobacterium* spp., *Clostridium* spp., and *Peptostreptococcus* spp., in addition to facultative anaerobes including *E. coli, Klebsiella* spp., *Enterobacter* spp., and *Enterococcus* spp. (Hopwood & Hampson., 2003, Vondruskova *et al*., 2010). The most frequently observed genera in the pig's GIT are *Streptococcus, Lactobacillus, Eubacterium, Fusobacterium, Bacteroides, Peptostreptococcus, Selenomonas, Clostridium, Butyrivibrio,* and *Escherichia* (Hopwood & Hampson., 2003).

2.2. Alteration of GIT normal flora in early-weaned pigs

The initial colonization of normal GIT flora in weaned piglets remains quite stable as long as they received the sow's milk, yet this can be changed in response to a new diet, environment and health conditions (Gaskins., 2001, Roselli *et al*., 2005). During weaning, factors such as short adaptation period to solid feed and environment, in combination with a lack of maternal antibodies can negatively affect the piglet's GIT microbiota increasing the vulnerability to enteric *E. coli* infections (Gaskins., 2001, Roselli *et al*., 2005, Vondruskova *et al*., 2010). It has been reported that the number of lactobacilli, a predominant bacterial group in the piglet's GIT, is markedly reduced around weaning (Janczyk *et al*., 2007, Savijoki *et al*., 2006). This reduction was even greater in piglets weaned at 17 days of age compared to piglets weaned at 24 days of age, suggesting the significance of weaning on the GIT microbiota (Franklin *et al*., 2002). Likewise, Castillo *et al.* (2007) reported an increase in the eubacteria:lactobacilli ratio. Franklin, *et al.* (2002) also reported no increase in the number of *E. coli* in piglet's GIT after weaning. Overall, studies show a reduction in beneficial bacteria with a shift towards a GIT microbial composition that contributes to increased susceptibility to infection (Janczyk *et al*., 2007).

3. Methods for determining gut microbial composition

With respect to numerous beneficial effects of the GIT microbiota, providing suitable techniques and tools to enumerate and characterize such a complex ecosystem is essential. Traditional culture-based techniques are limited due to the lack of selective media for anaerobes (predominant microorganisms in the GIT), inability to produce a phylogenetic classification, and

the inability to determine the true diversity of the GIT microbiota (Gaskins., 2001, Vaughan *et al*., 2000, Wang *et al*., 2003). It has been estimated that 40 to 90% of the GIT microorganisms are not cultivable due to culturing limitations (Richards *et al*., 2005). These drawbacks have led scientists to take advantage of more culture-independent techniques that are based on sequencing highly conserved molecules, such as 16S rRNA, as a marker to identify, characterize, and classify the diverse GIT microbiota.

To date, various culture-independent techniques have been developed to assess the entire GIT population and to evaluate the potential effects of any given treatment on that population. Such techniques include polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), PCR-temperature gradient gel electrophoresis (PCR-TGGE), single strand conformation polymorphism (SSCP), terminal-restriction fragment length polymorphisms (T-RFLP), DNA microarrays, DNA fingerprinting, fluorescence *in situ* hybridization (FISH), quantitative real-time PCR (Q-PCR) and high-throughput sequencing technologies (cyclic reversible termination (CRT), real-time sequencing, sequencing by ligation (SBL), and single-nucleotide addition (SNA) (pyrosequencing) (Metzker., 2009, Richards *et al*., 2005). Each method has its own utility and limitations, but for the sake of this study, we will focus on two frequently used methods, Q-PCR and pyrosequencing.

3.1. Real-time polymerase chain reaction

Real-time PCR or quantitative real-time PCR (Q-RT PCR/Q-PCR) are commonly used laboratory assays to evaluate the GIT microbiota. The Q-PCR uses DNA as starter material and relies on the quantitative relationship between the amount of mRNA and PCR amplicons used during each PCR cycle (Kubista *et al*., 2006, Richards *et al*., 2005, Wang *et al*., 2008). Despite

that standard PCR allows for detection of a final DNA product, Q-PCR quantifies amplified target DNA molecules during the course of a PCR reaction in real time (Kubista *et al*., 2006). To do that, non-sequence-specific DNA-binding dye (SYBR Green, a dye that specifically binds to the minor groove of DNA) or fluorescence-labled probes are introduced in the PCR reaction (Kubista *et al*., 2006, Richards *et al*., 2005). As amplification occurs, a machine detects the florescence signal during each cycle (real time). The amount of the fluorescence signal is proportional to the amount of target DNA being amplified; therefore, the Q-PCR can measure the amount of bacteria present and is considered as a quantitative method (Richards *et al*., 2005).

The Q-PCR can then be applied to investigate changes in the GIT microbiota, using universal or specific DNA (16S rDNA) primers. Universal primers complementary to the conserved region of the 16S rDNA sequence can be used to quantify the entire GIT microbiota. In contrast, specific primers complementary to variable regions of the 16S rDNA are more practical when quantifying specific species or genus within the GIT (Gaskins., 2001). Furthermore, this approach can be used in gene expression analysis to measure an up- or down-regulation of a particular gene in various conditions. In contrast to the microarray technique in which thousands of genes (selected and unselected) are investigated for their expression, the Q-PCR technique is more sensitive and cost efficient allowing us to assess expression of selected genes, for instance in a particular tissue, and exclude other genes (Kubista *et al*., 2006).

3.2. High-throughput sequencing - Pyrosequencing

Applying DNA sequencing technologies to the study of the GIT microbiota has markedly improved our understanding of its composition. High-throughput sequencing techniques are used to sequence unknown DNA in real time (Ahmadian *et al*., 2006).

Pyrosequencing technique belongs to the high-throughput sequencing technologies and is a combination of methods; template preparation, sequencing and imaging. Templates are prepared via an emulsion PCR (emPCR) method. For this method, a library of fragment/mate-pair templates is generated and adaptors with universal primers are ligated to the target ends. Next, DNA is separated into single strands then capturing each single strand onto a bead. In order to amplify the strands, the beads with captured strands are subjected to PCR, then immobilized in PicoTiterPlate (PTP) wells (Roche/454) and are subjected to sequencing, also referred to as "sequencing by synthesis". The sequencing and imaging method relies on the detection of light when a complementary nucleotide in incorporated onto the strand (Metzker., 2009). Next, DNA polymerase extends the primer and pyrophosphate is released. The released pyrophosphate undergoes a several enzymatic reactions in order to produce light which is detected by a charge-coupled device (Shendure & Ji., 2008).

Advantages to the pyrosequencing technique include longer DNA fragments, shorter run times (0.35 days) and lower crosstalk between adjacent wells containing amplified beads. However, compared with any real-time sequencing technique (~964 base pair), pyrosequencing results in overall shorter DNA fragments (~ 330 base pair) (Metzker., 2009).

3.3. Metagenomics

High-throughput sequencing technologies, like pyrosequencing, are used to produce high-quality sequence data from specific regions of the 16S rDNA gene of the GIT microbiota (Hamady & Knight., 2009). The resultant data can then be subjected to metagenomic studies that involve sampling the genome of a microbial population in any ecosystem. In contrast to genomics, that provides the complete sequence of an organism, metagenomics offers a powerful

means to reveal unbiased information regarding the community's structure (species richness and diversity) and the functional (metabolic) potential (Hugenholtz & Tyson., 2008). Hence, metagenomics allow us to gain a deeper view into the complex relationships between hosts and their GIT microbiota.

3.3.1. Biodiversity indices

The GIT is a dynamic ecosystem that is a habitat for approximately 10^{14} microbes (Ley *et al*., 2006) and like other ecosystems can be reviewed from biological diversity standpoint. The 16S rRNA sequencing technique associated with computational tools aids to identify the biodiversity of the GIT microbime.

Biological diversity or biodiversity is a complex phenomenon defined as the range of variation of organisms represented in an ecosystem or in a given habitat (Curtis & Sloan., 2004, Tilman., 1997) extending to various levels in microbes, genetics, and so on. Subjecting the GIT environment to the biodiversity concept helps to understand the fundamentals of host/microbial-microbial interactions and their associated functions in this ecosystem.

There is a strong relationship between structure and function in any given ecosystem. Hence, any disturbances to the host microbial ecosystem of the GIT, influences the species richness and diversity, which in turn significantly affects function (Cardinale & Palmer., 2002, Curtis & Sloan., 2004). In fact, the biodiversity reflects the health of an ecosystem. That is to say, a high level of species diversity in the GIT is an indication of a healthy and stable ecosystem, yet this may not be the case for increases in number of certain species. Biodiversity is a key component in GIT studies that look through the functional response of a microbial community to newly introduced materials such as probiotics and drugs (Curtis & Sloan., 2004).

Biodiversity has three components; alpha diversity, beta diversity, and gamma diversity, also called landscape diversity. Alpha diversity measures the diversity within an ecosystem, while beta diversity measures the diversity between ecosystems. Gamma diversity is combination of both alpha and beta diversity and measures the diversity within a larger region composed of several ecosystems (Sepkoski Jr., 1988). Various indices such as species richness estimators and diversity indices are used to measure the alpha diversity in an ecosystem (Chao *et al*., 2005).

3.3.1.1. Richness estimators (Chao1 and ACE)

Species richness, refers to the number of various types of species in an ecosystem, and is a commonly used measure of species abundance in a particular ecosystem. It is unlikely to estimate all species and their abundance in any ecosystem. Therefore, methods such as Chao1 (non-parametric method) are developed to estimate species (species missed from samples) with a total abundance of one (singleton) in abundance-based sample (Chao *et al*., 2005). Abundance-based coverage estimator (ACE) is a modified Chao1 method in which observed frequencies are divided into two groups, abundant and rare. The abundance-based coverage estimator uses only the rare group (10 or fewer species in a sample) to estimate the species richness (Chao *et al*., 2000, Chao *et al*., 2005).

3.3.1.2. Diversity indices (Simpson and Shannon indices)

Diversity is defined as a function of the number of species present and their evenness (Washington., 1984). Indeed, a population with higher species and evenness has more diversity (Pielou., 1966). Diversity indices, such as the Simpson and Shannon indices, are used to measure

the diversity of an ecosystem, differing from species richness, which only measures the number of species present in an ecosystem. Simpson's index measures the probability of randomly sampling two individuals from a population which belong to the same species (Peet., 1974, Washington., 1984) while the Shannon index is a heterogeneous index based on the information theory, which considers both species richness and evenness. The Shannon index measures the degree of uncertainty of randomly selecting a species from a population (Peet., 1974).

3.3.1.3. Effective number of species

Diversity indices (Simpson and Shannon indices) are considered as entropy of an ecosystem. However, they do not per se reflect the true diversity of that ecosystem. For example, if we consider radius of a sphere, it is only an index of the sphere volume but it is not itself the volume. Diversity indices use a nonlinear scale and interpretation of these indices can be misleading, therefore measuring the true diversity is an asset. Effective species number can be defined as the true diversity of a desired ecosystem. It uses a linear scale and can be calculated from the exponential of the Shannon index (exp(x)) and from the reciprocal of the Simpson index (1/x). Hence, calculation of effective species numbers helps to estimate the true diversity of the ecosystem in question (Jost., 2006).

3.3.2. Multivariate analysis

Data received from a microbial community is extensive, complex and multivariate as each sample within that community is affected by different factors such as the abundance of other species and environmental factors. Therefore, a mathematical method that deals with all these variables simultaneously is necessary. Multivariate analysis offers a means to treat the

multivariate data as a whole resulting in summarized data and revealing their structure, while reducing noise, finding correlations, identifying outliers and relating the data to other data, such as environmental information (Gauch., 1982). Such multivariate analyses that are utilized include Linear Discriminant Analysis (LDA), principal component analysis (PCA), and Canonical Correspondence Analysis (CCA).

4. Immune response of early-weaned pigs infected with entrotoxigenic *Escherichia coli*

Intestinal epithelial cells (IEC) separate contents of a harsh luminal environment from the layers of tissue comprising the internal milieu (Pitman & Blumberg., 2000). Besides that, IEC act as a physical barrier to inhibit bacterial colonization on the surface of the monolayer (Oswald., 2006, Pitman & Blumberg., 2000). Intestinal epithelial cells have been found to be an effective trigger in initiating the innate and adaptive immune systems in response to pathogens (Devriendt *et al*., 2010). Intestinal epithelial cells and immune cells such as lymphocytes, macrophages and dendritic cells recognize pathogen-associated molecular patterns (PAMP) through pathogen-recognition receptors (PRR), such as Toll-like receptors (TLR) and Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) (Kumar *et al*., 2009, Moue *et al*., 2008). This results in the expression of various cytokines and chemokines leading to attraction of immune cells to the site of infection, which changes the intestinal immune response to that infection (Devriendt *et al*., 2010). To date, 12 members of TLR have been identified. The receptor, TLR-2, recognizes the peptidoglycan from gram-positive bacteria, while TLR-4/MD-2 recognizes LPS from gram-negative bacteria. In addition, TLR5 and TLR9 are stimulated by flagellin and unmethylated CpG DNA from bacteria, respectively (Kumar *et al*., 2009).

Intracellular nucleic acids are recognized by TLR-3, TLR-7, TLR-8, and TLR-9 (Moue *et al*., 2008). Previous studies have demonstrated that ETEC infection induces an inflammation-associated response (Roselli *et al*., 2007). Expression of all TLR (1-9) have been reported in porcine IEC, yet treating these cells with ETEC resulted in an increased level of expression of TLR-2 and type I helper T cytokines such as IL-1α, IL-1ß, IL-12p35, and IL-6. In addition, chemokines such as monocyte chemoattractant protein (MCP-1) and neutrophil chemotaxis (IL-8) were all detected at increased levels. The expression of TLR-4 has been shown to decrease with a high concentration of ETEC (Moue *et al*., 2008, Roselli *et al*., 2007). An important indicator of pro-inflammatory responses is IL-1ß, mainly secreted by activated phagocytic cells (Burger & Dayer., 2002, Dube *et al*., 2001, Murtaugh., 1994). A high concentration of IL-1ß in serum of ETEC K88 challenged piglets has been previously reported (Opapeju *et al*., 2010).

Interleukin-6 secreted by IEC was found to be important for IgA secretion that may protect against ETEC adhesion (Asper *et al*., 2009, Pitman & Blumberg., 2000). Elevated levels of IL-6 have also been shown in response to cholera toxin (Pitman & Blumberg., 2000). An ETEC-induced increase of IL-8 was reported with low integrity porcine IEC, as anti-IL-8 ameliorated the membrane damages caused by ETEC K88 (Roselli *et al*., 2007). It also has been shown that K88 fimbriae are important in secretion of IL-6 and IL-8 by IEC (Devriendt *et al*., 2010). In parallel studies with human epithelial cell lines, increased expression of MCP-1 and IL-8 in response to Gram-negative bacteria such as *S. dublin, Y. enterocolitica* or *S. dysentariae* was observed (Pitman & Blumberg., 2000). Overall, secretion of chemokines and pro-inflammatory cytokines benefits the infected piglets by recruiting immune cells to the sites of infection. However, they may also have deleterious effects on optimization of dietary nutrients

required for growth or membrane integrity, as is the case for IL-8 (Klasing & Korver., 1997, Roselli *et al*., 2007).

5. Nutritional effects on the GIT microbiome in early-weaned pigs

The GIT functions as a site for absorption of nutrients and water, while secreting electrolytes, mucin and immunoglobulins. In addition, the GIT provides defence mechanisms against many foreign antigens including pathogenic bacteria (Lalles *et al*., 2007). During early weaning, the GIT is deleteriously affected by stresses caused by separation from mother, mixing with other litters, a new environment, withdrawal from milk and changing their diet to solids with lower digestibility. These stresses result in increased susceptibility of early-weaned pigs to GIT disorders, infection and diarrhea (Lallès *et al*., 2004). Nutrition can greatly affect environmental factors in the GIT and their resultant by-products, such as pH, VFA, and ammonia.

5.1. pH

Within the GIT, pH is one of the main environmental parameters influencing digestive enzymes and colonization of pathogenic bacteria. The high pH as a result of early weaning, contributes to colonization of pathogenic bacteria such as coliforms in the piglet's GIT. While low pH, by contrast, contributes to an increased proportion of resident bacteria such as lactobacilli (Mathew *et al*., 1996). The high pH found in the GIT of early-weaned pigs can be due to a lower secretion of hydrochloric acid by a less developed stomach, lack of sow's milk or

suppression of lactic acid production from lactobacilli in the upper alimentary tract (Snoeck *et al*., 2004). Lackeyram *et al.* (2010) reported that early weaning reduces the small intestinal alkaline phosphatase (IAC), which in turn mediates lumen pH.

5.2. VFA

Microbial fermentation of non-starch polysaccharides and oligosaccharides results in a greater production of volatile fatty acids (VFA) such as acetate, butyrate and propionate within the GIT (Gaskins., 2001, Richards *et al*., 2005). These fermentation by-products are important for water and sodium absorption, gut motility, vitamin production, energy supply and gut immunity. These VFAs also reduce the pH within the intestine, thereby increasing resistance to colonizing pathogens (Lalles *et al*., 2007). Factors, such as the content of fermentable carbohydrates within diet, retention time and microbial activity, greatly influence VFA concentration (Heo *et al*., 2010).

5.3. Ammonia

Ammonia is a toxic compound (Visek., 1978) and a high concentration can have a negative impact on gut health (Lin & Visek., 1991). It has been reported that excess protein in the diet, putrefaction of mucosal cells and urea are the main sources of nitrogen for intestinal bacteria. Moreover, amino acid deamination and urea hydrolysis are the principal sources for ammonia production in the small intestine, while in the large intestine the majority of ammonia production results from amino acid deamination. The role of intestinal bacteria has proven to be more valuable in recycling ammonia in pigs (Miller *et al*., 1991). Indeed, a high concentration of ammonia may promote viral and bacterial infections in the host (Visek., 1978).

6. Alternatives to antibiotics

Growth promoting antibiotics included in the diet have traditionally been used to improve growth performance and reduce enteric infections such as PWD in pigs. Usage of antibiotics at higher frequencies has resulted in antibiotic resistance bacteria (commensal and pathogens) in addition to well founded adverse effects on human health (Casewell *et al*., 2003, McEwen & Fedorka-Cray., 2002, Vondruskova *et al*., 2010). Frequently used dietary antibiotics within the swine industry are chlortetracycline-sulphametazine-penicillin and tylosin-furazolidone. Antibiotics such as gentamicin, apramicin and neomycin are mainly used in the treatment and prevention of diarrhea caused by *E. coli* and *C. perfringrns* (McEwen & Fedorka-Cray., 2002). In January 2006, the use of dietary antibiotics was banned in the European Union, therefore alternatives to antibiotics has become a necessity (Casewell *et al*., 2003). To date, a number of alternatives have been proposed to reduce or prevent PWD in piglets. Such alternatives include vaccines, spray-dried plasma, bacteriophages, organic acids, charcoal, prebiotics, probiotics and synbiotics in addition to different nutritional strategies, such as feeding a low-CP diet.

6.1. Vaccines

Several efforts have been made to produce a vaccine to protect against PWD. These vaccines may be comprised of inactivated whole cells, live attenuated bacteria, *Shigella*, *Vibrio cholera* and *Salmonella typhi* hybrids delivering ETEC antigens, purified fimbrial antigens, microencapsulated purified fimbriae, fimbrial conjugates and LT toxin sub-units (Hodgson & Barton., 2009). Parenteral vaccines administrated to sows are usually not very effective against

neonatal diarrhea as they mainly induce systemic rather than mucosal immunity to their piglets (Van den Broeck *et al*., 1999).

In general, a successful vaccine against PWD should induce specific antibodies inhibiting adhesion and/or neutralizing toxins while providing protection for a wide range of prevalent ETEC strains. To date, cross-protection between ETEC strains carrying different fimbriae has not shown much promise (Hodgson & Barton., 2009). Purified F4 fimbriae vaccine has demonstrated protection against ETEC F4 (Van den Broeck *et al*., 1999, Verdonck *et al*., 2005, Verdonck *et al*., 2008). Active oral immunization a live ETEC strain with F18 fimbriae has also been reported to be effective against PWD and edema disease (Bertschinger *et al*., 2000). Oral immunization of piglets with live attenuated avirulent *E. coli* carrying fimbrial adhesins contributes to protection against PWD. The vaccines induce local intestinal antibodies upon attachment to the host intestinal epithelium resulting in blocking the colonization of pathogenic *E. coli* (Fairbrother., 2006). In this sense, studies have shown some improvements in vaccine production against PWD, yet they are not very effective against ETEC (Hodgson & Barton., 2009).

6.2. Spray-dried plasma

Passive immunization with spray-dried plasma (SDP) is another method to reduce the incidence of PWD in young piglets. Previous studies have demonstrated the efficacy of a spray-dried porcine plasma (SDPP) diet to protect young pigs (Owusu-Asiedu *et al*., 2003, Van der Peet-Schwering & Binnendijk., 1997). This is primarily due to the presence of the anti-ETEC antibodies and complex protein fractions in the SDPP (Fairbrother., 2006, Owusu-Asiedu *et al*., 2003). On the other hand, Van Dijk et al. (2002) observed no protective effects against PWD

among young piglet models treated with a commercial dosage of SDPP however, improvements in weight gains and fecal scores were observed (Van Dijk *et al*., 2002). Overall, the SDPP-based diet is an expensive approach to protect young pigs against PWD and is currently banned in Europe due to its animal origin (Fairbrother *et al*., 2005).

6.3. Bacteriophages

Bacteriophages are viruses that infect only bacteria. They are non-toxic to other hosts and are majorly species specific or specific to one strain of bacterial host. Bacteriophages are able to invade the host and increase rapidly in number leading to lysis of the host. Commonly associated phages with Enterobacteriaceae belong to the order of *Caudovirales* (dsDNA tailed phages). They possess two life cycles; a virulent cycle in which they cause immediate death to the host and a temperate cycle in which their DNA integrates into the DNA of the host or stays as a plasmid (quiescent state) (Hodgson & Barton., 2009).

As bacteriophages are highly host specific, they can be used to reduce undesirable bacteria in the GIT. Smith et al., (1983) tested the effectiveness of phages P433/1, P433/2 and P433/1 against diarrhea. Theses phages were shown to successfully reduced diarrhea in piglets infected with *E. coli* 987P. Jamalludeen *et al.* (2007) evaluated the ability of nine bacteriophages against O149 ETEC. They reported that all nine phages were suitable for prophylaxis and therapy of PWD. Some concerns to bacteriophage therapy include transfer of virulence and antimicrobial drug-resistant genes or viruses to other non-pathogenic bacteria in the GIT, in addition to potential allergic reactions of the host to the phage (Fairbrother *et al*., 2005, Hodgson & Barton., 2009). Thereby, further experiments are necessary to confirm the potential use of bacteriophage therapy.

6.4. Organic acids

Organic acids (OA) possess antimicrobial properties in addition to their beneficial effects on digestibility, performance and nutrient resorption (Freitas *et al*., 2006, Roth & Kirchgessner., 1998). Antimicrobial effect of OA relies on either prevention of growth of pathogenic bacteria by lowering the pH in the GIT, or penetration in their non-disassociated form through the bacterial cell wall and destroying them (Eidelsburger *et al*., 1992, Hansen *et al*., 2007, Roth *et al*., 1992). Based on their antimicrobial properties, they are classified into two groups. The first group of OA (fumaric and citric acids) is characterized by an indirect effect on reducing bacterial population by lowering the pH in the stomach. The second group of OA (formic, acetic, propionic, and sorbic acids) is characterized by a direct effect of preventing deoxyribonucleic acid replication of the cell wall of Gram-negative bacteria by lowering the pH in the GIT (Vondruskova *et al*., 2010).

Previous studies have shown that addition of OA to young piglets was effective at reducing the population of enteric bacteria in the stomach (Hansen *et al*., 2007). In addition, Owusu-Asiedu *et al.* (2003) reported that fumaric acid was effective in reducing the incidence of PWD in young piglets infected with ETEC K88 (Owusu-Asiedu *et al*., 2003). Likewise, Tsiloyiannis *et al.* (2001) demonstrated that OA were effective in reducing the incidence and severity of PWD. However, one study failed to observe any positive effect of OA on diarrhea in pigs (Risley *et al*., 1993). Thereby, further studies are warranted to evaluate the effect of OA against PWD.

6.5. Charcoal

Charcoal is a black powder and considered an effective adsorbent in the removal of bacteria and their toxins both in *vitro* and in *vivo* (Naka *et al*., 2001, Pegues *et al*., 1979). It is primarily used for poison decontamination to prevent systemic absorption of toxic molecules from the GIT (Gaudreault., 2005, Tomaszewski., 1999). Charcoal is produced by pyrolysis of carbonaceous material such as wood, purified (removing non-carbon impurities) and then oxidized at high temperatures in order to form internal pores (Gaudreault., 2005, Mattson & Mark., 1971). The unique adsorption capacity of charcoal relies on its enormous internal pores that significantly increase its sorptive surface area. In addition, the pore size is important for charcoal's adsorptive capacity as bigger molecules require charcoal with large pores and smaller molecules need smaller pores (Chandy & Sharma., 1998, Chandy & Sharma., 1998, Mattson & Mark., 1971, Naka *et al*., 2001).

Bacteria and toxins with a negative-charged surface bind to the positive-charged surface of charcoal through an electrostatic attraction (Knutson *et al*., 2006, van der Mei *et al*., 2008). An in *vitro* study by Naka *et al.* (2001) demonstrated that charcoal has a lower binding affinity to the GIT bacteria, *Enterococcus faecium*, *Bifidobacterium thermofilum* and *Lactobacillus acidophilus,* than to verotoxin-producing *E. coli* (VTEC) O157:H7. They observed that the number of *E. coli* O157:H7 were reduced from 5.4-5.7 × 10^6 to 0.8 ± 0.46 × 10^3 within 5 min when charcoal was included in the diet at 5 mg/ml. They also reported that the reduction of *E. coli* O157:H7 with charcoal was dose dependent and effective in removal of verotoxin. In another study by Watarai *et al.* (2005), similar observations were reported on chickens fed charcoal. They reported that charcoal has potential for clinical use, as it is capable of binding more effectively to *Salmonella enteritidis* than to the normal GIT bacteria such as *E. faecium*. In

contrast, Knutson *et al.* (2006) observed no reduction in the number of *E. coli* O157:H7 and *Salmonella typhimurium* in the GIT of sheep. Charcoal has been recommended as an alternative treatment for diarrhea and as an adsorbent of gasses and harmful products within the GIT (Totusek & Beeson., 1953). In previous studies, it was reported that charcoal is efficient at reducing scours and increasing weight gains in pigs receiving charcoal (Frolich, G., and H. Luthge., 1934, Lentz., 1932). However, Totusek *et al.* (1953) failed to confirm those observations when pigs received a diet containing 0.5 or 3 percent charcoal.

Additional research is required regarding the adsorbent capacity of charcoal on other normal GIT and toxins before any solid conclusions can be made. As noted by Naka *et al.* (2001), they suggest that the binding capacity of activated charcoal to non-pathogenic *E. coli* and other serotypes of VTEC strains is almost the same as that to *E. coli* O157:H7 as charcoal is a non-specific adsorbent.

6.6. Probiotics

Probiotics are living, non-pathogenic microorganisms that possess beneficial effects for hosts (Gillor *et al*., 2008) and may not necessarily be part of the normal flora of the GIT. These organisms are largely members of the genera *Lactobacillus*, *Bifidobacterium* and *Bacillus*. Other bacteria such as avirulent *E. coli* and *E. faecium* and yeasts are also used as probiotics (O'sullivan *et al*., 1992). A large body of studies on probiotics demonstrates the role of these microorganisms in disease prophylaxis and improvement of GIT health. These microorganisms are able to out-compete pathogens by competing for nutrients or binding sites in the GIT, while some secrete antimicrobial substances such as SCFA and hydrogen peroxide or produce toxic compounds such as bacteriocins (Gillor *et al*., 2008, Sherman *et al*., 2009, Vondruskova *et al*.,

2010). Other beneficial effects include enhancing the integrity and function of the epithelial barrier, increasing the production of mucins and secretion of antibacterial peptides such as defensins and modulating host immune responses (Lebeer *et al*., 2010, Sherman *et al*., 2009).

A recent study by Zhang *et al.* (2010) demonstrated that *Lactobacillus rhamnosus* GG (LGG) was effective in reducing PWD in piglets challenged with ETEC K88. They observed an increase in the number of lactobacilli and bifidobacteria while decreasing the number of *E. coli* shed in the feces of piglets treated with LGG. They also reported higher serum IgA and lower IL-6 in piglets. They concluded that LGG is able to ameliorate diarrhea via modulation of intestinal microflora, enhancement of intestinal antibodies and regulation of systemic inflammatory cytokines (Zhang *et al*., 2010). Likewise, *E. faecium* has been found to have a protective function in piglets when administrated from birth to weaning. However, no benefit was observed when *E. faecium* was added to an electrolyte solution when diarrhea was occurring (Zeyner & Boldt., 2006). Shu *et al.* (2001) demonstrated that supplementation of the probiotic *Bifidobacterium lactis* strain HN019 in piglet diets reduced the severity of PWD associated with rotavirus and *E. coli* via an enhanced immune-mediated protection mechanism. They reported higher blood leukocyte phagocytic and T-lymphocyte proliferative responses and higher GIT pathogen-specific antibody titers in piglets receiving *B. lactis* HN019. Furthermore, *Bacillus subtilis* was found to be effective in reducing PWD at 24 h after challenge in piglets induced with ETEC K88 (Bhandari *et al*., 2008b). In another study, administration of spores of *B. licheniformis* and *B. subtilis* reduced morbidity and mortality in weaned piglets and improved performance parameters of finishing pigs (Alexopoulos *et al*., 2004).

6.6.1. Colicin-producing *Escherichia coli* as probiotics

Bacteria produce a range of allelopathic substances to exclude or displace other competing microorganisms. Allelopathic substances are toxic to other microorganisms, yet not to their producers. Such substances (mainly metabolic by-products) include ammonia, hydrogen peroxide, SCFA, bacitracin, polymyxin B, lysozyme-like bacteriolytic enzymes and bacteriocins. These substances play a major role in intra- and inter-specific interactions and in maintaining biodiversity (Gillor *et al*., 2008, Gordon *et al*., 2007).

Colicins are subset of bacteriocins that is produced by *E. coli* bacteria. Colicin enables a strain of *E. coli* to out-compete other strains and closely related species in the GIT (Gillor *et al*., 2009). Colicins differ from antibiotics in their narrow range of activity. This relies on the limited receptor-bearing strains of the same species, *E. coli*, and closely related species of Enterobacteriaceae (Trcka & Smarda., 2003). It has been reported that 24% of human *E. coli* isolates and 33% of mammalian isolates were colicin producers (Gordon *et al*., 2007). Previous studies have demonstrated higher colicinogenecity in ETEC strains rather than commensal *E. coli* strains (up to 80%). However, the vast majority of saprophytic strains of commensal *E. coli* were resistant to the ETEC colicin (mainly colicin Ia). In addition, no effect of ETEC colicins was observed during establishment in the GIT. Therefore, it can be concluded that ETEC colicins have no significant role in the pathogenesis of PWD infection (Trcka & Smarda., 2003).

To date, more than 30 colicins have been identified with different modes of action. These modes of action include formation of ion-permeable pores through the inner cell membrane, DNase activity, RNase activity (against rRNA or tRNA) and interference with cell wall peptidoglycan synthesis (Gillor *et al*., 2009, Trcka & Smarda., 2003). Colicins are classified

into two groups, enzymatic colicins (E2, E7, E8, E9, E3, E4, E6, D and E5) and channel-forming colicins (A, B, E1, Ia, Ib, K and N). Colicins E2, E7, E8 and E9 are endodeoxyribonucleases, E3, E4 and E6 are endoribonucleases, and D and E5 are protein synthesis inhibitors. Channel-forming colicins bind to their receptor on the target cell and then are translocated through the cell envelope. Following that, they become inserted in the membrane and form voltage-dependent ion channels on the cytoplasmic membrane resulting in transmembrane electrochemical gradient disruption (Alonso, Guillermina 2000). Colicins are mainly plasmid encoded and contain three functional domains, receptor recognition domain, protein translocation domain and a killing domain.

Colicins hold a great deal of promise in reducing PWD. Cutler, et al. (2007) reported the efficacy of dietary inclusion of a high dose (16.6 mg/per kg) of colicin E1 in reducing PWD in piglets infected by ETEC F18. However, the dietary inclusion of a low dose (11 mg/kg) of colicin E1 resulted in a slight delay of the onset of PWD. An in *vitro* study by Stahl *et al.* (2004) demonstrated that both colicin E1 and N were effective against ETEC K88. In another in *vitro* study, Setia *et al.* (2009) screened a large collection of environmental *E. coli* strains and reported that 14 of them to inhibit the ETEC K88. They also concluded that two of those strains (UM2 and UM7) producing colicin N, S4, B, and D were able to grow on starch and inulin, commonly used prebiotics in weaned piglet diets, and were able to out-compete the ETEC K88. Krause *et al.* (2010) reported that those same two strains (UM2 and UM7) in combination with raw potato starch reduced the negative effects of ETEC K88 in piglet models.

In conclusion, bacteriocins such as colicins are highly specific to their target bacteria without adverse effects on the mammalian host (no residual absorbance). Thereby, they hold particular promise as alternatives to antibiotics used for PWD (Gillor *et al*., 2008).

6.7. Prebiotics

Prebiotics are non-digestable, fermentable carbohydrates included as part of the diet. They selectively promote the proliferation of beneficial bacteria, such as lactobacilli and bifidobacteria, associated with a healthy GIT. Therefore, prebiotics can influence VFA and lactic acid production, branched-chain ratio and the pH in the GIT to improve gut health. Such prebiotics commonly used include lactulose, inulin, mannanoligosaccharides, galactooligosaccharides, fructooligosaccharides, soybeanoligosaccharides, isomaltooligosaccharides and xylooligosaccharides (Hodgson & Barton., 2009). Fibrous components and resistant starch are also recommended as prebiotics (Topping *et al*., 2003, Verstegen & Williams., 2002). Kiarie *et al.* (2008) found that non-starch polysaccharide (NSP) products reduced the severity of PWD in piglets challenged with ETEC K88. In another study, raw potato starch was found to be effective in prevention of PWD in weaned piglets infected by ETEC K88 (Bhandari *et al*., 2008a). Prebiotics are also recommended in conjunction with probiotics to support the growth and activity of probiotics (Hodgson & Barton., 2009). Shim *et al.,* (2005) demonstrated that feeding 0.2% oligofructose and 0.5% synbiotics (0.3% probiotics and 0.2% oligofructose) in combination markedly reduced the number of *E. coli* in the colon. They also observed a significant increase in the number of bifidobacteria in the ileum and colon of pigs fed oligofructose and synbiotics.

6.8. Dietary crude protein

An alternative nutritional strategy to reduce the incidence and severity of PWD is through manipulating dietary levels of CP. It has been suggested that protein availability in the GIT directly affects the proliferation of its microbiota. One study demonstrated that an increase

in dietary levels of CP favours the growth of pathogenic bacteria such as ETEC (Wellock *et al*., 2006). The effect of higher CP may be attributed to the fact that higher levels increase undigested protein that reaches the distal small intestine and colon, thus increasing the growth of nitrogen utilizing bacteria (Stein & Kil., 2006). This then leads to the production of toxic compounds such as ammonia, amines, indoles, phenols and BCFA, which are harmful to gut health (de Lange *et al*., 2010, Gaskins., 2001, Stein & Kil., 2006). Therefore, it would be anticipated that a reduction in dietary CP levels might inhibit the proliferation of pathogenic bacteria, including ETEC, and reduce the incidence of PWD. A study by Heo, *et al.* (2008) revealed that a reduction in dietary CP from 243 d CP/kg to 173 g CP/kg decreased the incidence of diarrhea without affecting growth rate.

Data on the effects of dietary CP inclusion levels on microbial populations are controversial. A study by Prohaszka, *et al.* (1980) revealed that piglets fed a high-CP diet (21%) had higher hemolytic *E. coli* counts in the small intestine. In addition, an increase in the population of *Bifidobacteria* was reported in the colon of piglets fed low-CP diet (1s40 g/kg) in comparison to piglets fed high-CP diets (200 g/kg) (Lynch *et al*., 2007). On the contrary, Bikker *et al.* (2006) found no influence of CP levels on coliforms and lactobacilli counts in the jejunum and colon of piglets fed either high or low-CP diets. In addition, they also failed to observe any changes in VFA concentration in the jejunum and colon. Likewise, Nyachoti *et al.* (2006) demonstrated that low-CP diets had no effect on coliform counts, including *E. coli*, in ileal digesta. This indicates that other factors such as environment cleanliness where piglets were housed may play a role. Most of our knowledge on the effect of dietary CP on the GIT microbiota is limited to culture-based techniques. Therefore, further studies using culture-

independent techniques may resolve any obstacles and complications regarding this production issue.

HYPOTHESIS AND OBJECTIVES

The main objective of this thesis was to develop new alternatives to antibiotics in order to reduce the incidence and severity of ETEC K88 infection in early-weaned pigs. To achieve this, secondary objectives were considered and listed as follows:

1. To evaluate the potential effects of charcoal on incidence and severity of PWD, performance, and gut microbial population of early-weaned pigs experimentally infected with ETEC K88, using indices of the GIT health and culture-dependent and culture-independent techniques.
2. To evaluate the potential synergistic effects of different levels of CP and colicin-producing *E. coli* strains on incidence and severity of PWD, gut microbial population, and inflammatory immune response of early-weaned pigs experimentally infected with ETEC K88.

MATERIALS AND METHODS

This study was reviewed and approved by the University of Manitoba Animal Care Committee. Animals were cared for according to the Canadian Council on Animal Care (Canadian Council on Animal Care (CCAC)., 1993). The experiment on evaluating any synergistic effects of different dietary CP levels and colicin-producing *E. coli* probiotic strains was part of a larger study performed by Bhandari *et al.* (2010). For further information regarding animals, housing, experimental design, experimental diets (Table 2), bacterial culture and animal performance refer to the journal article by Bhandari *et al.* (2010).

1. Animals, maintenance, and experimental design – Charcoal experiment

A total of 36 healthy weaned piglets (19 ± 21 days old) with an initial BW of 6.19 ± 0.22 kg were obtained from the University of Manitoba Glenlea Swine Research Farm (Winnipeg, MB, Canada). On arrival, piglets were allowed to adapt to the new environment for 4 days before beginning the experiment in a Level 2 animal facility at the University of Manitoba (Winnipeg, MB, Canada). During adaptation, piglets received a non-treated corn, wheat and soybean meal diet. On the first day of the trial, each piglet was marked, weighed and assigned to an individual cage. Piglets were randomly assigned to experimental treatments based on their sex and initial BW. This trial was conducted as a completely randomized design. The experimental design was based on six dietary treatments with six replicates (piglets) for each diet. Piglets had unlimited access to feed and water throughout the two week experimental period. Cages were equipped

with a plastic feeder, a low-pressure nipple-type drinker and a plastic-cover expanded metal floors. Room temperature was maintained at 29 ± 1°C.

2. Experimental diets – Charcoal experiment

Piglets were fed acorn-wheat-soybean meal-based diet formulated to meet NRC (1998), nutrient requirements for piglets weighing 7 to 12 kg for a 14-d period (Table 1). The Eucalyptus charcoal (Pancosma SA, Geneva, Switzerland), premix vitamins and minerals were added to the diets one week before the start of the experiment. The composition of the diets was:

- NA = basal diet with no additives
- AB =basal diet + antibiotics (0.25% of diet) (Aureo SP250: chlortetracycline, Penicillin (as penicillin G Porcine), Sulfamethazine, Alpharma Inc., Fort Lee, New Jersey, USA)
- 0.1% C = basal diet + 0.1% charcoal
- 0.5% C =basal diet + 0.5 % charcoal
- 1% C = basal diet + 1% charcoal
- 2% C =basal diet + 2% charcoal

Table 1. Composition of experimental diets – Charcoal Experiment

Composition	Diets[1]					
	NA	AB	0.1% C	0.5% C	1% C	2% C
Ingredients %						
Corn	24.61	24.36	24.51	24.41	24.11	24.11
Wheat	20	20	20	20	19.5	19
Soybean meal	20.66	20.66	20.66	20.36	20.66	20.16
Dried whey	20	20	20	20	20	20
Select menhaden fish-meal	5	5	5	5	5	5
Vegetable oil	4.18	4.18	4.18	4.18	4.18	4.18
Spray dried blood plasma	3	3	3	3	3	3
Vitamin and mineral Premix[2]	1	1	1	1	1	1
Limestone	0.65	0.65	0.65	0.65	0.65	0.65
Biolysine[3]	0.41	0.41	0.41	0.41	0.41	0.41
Biofos[4]	0.27	0.27	0.27	0.27	0.27	0.27
DL-Methionine	0.17	0.17	0.17	0.17	0.17	0.17
L-Threonine	0.05	0.05	0.05	0.05	0.05	0.05
ASP 250[5]	0	0.25	0	0	0	0
Charcoal	0	0	0.1	0.5	1	2

[1] Diets: NA = No additive, basal diet with no additives; AB = basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5 % charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal.

[2]Provided per Kg of diet: 9,000 IU of vitamin A; 1,500 IU of vitamin D3; 18 mg of vitamin E; 1.5 mg of vitamin K; 250 mg of choline; 30 mg of niacin; 27.5 mg of calcium pantothenate; 9.4 mg of B2; 2 mg of B6; 25 µg of B12; 80 µg of biotin; 0.5 mg of folic acid; 18 mg of copper, 110 mg zinc, 0.2 mg iodine, 110 mg iron, 50 mg manganese, and 0.3 mg selenium.

[3]Contains 50.7% L-Lysine; Evonik Degussa GmbH, Rodenbacher Chaussee 4, Hanau-Wolfgang, Germany.

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

[5]Aureo SP250: chlortetracycline, Penicillin (as penicillin G Porcine), Sulfamethazine; Alpharma Inc., Fort Lee, New Jersy, USA.

Table 2. Composition of experimental diets – Crude Protein Experiment

Composition	Diets[1]	
	High-CP	Low-CP
Ingredients %		
Corn	60.417	66.281
Wheat	5.000	5.000
Soybean meal	4.000	4.000
Fish meal	3.000	3.000
Whey powder	7.000	7.000
Spray-dried blood plasma	3.000	3.000
Casein	10.800	2.500
Canola oil	3.900	3.680
Limestone	0.970	0.950
Biophos[2]	0.730	1.050
Vitamin and mineral premix[3]	1.000	1.000
Biolysine [4]	0.080	1.111
DL-Methionine	0.093	0.300
L-Threonine	0.001	0.283
L-Tryptophan	-	0.092
L-Isoleucine	-	0.337
L-Valine	-	0.416
Calculated nutrient composition		
ME, MJ/kg	14.50	14.50
CP, %	22.22	17.31
Fiber, %	1.79	1.92
Lys, %	1.45	1.45
Met, %	0.59	0.54
Ile, %	0.97	0.95
Leu, %	2.09	1.49
Thr, %	0.94	0.93
Trp, %	0.28	0.28
Val, %	1.24	1.22

Table 2 (continued). Composition of experimental diets

Composition	Diets[1]	
	High-CP	Low-CP
Standardized ileal digestible AA, %		
Lys	1.35	1.35
Met + Cys	0.81	0.81
Thr	0.85	0.85
Trp	0.25	0.25
Ile	0.89	0.89
Val	1.14	1.14
Ca, %	0.80	0.80
Total P, %	0.65	0.65

[1]Diets; High-CP= High crude protein, Low-CP = Low crude protein
[2]Ca, 17%; P, 21% (Feed-Rite, Winnipeg, Manitoba, Canada)
[3]Supplied the following per kg of diet: 8,255 IU of vitamin A, 1,000 IU of vitamin D_3, 20 IU of vitamin E, 25 μg of vitamin B_{12}, 1.5 mg of vitamin K, 30 mg of niacin, 781 mg of choline chloride, 7.5 mg of riboflavin, 200 μg of biotin, 4.5 mg of pyridoxine, 1 mg of folic acid; 4 mg of thiamin, 40 mg of Mn (as MnO), 130 mg of Zn (as ZnO), 130 mg of Fe (as $FeSO_4.H_2O$), 10 mg of Cu (as CuO), 0.30 mg of Se (as Na_2SeO_3), 0.6 mg of I (as $Ca(IO_3)_2$).
[4]Contains 50.7% L-Lysine; Evonik Degussa GmbH, Rodenbacher Chaussee 4, Hanau-Wolfgang, Germany.

3. Performance monitoring – Charcoal experiment

Appearance, behavior and body temperature of piglets were recorded twice per day. Individual BW of piglets was measured on the day of assigning them to the cages (d 1), at the end of the first week (d 7) (pre-inoculation) and at the end of the second week (d 14) (post-inoculation). Feed disappearance was determined at the end of the pre-infection and post-infection period. The severity of PWD in sick weaned piglets was determined using a fecal consistency (FC) scoring system as described by Marquardt *et al.* (1999). The FC scoring was numerical described as follows: 0-normal; 1-soft feces; 2-mild diarrhea; 3-severe diarrhea. Two people with no prior knowledge of the dietary treatments were trained to distinguish different type, scoring and presence of blood in feces (twice per day). The total FC score of each piglet was used as the total score for 7 days.

4. *E. coli* strains and inoculation – Charcoal experiment

Two *E. coli* K88 strains (2-12 and I-36) that were used were kindly gifted from Dr. Carlton Gyles (University of Guelph, Guelph, ON, Canada). The strains were made resistant to levofloxacin (up to 2 mg/ml), which could then be subsequently recovered from the ileum and colon samples. Each strain was separately maintained in Luria-Bertani (LB) broth and incubated at 38°C with agitation for 16 h. Following that, 300 µl of each culture was transferred daily to 9.7 ml of fresh LB broth containing levofloxacin (starting at 0.1 µg/ml). These fresh cultures were used to inoculate 2 liter LB broth and incubated at 38°C with agitation for 16 h. To

determine colony forming units (CFU), a subsample of each strain was taken on day 8 of the experiment (inoculation day) and then serially diluted and cultivated on Eosin Methylene Blue (EMB) agar (Becton Dickson and company, Sparks, MD). The CFU of the two strains were found to be 7.1 $\times 10^9$ and 6.6 $\times 10^9$ per ml, respectively. On day 8 of the trial, healthy weaned piglets were orally challenged with a freshly mixed *E. coli* (6 ml) suspension, using a 10 ml modified syringe attached to a polyethylene tube to facilitate the inoculation towards the back of oral cavity.

5. Tissue and digesta sampling – Charcoal and crude protein experiments

At the end of experiment, piglets were sedated and euthanized by an intra-cardiac injection of sodium pentobarbital (50 mg/kg BW) (Bimeda-MTC Animal Health Inc., ON, Canada). Following that, the abdominal cavities were incised and visceral organs excised aseptically and weighed. Ileal tissue segments (proximal) (~5 cm) were cut open longitudinally, rinsed with 0.9% physiological saline to remove blood and debris, and stored in sterile sampling bags. Ileal tissue samples were transferred immediately to the laboratory for culturing of adherent bacteria. Care was taken to avoid cross contamination. Digesta samples of the ileum (proximal) were emptied into two sterile sampling bags and pH measured (Accumet Basic 15, Fisher Scientific, Pittsburgh, PA, USA). Following that, one bag was frozen in liquid nitrogen and stored at -80°C for further analysis of VFA's, lactic acid and ammonia nitrogen. The other bag was kept on ice and immediately transferred to the laboratory for further microbial analysis. The same procedure was conducted for colonic digesta samples.

Organic acid concentrations in digesta samples were determined using gas chromatography as described by Erwin *et al.* (1961). Ammonia concentration in digesta samples was determined using the indole phenol-blue method as described by and Novozamsky *et al.* (1974). Subsamples of ileal and colonic digesta (~ 2g) were suspended in 10 ml of 0.1 *M* HCl (1:5, wt/vol) to stop microbial activity and shaken over night at 180 RPM (room temperature) using an incubator shaker (New Brunswick Scientific Inc., Edison, NJ, USA). Subsequently, a 10 ml aliquot of each sample was pipetted into a polypropylene tube and kept at -25°C for VFA, lactic acid and ammonia nitrogen analysis. To measure OA concentrations, ileal and colonic digesta samples were allowed to thaw, and a 2.5 ml aliquot of each sample was transferred to a centrifuge tube. Each sample (2.55 ml) was then mixed with 0.5 ml of 25% meta-phosphoric acid, caped tightly, and frozen overnight. The next day, thawed samples were vortexed and mixed with 0.2 ml of 25% sodium hydroxide (NaOH), followed by vortexing with 320 µl of 0.3 M oxalic acid. Samples were centrifuged at 3000 RPM for 20 minutes and 1 ml of supernatant was transferred into a GC vial using a Pasteur pipette. A gas chromatography (Varian Chromatography System, Model Star 3400, Walnut, Creek, CA, USA) was used to determine OA concentrations. The column conditions were as follows: initial temperature, 175°C; initial hold time, 20 min; final temperature, 215°C; gradient, 20°C/min; carrier gas, pre purified low helium with a flow rate of 24 mL/min. To determine ammonia concentration, 50 µl of each thawed and vortexed digesta sample was transferred into 10 ml tubes followed by addition of 1.5 ml of reagent I (100 ml alkaline phenolate, 200 ml 0.05% sodium nitroprusside, 10 ml 4% Na_2EDTA) and 2.5 ml of reagent II (400 ml phosphate buffer and 100 ml 10% NaCl) and vortexing for 2 seconds at each step. Samples were incubated for 30 minutes in darkness.

Subsequently, samples were read at 630 nm using Ultraspec 3100 pro spectrophotometer (Biochrom Ltd, Cambridge, England). All analyses were conducted in duplicate.

6. Gastrointestinal microbial analysis

6.1. Culture-dependent analysis – Charcoal experiment

Ileal tissue samples were rinsed with sterile 0.9% physiological saline to remove any non-attached bacteria. Subsequently, tissue samples were scraped with a blunt knife and mucosal samples (1 g) were serially diluted in sterile peptone water (0.1%, 9 ml). Then 10 µL from each dilution 10^1 to 10^{10} of each sample were pipetted onto supplemented EMB agar with 0.4 µg/ml levofloxacin and generic EMB agar (free of antibiotic) in order to count and distinguish levoflaxecin resistant ETEC K88 from other coliforms. Plates were kept at 39 ± 1 ºC for 24 to 36 h. Colonies with a metallic green sheen on the EMB were enumerated as CFU per gram of mucosa (cfu/g mucosa).

Fresh fecal samples were randomly collected from piglets on day 7 (pre-infection) and day 8 (post-infection). Fecal sampling was conducted with caution to prevent cross contamination. One gram of each sample was serially diluted in sterile peptone water (0.1%, 9 ml) and 10 µL from each dilution 10^1 to $10^{10}$of each sample were applied to the dry surface of EMB agar containing 0.4 µg/ml levofloxacin and generic EMB agar. After incubation at 39 ± 1ºC for 24 to 36 h violet colonies with a metallic green sheen on the EMB were counted. The same procedure was conducted for ileal and colonic digesta samples.

6.2. Culture-independent analysis

6.2.1. Bacterial DNA extraction – Charcoal and crude protein experiments

The DNA was extracted from the ileal and colonic digesta samples using the ZR Fecal DNA Kit™ (Zymo Research, Orange, CA, USA) according to the manufactuer's protocol. The DNA concentration (A_{260}) and purity ($A_{260/280}$) were confirmed by spectrophotometer (Beckman DU/800; Beckman Coulter, Inc., Fullerton, CA, USA). DNA quality was further verified by PCR amplification of the variable regions (V1 and V2) of the 16S rRNA gene. Amplification occurred using the forward primer 27F (AGAGTTTGATCMTGGCTCAG) and reverse primer 342R (CTGCTGCSYCCCGTAG) (Khafipour *et al*., 2009a) and visualized on a 1% agarose gel.

6.2.2. High-throughput compositional sequencing (Pyrosequencing) – Charcoal and crude protein experiments

The effect of alternatives to antibiotics on the GIT microbiome of piglets was evaluated using a pyrosequencing-based strategy. Isolated DNA from two animals per treatment was pooled (20 ng/µl). All pyrosequencing procedures were carried out at the Research and Testing Laboratory (Lubbock, TX; http://researchandtesting.com). Bacterial tag-encoded GS FLX-Titanium amplicon pyrosequencing was conducted on 12 composite samples as described by Dowd *et al.* (2008) with some modifications. In this new approach, the 454 Genome Sequencer FLX Titanium platform (Roche Diagnostics Ltd, West Sussex, UK) using titanium reagents, titanium procedures, a one-step PCR (35 cycles), and a mixture of Hot Start and HotStart high fidelity taq polymerases was employed. Amplicons that originated and extended 250-550 bp

from the 27F region were numbered according to *E. coli* 16S rRNA, which in turn covers the variable regions, V1 to V3, of the bacterial 16S rRNA gene.

6.2.2.1. Sequence editing

Sequence editing from the ileal and colonic digesta samples was the first step in analyzing the pyrosequenced data. Sequences that were removed included non-bacterial ribosome sequences, chimeras, failed sequence reads, low quality sequence ends and tags by using a custom software, previously described by Dowd *et al.*, (2008). The resultant sequences were then quality trimmed and aligned using an open-source, platform-independent software package called MOTHUR (Schloss., 2009). Following a pipeline, commands used in MOTHER were as follows: 1) trim.seqs command (removing all sequences shorter than 200 bp, sequences containing an ambiguous base pair or a homopolymer length 8 bp), 2) unique.seqs command (removing redundant sequences, and retaining the number of times that each sequence was observed), 3) aligning of the unique sequences against 16S bacterial sequences derived from SILVA database followed by screening, filtering, and pre-clustering processes to remove columns that contained gap or "."and to reduce the noise from pyrosequencing dataset, 4) generating a distance matrix with a distance threshold of 0.1, 5) assigning retained sequences to OTU using furthest neighbor algorithm with a cutoff of 95% similarity, 6) assigning representative sequences from each to a taxonomical hierarchy with a confidence level of 60% using a Baysian approach utilized by RDP (Wang *et al*., 2007).

6.2.2.2. Alpha diversity

The MOTHUR software (Schloss., 2009) was also used to calculate sequence coverage, richness (Chao1 and ACE) and diversity indices (Simpson and Shannon) at an OTU distance of 0.05 or 5%. A rarefaction curve was also generated for treatment groups and plotted based on a re-sampling without replacement, described by Schloss (2009).

6.2.2.3. Principal component and canonical correspondence analyses

Principal component analysis was performed with genus data (> 0.1% prevalence) using the JMP software (version 8; SAS Institute Inc., Cary, NC, USA). This approach was used to identify dietary patterns. Canonical correspondence analysis was conducted with genus data and biophysical variables (FC score, pH, ammonia, VFA, and lactic acid) using CANOCO software (version 4.53; Biometris-plant research international; Wageningen, The Netherlands) to identify any correlations between ileal and/or colonic microbiota with the biophysical variables in multivariate data sets.

7. Quantitative real-time PCR – Crude protein experiment

Primers used in the present study are outlined in Table 3. Real-time PCR was carried out with an AB 7300 system (Applied Biosystems, Foster City, CA, USA). A final reaction mixture of 25 µL, contained 12.5 µL of Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA), 1.25 µL (0.5 µM) of each primer and 2 µL (10 ng) of genomic DNA. Water was used as a negative control. Each reaction mixture was completed in triplicate within an optical

Table 3. Primers used in the present study for quantitative real-time PCR

Target Group	Primer	Sequence (5´ → 3´)	Amplicon size (bp)	Reference
Eubacteria	341-357F 518-534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	189	(Muyzer *et al*., 1993)
Escherichia coli	EcoliFimH2F EcoliFimH2R	GCCGGTGGCGCTTTATTTG TCATCGCTGTTATAGTTGTTGGTCT	114	(Khafipour *et al*., 2009b)
Lactobacillus spp.	Ulac16S1F Ulac16S1R	AGCAGTAGGGAATCTTCCA ATTCCACCGCTACACATG	345	(Lan *et al*., 2004,Muyzer *et al*., 1993,Walter *et al*., 2001)
Bacteroides spp.(Bact)	Bac303F Bfr-Fmrev	GAAGGTCCCCCACATTG CGCKACTTGGCTGGTTCAG	103	(Ramirez-Farias *et al*., 2009)
Roseburia spp. and Eubacterium rectal (Rrec1)	RrecF Rrec630mR	GCGGTRCGGCAAGTCTGA CCTCCGACACTCTAGTMCGAC	81	(Ramirez-Farias *et al*., 2009)
Clostridial cluster IV (Clep)	Clep866mF Clept1240mR	TTAACACAATAAGTWATCCACCTGG ACCTTCCTCCGTTTTGTCAAC	314	(Ramirez-Farias *et al*., 2009)
Cluster IV *Ruminococcus spp.*(Rum)	Rflbr730F Clep866mR	GGCGGCYTRCTGGGCTTT CCAGGTGGATWACTTATTGTGTTAA	157	(Ramirez-Farias *et al*., 2009)
Bifidobacterium adolescentis (Bad)	Bif164F BiADO-2	GGGTGGTAATGCCGGATG CGAAGGGCTTGCTCCCAGT	298	(Ramirez-Farias *et al*., 2009)

reaction plate (Applied Biosystems, Foster City, CA, USA) covered with optical adhesive film (Applied Biosystems, Foster City, CA, USA). Cycling conditions for PCR amplification for *Lactobacillus spp., E. coli* and *Eubacteria* were as follows: one cycle of 95°C (10 min), 40 cycles of denaturation at 95°C (15 sec) and annealing/extension at 60°C (1 min), except for *Eubacteria*, which annealed at 58.5°C (1 min). Cycling conditions for *Bacteroides spp., Roseburia spp. and Eubacterium rectal*, Clostridial cluster IV, Cluster IV *Ruminococcus spp.,* and *Bifidobacterium adolescentis* were as follows: one cycle of 95°C (10 min), 40 cycles of denaturation at 95°C (15 sec), and annealing/extension at 60°C (30 sec), except for Clostridial cluster IV, Cluster IV *Ruminococcus spp.*, and *B. adolescentis*, where extension was 72°C (30 s). The efficiency (E) of each primer set was determined using pooled DNA samples (40 ng/reaction mixture) serially diluted eight fold. The PCR efficiency was calculated using the slope of the generated standard curve (the threshold cycle (CT) versus logarithmic values of different DNA concentrations) from the following equation (Denman & McSweeney., 2006): $E = 10^{(-1/slope)}$. The relative gene expression of target genes was calculated using the following equation (Pfaffl., 2001): $R_i = [(E_{target})^{\Delta CT\ target\ (controli\ -\ samplei)}]/[(E_{reference}^{\Delta CT\ ref\ (controli\ -\ samplei)}]$. All results were normalized to the 16S rRNA gene of *Eubacteria*.

8. Inflammatory cytokines and receptors PCR array – Crude protein experiment

8.1. RNA extraction

Total RNA was extracted from ileal tissue samples using the TRIzol reagent (Invitrogen^{TM}, Carlsbad, CA, USA). In brief, approximately 100 mg of tissue sample was

transferred to a 2-ml centrifuge tube containing 1 ml of TRIzol reagent and ~2 g of 1-mm Zirconial/silica beads (Biospec Products Inc., Bartlesville, OK, USA). The mixture was placed in a bead-beater (Biospec Products Inc., Bartlesville, OK, USA) and processed at maximum speed for 3 min. Once the tissue was lysed, 0.2 ml of chloroform was added to the mixture. Subsequently, the mixture was shaken (15 s), incubated at 25°C for 2 min and centrifuged at 12,000 × g for 15 min at 6°C. The upper aqueous phase was transferred to a fresh 1.5 ml centrifuge tube and 0.5 ml of isopropyl alcohol was added in order to precipitate the RNA. Following that, the mixture was incubated at 24 °C for 10 min and centrifuged at 12,000 × g for 10 min at 6°C. The supernatant was removed, the RNA pellet was washed with 1 ml of 75% ethanol and then the mixture was centrifuged at 7,500 × g for 5 min at 6°C. Following centrifugation, the RNA pellet was allowed to air-dry for 10 min and redissolved in 50 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™, Carlsbad, CA, USA).

The total RNA was further purified using the RT^2 qPCR-Grade RNA Isolation Kit (SABiosciences, A Qiagen Company, Frederick, MD, USA) according to the manufacturer's protocol. Total RNA concentration (A_{260}) and purity ($A_{260/280}$) were determined using spectrophotometer (Beckman DU/800; Beckman Coulter, Inc., Fullerton, CA, USA).

8.2. Reverse transcription (cDNA synthesis)

Reverse transcription was conducted using the RT^2 First Strand Kit (SABiosciences; A Qiagen Company, Frederick, MD, USA) following the manufacture's protocol. In brief, 8 µL (1 µg) of total RNA was mixed with Genomic DNA Elimination Buffer followed by adding 10 µL of reverse transcription cocktail containing 4 µL of reverse transcription buffer, 1 µL of primers, 2 µL of reverse transcription enzyme and 3 µL of UltraPure™ DNase/RNase-Free Distilled

Water (Invitrogen™, Carlsbad, CA, USA). The mixture was incubated at 42°C for 15 min and then immediately heated at 95°C for 5 min to stop the reaction. Subsequently, 91 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™, Carlsbad, CA, USA) was added to the mixture.

8.3. Real-Time PCR

Investigated genes in this study are outlined in Table 4. The Q-PCR was performed using a RT^2 Profiler PCR Array System Kit – Pathway-Focused Gene Expression Profiling Using Real-Time PCR (SABiosciences; A Qiagen Company, Frederick, MD, USA) and an AB 7300 system (Applied Biosystems, Foster City, California, USA). Briefly, 102 µL of diluted first strand cDNA was mixed with 1350 µL SABiosciences RT^2 qPCR Master Mix (SABiosciences; A Qiagen Company, Frederick, MD, USA) and 1248 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™, Carlsbad, CA, USA). A total reaction mixture of 25 µL was added to each well of a 96-well PCR Array Plate (SABiosciences; A Qiagen Company, Frederick, MD, USA) containing with primers of interest. Subsequently, the plate was sealed with optical thin-wall 8-cap strips (SABiosciences; A Qiagen Company, Frederick, MD, USA) and centrifuged at 1000 × g for 1 min at room temperature. Cycling conditions for PCR were as follows: one cycle of 10 min at 95°C for HotStart DNA polymerase activation, 40 cycles of 15 s denaturation at 95°C and 40 cycles of 1 annealing min at 60°C. Each reaction mixture was analyzed in triplicate.

ble 4. Genes of inflammatory cytokines and receptors

Symbol	Description	Gene Name
ABCF1	ATP-binding cassette, sub-family F (GCN20), member 1	ABC27, ABC50
BCL6	B-cell CLL/lymphoma 6	BCL5, BCL6A, LAZ3, ZBTB27, ZNF51
C3	Complement component 3	ARMD9, ASP, CPAMD1
C4A	Complement component 4A (Rodgers blood group)	C4, C4A2, C4A3, C4A4, C4A6, C4S, CO4, CPAMD2, MGC164979, RG
C5	Complement component 5	CPAMD4, FLJ17816, FLJ17822, MGC142298
CCL1	Chemokine (C-C motif) ligand 1	I-309, P500, SCYA1, SISe, TCA3
CCL11	Chemokine (C-C motif) ligand 11	MGC22554, SCYA11
CCL13	Chemokine (C-C motif) ligand 13	CKb10, MCP-4, MGC17134, NCC-1, NCC1, SCYA13, SCYL1
CCL15	Chemokine (C-C motif) ligand 15	HCC-2, HMRP-2B, LKN1, Lkn-1, MIP-1d, MIP-5, NCC-3, NCC3, SCYA15, SCYL3, SY15
CCL16	Chemokine (C-C motif) ligand 16	CKb12, HCC-4, ILINCK, LCC-1, LEC, LMC, MGC117051, Mtn-1, NCC-4, NCC4, SCYA16, SCYL4
CCL17	Chemokine (C-C motif) ligand 17	A-152E5.3, ABCD-2, MGC138271, MGC138273, SCYA17, TARC
CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	AMAC-1, AMAC1, CKb7, DC-CK1, DCCK1, MIP-4, PARC, SCYA18
CCL19	Chemokine (C-C motif) ligand 19	CKb11, ELC, MGC34433, MIP-3b, MIP3B, SCYA19
CCL2	Chemokine (C-C motif) ligand 2	GDCF-2, HC11, HSMCR30, MCAF, MCP-1, MCP1, MGC9434, SCYA2, SMC-CF
CCL20	Chemokine (C-C motif) ligand 20	CKb4, LARC, MIP-3a, MIP3A, SCYA20, ST38
CCL21	Chemokine (C-C motif) ligand 21	6Ckine, CKb9, ECL, MGC34555, SCYA21, SLC, TCA4
CCL23	Chemokine (C-C motif) ligand 23	CK-BETA-8, CKb8, Ckb-8, Ckb-8-1, MIP-3, MIP3, MPIF-1, SCYA23
CCL24	Chemokine (C-C motif) ligand 24	Ckb-6, MPIF-2, MPIF2, SCYA24
CCL25	Chemokine (C-C motif) ligand 25	Ckb15, MGC150327, SCYA25, TECK
CCL26	Chemokine (C-C motif) ligand 26	IMAC, MGC126714, MIP-4a, MIP-4alpha, SCYA26, TSC-1
CCL3	Chemokine (C-C motif) ligand 3	G0S19-1, LD78ALPHA, MIP-1-alpha, MIP1A, SCYA3
CCL4	Chemokine (C-C motif) ligand 4	ACT2, AT744.1, G-26, LAG1, MGC104418, MGC126025, MGC126026, MIP-1-beta, MIP1B, MIP1B1, SCYA2, SCYA4

Table 4 (continued). Genes of inflammatory cytokines and receptors

Symbol	Description	Gene Name
CCL5	Chemokine (C-C motif) ligand 5	D17S136E, MGC17164, RANTES, SCYA5, SISd, TCP228
CCL7	Chemokine (C-C motif) ligand 7	FIC, MARC, MCP-3, MCP3, MGC138463, MGC138465, NC28, SCYA6, SCYA7
CCL8	Chemokine (C-C motif) ligand 8	HC14, MCP-2, MCP2, SCYA10, SCYA8
CCR1	Chemokine (C-C motif) receptor 1	CD191, CKR-1, CKR1, CMKBR1, HM145, MIP1aR, SCYAR1
CCR2	Chemokine (C-C motif) receptor 2	CC-CKR-2, CCR2A, CCR2B, CD192, CKR2, CKR2A, CKR2B, CMKBR2, FLJ78302, MCP-1-R, MGC103828, MGC111760, MGC168006
CCR3	Chemokine (C-C motif) receptor 3	CC-CKR-3, CD193, CKR3, CMKBR3, MGC102841
CCR4	Chemokine (C-C motif) receptor 4	CC-CKR-4, CD194, CKR4, CMKBR4, ChemR13, HGCN:14099, K5-5, MGC88293
CCR5	Chemokine (C-C motif) receptor 5	CC-CKR-5, CCCKR5, CD195, CKR-5, CKR5, CMKBR5, FLJ78003, IDDM22
CCR6	Chemokine (C-C motif) receptor 6	BN-1, CD196, CKR-L3, CKR6, CKRL3, CMKBR6, DCR2, DRY-6, GPR-CY4, GPR29, GPRCY4, STRL22
CCR7	Chemokine (C-C motif) receptor 7	BLR2, CD197, CDw197, CMKBR7, EBI1
CCR8	Chemokine (C-C motif) receptor 8	CDw198, CKR-L1, CKRL1, CMKBR8, CMKBRL2, CY6, GPR-CY6, MGC129966, MGC129973, TER1
CCR9	Chemokine (C-C motif) receptor 9	CDw199, GPR-9-6, GPR28
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	C, EBP-beta, CRP2, IL6DBP, LAP, MGC32080, NF-IL6, TCF5
CRP	C-reactive protein, pentraxin-related	MGC149895, MGC88244, PTX1
CX3CR1	Chemokine (C-X3-C motif) receptor 1	CCRL1, CMKBRL1, CMKDR1, GPR13, GPRV28, V28
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	FSP, GRO1, GROa, MGSA, MGSA-a, NAP-3, SCYB1
CXCL10	Chemokine (C-X-C motif) ligand 10	C7, IFI10, INP10, IP-10, SCYB10, crg-2, gIP-10, mob-1
CXCL11	Chemokine (C-X-C motif) ligand 11	H174, I-TAC, IP-9, IP9, MGC102770, SCYB11, SCYB9B, b-R1
CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	PBSF, SCYB12, SDF-1a, SDF-1b, SDF1, SDF1A, SDF1B, TLSF-a, TLSF-b, TPAR1
CXCL13	Chemokine (C-X-C motif) ligand 13	ANGIE, ANGIE2, BCA-1, BCA1, BLC, BLR1L, SCYB13

Table 4 (continued). Genes of inflammatory cytokines and receptors

Symbol	Description	Gene Name
CXCL14	Chemokine (C-X-C motif) ligand 14	BMAC, BRAK, KS1, Kec, MGC10687, MIP-2g, NJAC, SCYB14, bolekine
CXCL2	Chemokine (C-X-C motif) ligand 2	CINC-2a, GRO2, GROb, MGSA-b, MIP-2a, MIP2, MIP2A, SCYB2
CXCL3	Chemokine (C-X-C motif) ligand 3	CINC-2b, GRO3, GROg, MIP-2b, MIP2B, SCYB3
CXCL5	Chemokine (C-X-C motif) ligand 5	ENA-78, SCYB5
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CKA-3, GCP-2, GCP2, SCYB6
CXCL9	Chemokine (C-X-C motif) ligand 9	CMK, Humig, MIG, SCYB9, crg-10
CARD18	Caspase recruitment domain family, member 18	ICEBERG, UNQ5804, pseudo-ICE
IFNA2	Interferon, alpha 2	IFNA, INFA2, MGC125764, MGC125765
IL10	Interleukin 10	CSIF, IL-10, IL10A, MGC126450, MGC126451, TGIF
IL10RA	Interleukin 10 receptor, alpha	CDW210A, HIL-10R, IL-10R1, IL10R
IL10RB	Interleukin 10 receptor, beta	CDW210B, CRF2-4, CRFB4, D21S58, D21S66, IL-10R2
IL13	Interleukin 13	ALRH, BHR1, IL-13, MGC116786, MGC116788, MGC116789, P600
IL13RA1	Interleukin 13 receptor, alpha 1	CD213A1, IL-13Ra, NR4
IL17C	Interleukin 17C	CX2, IL-17C, IL-21, MGC126884, MGC138401
IL1A	Interleukin 1, alpha	IL-1A, IL1, IL1-ALPHA, IL1F1
IL1B	Interleukin 1, beta	IL-1, IL1-BETA, IL1F2
IL1F10	Interleukin 1 family, member 10 (theta)	FIL1-theta, FKSG75, IL-1HY2, IL1-theta, MGC119831, MGC119832, MGC119833
IL1F5	Interleukin 1 family, member 5 (delta)	FIL1, FIL1(DELTA), FIL1D, IL1HY1, IL1L1, IL1RP3, MGC29840
IL1F6	Interleukin 1 family, member 6 (epsilon)	FIL1, FIL1(EPSILON), FIL1E, IL-1F6, IL1(EPSILON), MGC129552, MGC129553
IL1F7	Interleukin 1 family, member 7 (zeta)	FIL1, FIL1(ZETA), FIL1Z, IL-1F7, IL-1H4, IL-1RP1, IL1H4, IL1RP1
IL1F8	Interleukin 1 family, member 8 (eta)	FIL1, FIL1-(ETA), FIL1H, IL-1F8, IL-1H2, IL1-ETA, IL1H2, MGC126880, MGC126882
IL1F9	Interleukin 1 family, member 9	IL-1F9, IL-1H1, IL-1RP2, IL1E, IL1H1, IL1RP2
IL1R1	Interleukin 1 receptor, type I	CD121A, D2S1473, IL-1R-alpha, IL1R, IL1RA, P80
IL1RN	Interleukin 1 receptor antagonist	ICIL-1RA, IL-1ra3, IL1F3, IL1RA, IRAP, MGC10430

Table 4 (continued). Genes of inflammatory cytokines and receptors

Symbol	Description	Gene Name
IL22	Interleukin 22	IL-21, IL-22, IL-D110, IL-TIF, IL21, ILTIF, MGC79382, MGC79384, TIFIL-23, TIFa, zcyto18
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	EDF, IL-5, TRF
IL5RA	Interleukin 5 receptor, alpha	CD125, CDw125, HSIL5R3, IL5R, MGC26560
IL8	Interleukin 8	CXCL8, GCP-1, GCP1, LECT, LUCT, LYNAP, MDNCF, MONAP, NAF, NAP-1, NAP1
IL8RA	Interleukin 8 receptor, alpha	C-C, C-C-CKR-1, CD128, CD181, CDw128a, CKR-1, CMKAR1, CXCR1, IL8R1, IL8RBA
IL8RB	Interleukin 8 receptor, beta	CD182, CDw128b, CMKAR2, CXCR2, IL8R2, IL8RA
IL9	Interleukin 9	HP40, IL-9, P40
IL9R	Interleukin 9 receptor	CD129
LTA	Lymphotoxin alpha (TNF superfamily, member 1)	LT, TNFB, TNFSF1
LTB	Lymphotoxin beta (TNF superfamily, member 3)	TNFC, TNFSF3, p33
LTB4R	Leukotriene B4 receptor	BLT1, BLTR, CMKRL1, GPR16, LTB4R1, LTBR1, P2RY7, P2Y7
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	GIF, GLIF, MMIF
SCYE1	Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	AIMP1, EMAP2, EMAPII, p43
SPP1	Secreted phosphoprotein 1	BNSP, BSPI, ETA-1, MGC110940, OPN
TNF	Tumor necrosis factor (TNF superfamily, member 2)	DIF, TNF-alpha, TNFA, TNFSF2
CD40LG	CD40 ligand	CD154, CD40L, HIGM1, IGM, IMD3, T-BAM, TNFSF5, TRAP, gp39, hCD40L
TOLLIP	Toll interacting protein	FLJ33531, IL-1RAcPIP
XCR1	Chemokine (C motif) receptor 1	CCXCR1, GPR5

9. Statistical analysis

The PROC MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC, USA) was used to analyze all data except for the pyrosequencing data. Data from the charcoal experiment were analyzed as a completely randomized design. Enumerated bacterial data were $\log_{10}$-transformedprior to statistical analysis. Data from the CP experiment were analyzed as a randomized complete block design with 2 × 3 factorial arrangement of treatments. The main effect of protein level and supplement type and their interactions were then determined. Each pig was considered as an experimental unit (n = 6). Tukey's multiple comparison test was used to determine significance at $P < 0.05$ among experimental groups. Fisher's exact test was used to determine significance at $P < 0.05$ in expression of inflammatory cytokines and receptors of ETEC K88 infected piglets fed different levels of dietary CP supplemented with probiotics and antibiotics. Tendencies were accepted at $0.05 < P < 0.10$.

9.1. Categorical analysis of pyrosequenced data

The raw abundance values of each phylum and genus taxon were converted to percentages of that taxon in each individual sample. These data values were used to determine the statistical difference among treatments at each taxanomical level. The UNIVARIATE procedure of SAS (version 9.2; SAS Institute Inc.) was used to test for a normal distribution of the percentage data. This data was further analyzed using the PROC GLIMMIX procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC, USA) by fitting normal, Poisson and negative binomial distributions. In the case of the Poisson and negative binomial distributions, a log link

function was applied. The distribution with the best fit was determined by comparing the -2 log likelihood, Akaike information criterion (AIC) and Pearson chi-square over the degree of freedom ratio.

The UNIVARIATE procedure of SAS (version 9.2, SAS Inst., Inc., Cary, NC, USA) was applied to test the normality of distribution of the biodiversity indices. Non-normal data was transformed using the Box-Cox power transformation macro (http://www.datavis.ca/sasmac/boxcox.html) in SAS (version 9.2, SAS Inst., Inc., Cary, NC,USA) according to the model: BoxCox (y) = $(y^{\lambda} - 1)/\lambda$, if $\lambda \neq 0$ OR BoxCox (y) = log(y), if $\lambda = 0$. The best fitting value of λ for each parameter was determined using a maximum likelihood method. Subsequently, the normalized data was then analyzed using the PROC MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC, USA).

RESULTS

1. Charcoal experiment

1.1. Animal performance

The performance data for pre- and post-inoculation periods are listed in Table 5. Initial BW was similar for all weaned pigs in all dietary treatments ($P > 0.05$). No changes were observed in BW during pre- and post-infection periods ($P > 0.05$). Piglets fed the 2% charcoal diet had the lowest BW throughout the2-week experiment, although this was not statistically significant ($P > 0.05$). There was a tendency towards a decrease in the ADG of piglets fed charcoal diets during pre-infection period ($P < 0.1$) compared to those fed the AB diet. Piglets fed AB diet had the best ADG during post-infection ($P < 0.05$) and during the 2-week experiment ($P < 0.05$). There was no effect on the ADG of piglets fed charcoal during post-infection and throughout the2-week experiment compared to piglets fed the NA diet. Piglets fed the AB diet had the greatest ADFI during pre- and post-infection periods ($P < 0.05$). There was no effect on ADFI of piglets fed charcoal during post-infection and during the 2-week experiment compared to piglets fed the NA diet. The feed efficiency, represented as a G:F ratio, was not influenced by charcoal during pre- and post-infection periods ($P > 0.05$).

1.2. Fecal consistency score

Fecal scores for the post-infection period are presented in Table 5. Fecal scores for

Table 5. Performance and fecal consistency (FC) score of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Items	Diets[1]							
	NA	AB	0.1% C	0.5% C	1% C	2% C	SEM[2]	*P*-value[3]
Initial BW, kg	5.36	5.30	5.32	5.38	5.24	5.35	0.15	NS
Pre-infection BW, kg	6.70	6.83	6.30	6.48	6.34	6.19	0.22	NS
Post-infection BW, kg	8.66	9.44	8.14	8.73	8.56	8.00	0.39	NS
ADG[4], g/d								
d 0 to 7	191^{ab}	219^{a}	140^{b}	157^{ab}	158^{ab}	119^{b}	23.70	(*)
d 7 to 14	281^{b}	420^{a}	263^{b}	283^{b}	317^{b}	259^{b}	31.44	*
d 0 to 14	236^{b}	321^{a}	201^{b}	210^{b}	237^{b}	189^{b}	21.82	*
ADFI[5], g/d								
d 0 to 7	269^{ab}	296^{a}	207^{bc}	212^{bc}	212^{bc}	160^{c}	19.83	*
d 7 to 14	419^{b}	540^{a}	347^{b}	395^{b}	386^{b}	372^{b}	26.11	*
d 0 to 14	351^{ab}	418^{a}	277^{b}	304^{b}	299^{b}	271^{b}	16.93	*
Gain:Feed, g/g								
d 0 to 7	0.70	0.74	0.64	0.70	0.72	0.74	0.05	NS
d 7 to 14	0.65	0.68	0.74	0.79	0.82	0.82	0.05	NS
d 0 to 14	0.67	0.71	0.69	0.75	0.77	0.78	0.04	NS
FC score[6]								
24 h	1.08	0.47	0.94	0.82	0.67	0.44	0.22	NS
48h	1.73^{a}	0.50^{b}	1.72^{a}	1.64^{a}	0.94^{b}	0.96^{b}	0.16	*
72h	1.62	0.63	1.41	1.38	1.12	1.07	0.28	NS
104h	1.60^{a}	0.63^{b}	1.27^{ab}	0.94^{ab}	0.79^{b}	0.65^{b}	0.25	*
d 7 to d 14	1.46^{a}	0.58^{c}	1.33^{ab}	1.22^{ab}	1.08^{bc}	0.83^{bc}	0.19	*

[1]Diets; NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal
[2]Pooled standard error of the mean
[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$
[4]ADG; Average daily gain
[5]ADFI; Average daily feed intake
[6]FC; Fecal consistency score: 0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea
[a,b,c] Means with different subscripts within the same row are different.

piglets fed the NA diet were the greatest during the7 days post infection ($P < 0.05$). Piglets fed AB, 1% C and 2% C diets had the lowest FC score ($P < 0.05$) at 48, 104 h and 7 days postchallenge. A correlation was observed between charcoal doses and FC scores ($P < 0.05$) (Figure 1).

1.3. Visceral organ weights

Dietary treatments had no influence on the weights of the stomach, small intestine, caecum, colon, liver, and spleen ($P > 0.05$) (Table 6).

1.4. pH, ammonia, and organic acids

Dietary treatments had no effect on pH, ammonia and OA concentrations within the ileum and colon of ETEC K88 infected piglets ($P > 0.05$) (Table 7). There was however, a decrease in pH and ammonia concentration in the ileum of piglets fed 1% C and 2% C diets compared with those fed the NA diet. Acetic acid and total VFA concentration were considerably lower in the ileum of piglets fed AB and charcoal diets compared with those fed the NA diet. Lactic acid concentration was numerically higher in the ileum of piglets fed 0.5 to 2% C compared to piglets fed other diets. In addition, insignificant lower pH, and ammonia concentration were observed in the colon of piglets fed the AB diet compared with those fed the NA diet. There was a numerical decrease in the lactic acid concentration of 2% C fed piglets compared to piglets fed other diets. Butyric acid concentration was insignificantly higher in the colon of piglets fed charcoal compared with those fed the NA diet.

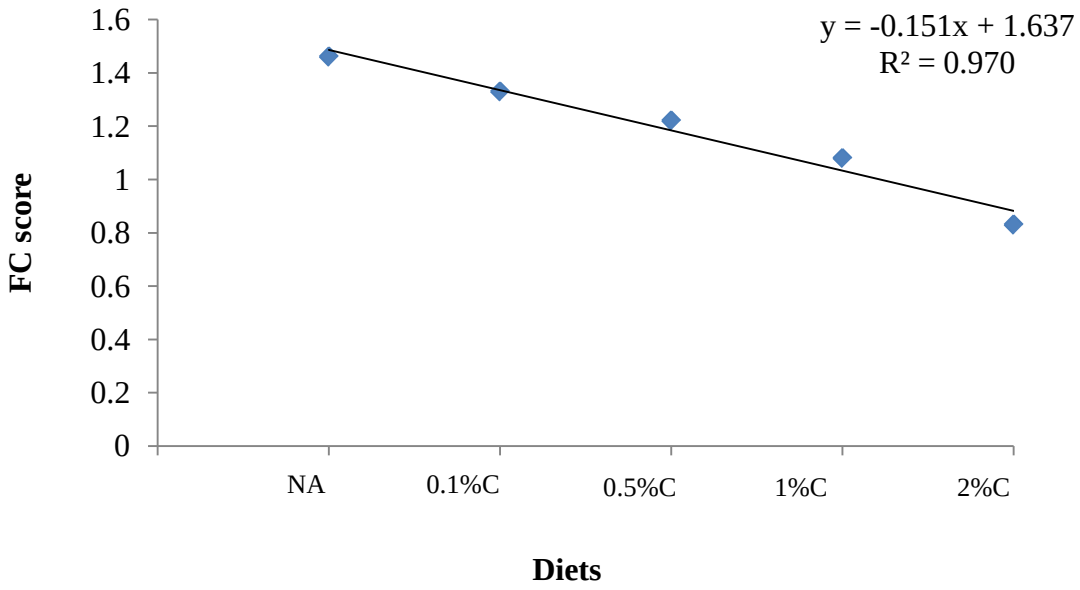

Figure 1. Effect of different doses of charcoal on fecal consistency (FC) score of ETEC K88 infected piglets. NA = no additive, basal diet with no additives; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal.

Table 6. Visceral organ weights of ETEC K88 infected piglets fed charcoal and charcoal and noncharcoal-based diets

Items	Diets[1]							
	NA	AB	0.1% C	0.5% C	1% C	2% C	SEM[2]	*P*-value[3]
% of BW								
Stomach[δ]	0.82	0.97	0.97	0.83	0.91	0.89	0.05	NS
Small Intestine[δ]	6.14	6.40	5.79	6.60	6.10	7.25	0.34	NS
Cecum[δ]	0.28	0.31	0.31	0.36	0.27	0.34	0.02	NS
Colon[δ]	1.70	1.68	1.83	1.44	1.90	1.64	0.12	NS
Liver	3.51	3.56	3.34	3.52	3.46	3.45	0.14	NS
Spleen	0.27	0.28	0.32	0.33	0.35	0.32	0.03	NS

[1]Diets: NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[δ] Emptied dried organs were weighed.

[a,b,c] Means with different subscripts within the same row are different.

Table 7. Levels of pH, ammonia, and OA in the ileum and colon of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Items	Diets[1]							
	NA	AB	0.1% C	0.5% C	1% C	2% C	SEM[2]	*P*-value[3]
pH								
Ileum	6.65	6.51	6.72	6.76	6.14	6.22	0.18	NS
Colon	6.15	5.95	5.82	6.10	5.91	6.06	0.09	NS
Ammonia, mg/L								
Ileum	12.60	12.30	12.73	13.32	9.72	9.84	1.45	NS
Colon	37.67	32.95	32.25	33.14	33.92	32.57	7.30	NS
Ileum OA concentration, mmol/L								
Acetic acid	1.52	1.27	1.07	1.07	1.14	0.86	0.21	NS
Propionic acid	0.10	0.08	0.05	0.08	0.06	0.07	0.01	NS
Isobutyric acid	0.01	0.01	0.00	0.01	0.01	0.01	0.01	NS
Butyric acid	0.03	0.02	0.02	0.02	0.03	0.02	0.01	NS
Isovaleric acid	0.00	0.00	0.00	0.00	0.01	0.00	<0.01	NS
Valeric acid	0.00	0.00	0.00	0.00	0.13	0.00	0.05	NS
Lactic acid	6.81	5.01	5.31	7.53	7.20	8.01	1.25	NS
Total	1.67	1.42	1.20	1.20	1.30	0.91	0.22	NS
Colon OA concentration, mmol/L								
Acetic acid	11.56	11.01	12.39	11.07	12.18	11.03	0.55	NS
Propionic acid	4.64	4.34	5.01	4.73	5.01	4.64	0.41	NS
Isobutyric acid	0.17	0.12	0.10	0.23	0.15	0.18	0.03	NS
Butyric acid	3.46	2.07	2.69	2.67	3.49	2.84	0.34	NS
Isovaleric acid	0.10	0.10	0.04	0.14	0. 06	0.10	0.03	NS
Valeric acid	0.73	0.64	0.56	0.62	0.90	0.69	0.10	NS
Lactic acid	1.34	0.11	0.19	0.16	0.62	0.04	0.40	NS
Total	20.99	20.02	20.74	19.45	21.87	19.49	0.97	NS

[1]Diets: NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[a,b,c] Means with different subscripts within the same row are different.

1.5. Gastrointestinal microbial analysis

1.5.1. Culture-dependent analysis

Data for enumerated *E. coli* from ileal mucosa, ileal and colonic digesta and faeces of ETEC infected piglets fed charcoal and non charcoal-based diets are presented in Table 8. Prior to inoculation with ETEC K88, levofloxacin resistant ETEC K88 was not observed in the faeces of all piglets. The ETEC K88 persisted in faeces of all piglets post infection, with counts tending to be lower in dietary treatments with antibiotics and 0.5 to 2% charcoal ($P < 0.1$). The ileal mucosal counts of ETEC K88 were lower in dietary treatments with antibiotics and charcoal ($P < 0.05$). There was a decrease in the number of ETEC K88 in the ileal and colonic digesta of piglets fed charcoal compared with those fed the NA diet ($P > 0.1$).

Table 8. *Escherichia coli* counts from the ileal mucosa, ileal and colonic digesta, and faeces of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Items	Diets[1]						SEM[2]	*P*-value[3]
	NA	AB	0.1% C	0.5% C	1% C	2% C		
Ileal mucosa								
Generic *E. coli*	5.80[a]	4.63[b]	5.28[ab]	4.58[b]	4.54[b]	4.28[b]	0.34	(*)
ETEC K88	4.79[a]	3.15[b]	3.74[b]	3.00[b]	3.03[b]	2.90[b]	0.30	*
Ileal digesta								
Generic *E. coli*	5.03	6.06	5.77	5.20	5.23	5.20	0.36	NS
ETEC K88	4.36	3.47	4.09	3.50	3.03	3.20	0.58	NS
Colonic digesta								
Generic	6.52	5.59	6.45	6.62	5.54	6.08	0.33	NS
ETEC K88	4.94	3.70	3.96	3.85	3.55	3.02	1.24	NS
Faeces (pre-infection)								
Generic *E. coli*	6.34	6.07	5.77	5.36	5.85	5.53	0.89	NS
ETEC K88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
Faeces (post-infection)								
Generic *E. coli*	7.33	6.23	7.20	6.70	7.24	5.43	0.81	NS
ETEC K88	7.55[a]	4.51[bc]	5.88[ab]	3.48[c]	4.29[bc]	3.00[c]	0.33	(*)

[1]Diets: NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[a,b,c] Means with different subscripts within the same row are different.

1.1. Culture-independent analysis - Pyrosequencing

Following trimming, sequence averages of 23,485 and 16,736, were obtained from ileal (Table 9) and colonic (Table 10) digesta, respectively. Sampling coverage of ileal and colonic digesta of piglets were ~98.5% and 92.56%, respectively. Rarefraction curves of individual and pooled pyrosequenced 16S rRNA data from the ileal (Figures 2 and 3) and colonic (Figures 4 and 5) digesta of ETEC K88 infected piglets fed charcoal and non charcoal-based diets were plotted.

In the ileal digesta, the number of estimated individual OTUs varied from 161 to 203 (Table 9) and in the colonic digesta this ranged from 458 to 855 (Table 10). The number of OTUs from ileal digesta was not affected by diets ($P > 0.05$), but this number was lower in colonic digesta of piglets fed the AB diet compared to piglets fed the NA diet ($P < 0.05$).

Richness estimators (Chao1 and ACE), diversity indices (Shannon and Simpson) and effective species (conversion of diversity indices) were determined to describe the microbial population within the ileal (Table 9) and colonic (Table 10) digesta. There were no statistical significance in richness, diversity and effective species for the ileal digesta of piglets ($P > 0.05$) (Table 9). However, AB fed piglets had numerically higher richness and diversity in the ileum compared to piglets fed other diets. Chao1 was numerically lower in the colonic digesta of piglets fed the AB diet compared with piglets fed the NA diet ($P < 0.05$) (Table 10). Piglets fed the 0.1% C diet tended to have a greater microbial diversity in the colonic digesta compared with those fed the AB diet ($P < 0.1$) (Table 10). However, no statistical significance was observed between piglets fed AB and charcoal diets compared with piglets fed the NA diet ($P > 0.1$)

(Table 10). Effective species indices were insignificantly lower for the colonic digesta of piglets fed the AB diet compared with piglets fed all other diets ($P < 0.1$ and $P < 0.05$) (Table 10).

Table 9. Statistical analysis of pyrosequenced bacterial 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Statistics	Diets[1]						SEM[2]	*P*-value[3]
	NA	AB[δ]	0.1% C[δ]	0.5% C	1% C	2% C		
Sequence Matrices								
Trimmed[4]	25,396	21,714	22,100	19,780	23,789	28,133	-	-
OTU[5]	161	203	162	162	176	178	27.98	NS
Coverage (%)	99.00	99.00	99.00	99.00	96.00	99.00	0.01	NS
Richness[6]								
Chao1	223.03	350.96	236.62	250.20	291.05	268.29	49.14	NS
ACE	279.86	435.08	288.10	296.35	391.62	304.96	66.63	NS
Diversity[7]								
Shannon	1.61	2.44	1.70	1.80	1.80	1.89	0.34	NS
Simpson	0.43	0.20	0.42	0.43	0.39	0.41	0.11	NS
Effective Species[8]								
Simpson's reciprocal index	2.50	5.01	2.76	3.02	3.87	2.67	0.99	NS
Exponential of shannon's index	5.08	11.52	6.27	7.45	7.79	6.94	2.42	NS

[1]Diets: NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[4]Number of trimmed sequences

[5]Number of operational taxonomic units (OTU)

[6]Determined by Chao1 and abundance based coverage estimation (ACE) richness estimators

[7]Determined by Shannon and Simpson diversity indices

[8]Conversion of diversity indices to true diversity (Jost., 2006)

[δ] Two replicate samples were analyzed.

Table 10. Statistical analysis of pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Statistics	Diets[1]						SEM[2]	*P*-value[3]
	NA	AB	0.1% C	0.5% C	1% C	2% C		
Sequence Matrices								
Trimmed[4]	18,238	17,521	14,525	18,371	16,843	14,916	-	-
OTU[5]	855[a]	458[b]	743[ab]	640[ab]	662[ab]	708[ab]	67.10	*
Coverage (%)	91.67	95.33	91.00	93.33	93.33	90.67	0.01	NS
Richness[6]								
Chao1	1724.78[a]	894.35[b]	1497.91[ab]	1397.47[ab]	1302.83[ab]	1451.05[ab]	148.37	*
ACE	2575.25	1390.11	2328.01	2146.41	2068.66	2263.01	343.91	NS
Diversity[7]								
Shannon	4.76[ab]	3.42[b]	4.99[a]	3.98[ab]	4.48[ab]	4.56[ab]	0.33	(*)
Simpson	0.07[ab]	0.19[a]	0.04[b]	0.13[ab]	0.07[ab]	0.10[ab]	0.03	(*)
Effective Species[8]								
Simpson's reciprocal index	15.22[ab]	6.13[b]	26.72[a]	10.85[ab]	22.58[ab]	20.78[ab]	4.27	(*)
Exponential of shannon's index	118.83[a]	38.90[b]	146.49[a]	75.03[ab]	119.51[a]	139.33[a]	18.50	*

[1]Diets: NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$

[4]Number of trimmed sequences

[5]Number of operational taxonomic units (OTU)

[6]Determined by Chao1 and abundance based coverage estimation (ACE) richness estimators

[7]Determined by Shannon and Simpson diversity indices

[8]Conversion of diversity indices to true diversity (Jost., 2006)

[a,b,c] Means with different subscripts within the same row are different.

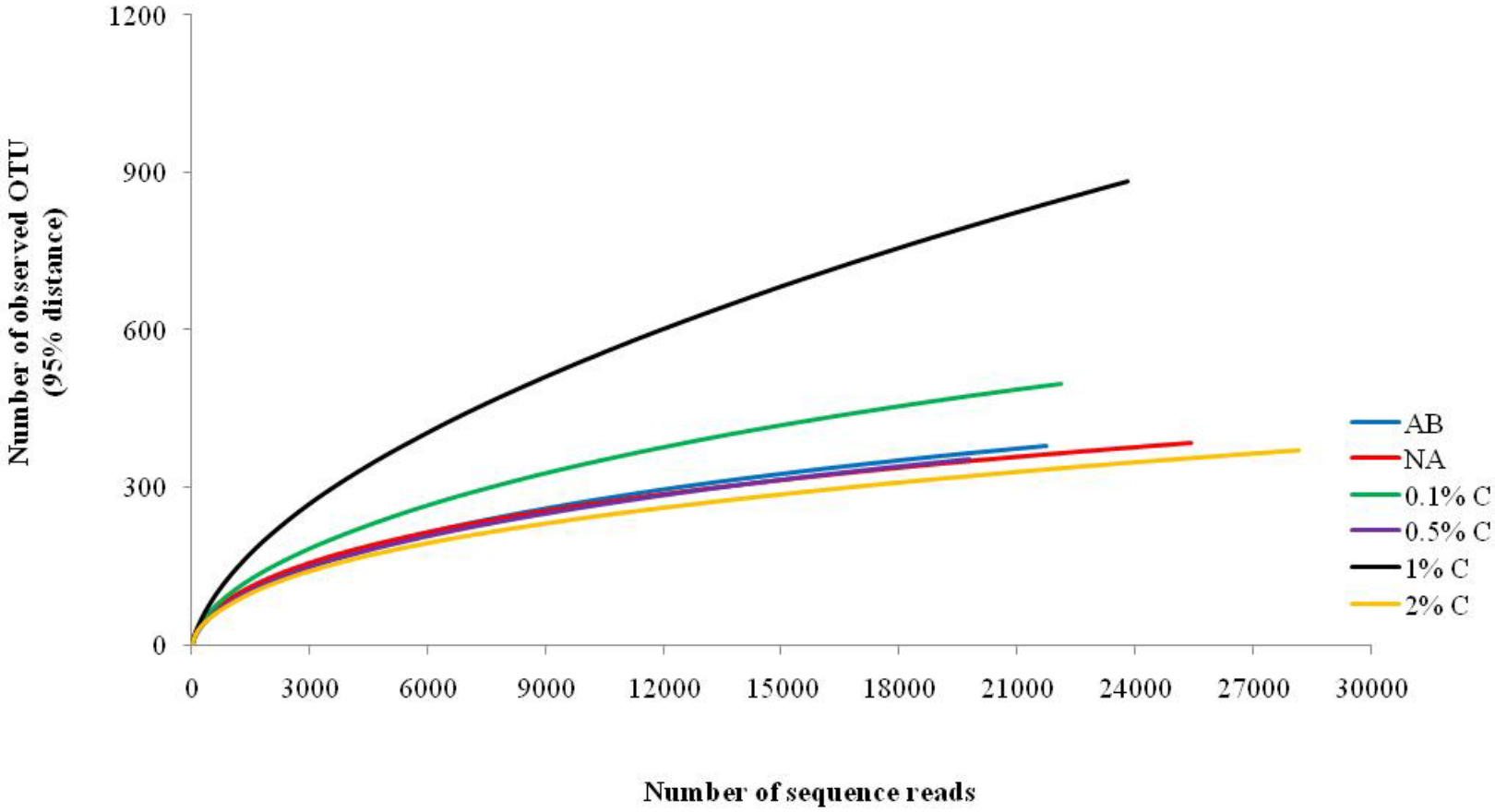

Figure 2. Rarefaction curve analysis of pooled pyrosequenced bacterial 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed charcoal and non charcoal-based diets. Diets; NA = no additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal. Operational toxonomic units (OTU) were defined at 95% similarity. The curves approximate to a plateau state, implying that the majority of the GIT microbiota present in these samples was covered.

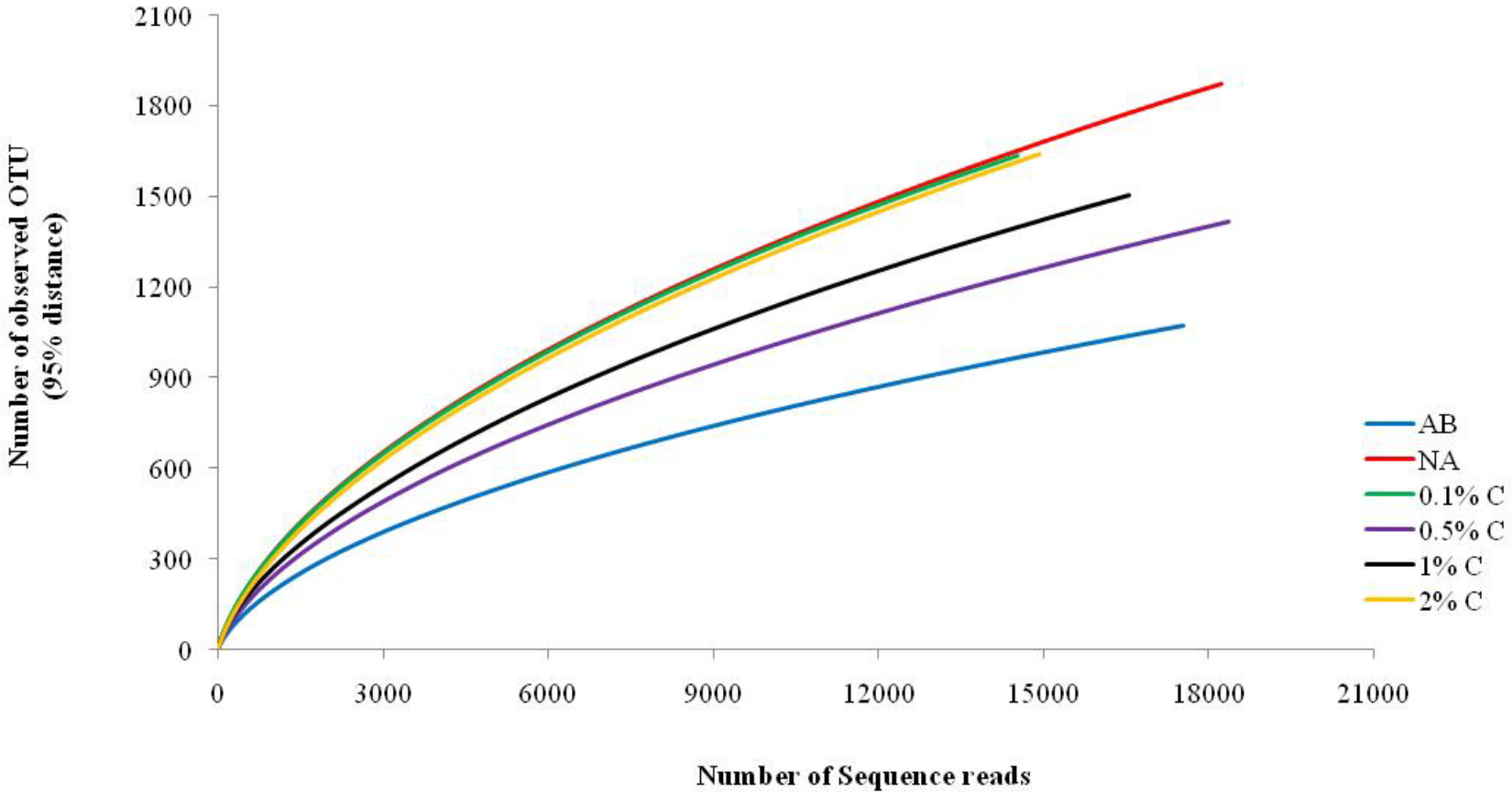

Figure 3. Rarefaction curve analysis of pooled pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed charcoal and non charcoal-based diets. Diets; NA = no additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal. Operational toxonomic units (OTU) were defined at 95% similarity. The curves approximate to a plateau state, implying that the majority of the GIT microbiota present in these samples was covered.

In the ileal digesta, five phyla were observed with the most predominant belonging to Firmicutes (Table 11). The most predominant genus was *Lactobacillus,* followed by *Clostridium, Streptococcus* and *Actinobacillus* (Table 12). Dietary treatment had no effect on the microbiota of the ileal digesta at the phylum level ($P > 0.05$). Charcoal also had no effect on ileal microbiota at the genus level. However, *Clostridium* and unclassified genus of family Rs-D42 significantly increased in the ileal digesta of piglets fed the AB diet.

Principal component analysis of the genera data from ileal digesta of ETEC K88 infected piglets fed diets with or without charcoal is presented in Figure 6. The first two principal components (PC) explained 67.75% of the total variance. The results from the PCA suggest that charcoal had no effect on the variability of bacterial genera in the ileal digesta of piglets. On the other hand, dietary antibiotics did change the bacterial genera in the ileal digesta of piglets.

Calculation of CCA for ileal digesta microbiota revealed no correlation between dietary treatments and biophysical variables ($P > 0.05$) (Figure 7). However, a trend was observed between a lower FC score and a higher percentage of charcoal in piglets fed 1% C and 2% C diets. Piglets fed the AB diet also displayed a similar effect. The genera *Clostridium, Blautia, Actinobacillus,* and unclassified genus of families Rs-D42, Clostridiaceae, and Peptostreptococcaceae were generally associated with ileal digesta of piglets fed the AB diet. The genera *Escherchia* and *Sarcina* were found to be associated with higher FC score in piglets fed the NA diet.

Table 11. Phylogenetic composition of bacterial phyla from pyrosequenced 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Phylum	Percentage of sequences in:						SEM[2]	*P*-value[3]
	Diets[1]							
	NA	AB[δ]	0.1% C[δ]	0.5% C	1% C	2% C		
Actinobacteria	†	†	0.2	0.5	†	0.1	0.2	NS
Bacteroidetes	†	0.2	0.1	†	0.4	†	0.2	NS
Cyanobacteria	0.1	†	0.1	0.1	†	†	<0.1	NS
Firmicutes	99.5	99.2	99.1	99.2	99.4	99.9	0.4	NS
Proteobacteria	0.4	0.4	0.5	0.2	0.1	0.1	0.1	NS

[1]Diets: NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[†]The phyla with lower abundance of 0.10% are omitted.

[δ]Two replicate samples were analyzed.

Table 12. Phylogenetic composition of bacterial genus from pyrosequenced 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Phylum;Family;Genus	Percentage of sequences in: Diets[1]						SEM[2]	*P*-value[3]
	NA	AB[δ]	0.1% C[δ]	0.5% C	1% C	2% C		
Firmicutes								
Lactobacillaceae; *Lactobacillus*	96.8	76.1	97.5	98.2	95.2	92.5	6.0	NS
Lactobacillaceae; *Pediococcus*	†	†	†	†	†	0.4	<0.1	NS
Rs-D42; unclassified	0.1[b]	2.8[a]	0.1[b]	0.1[b]	0.2[b]	0.4[b]	0.3	*
Streptococcaceae; *Streptococcus*	2.1	1.6	1.3	0.6	2.5	0.4	0.9	NS
Clostridiaceae; *Clostridium*	0.1[b]	15.9[a]	0.2[b]	0.2[b]	1.0[b]	0.1[b]	0.6	*
Clostridiaceae; unclassified	†	0.2	†	†	†	0.1	0.1	NS
Clostridiaceae; *Sarcina*	0.4	†	0.3	0.2	†	†	0.2	NS
Lachnospiraceae; *Blautia*	†	0.2	†	†	†	†	0.1	NS
Lachnospiraceae; Incertae Sedis	†	0.1	†	†	†	†	<0.1	NS
Lachnospiraceae; uncultured	†	0.1	†	†	†	†	<0.1	NS
Peptostreptococcaceae; unclassified	†	0.6	†	†	0.1	†	0.2	NS
Peptostreptococcaceae; uncultured	†	0.4	†	†	0.3	0.1	0.2	NS
Ruminococcaceae; uncultured	†	0.1	†	†	†	†	0.1	NS
Veillonellaceae; *Megasphaera*	†	†	†	†	†	0.1	0.1	NS
Erysipelotrichaceae; *Turicibacter*	†	0.2	†	†	0.5	0.1	0.2	NS
Proteobacteria								
Enterobacteriaceae; *Escherichia*	0.2	†	0.1	†	†	†	0.1	NS
Pasteurellaceae; *Actinobacillus*	†	0.1	†	†	†	†	<0.1	NS

[1]Diets: NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[a,b,c] Means with different subscripts within the same row are different.

[†]The genera with lower abundance of 0.10% are omitted.

[δ]Two replicate samples were analyzed.

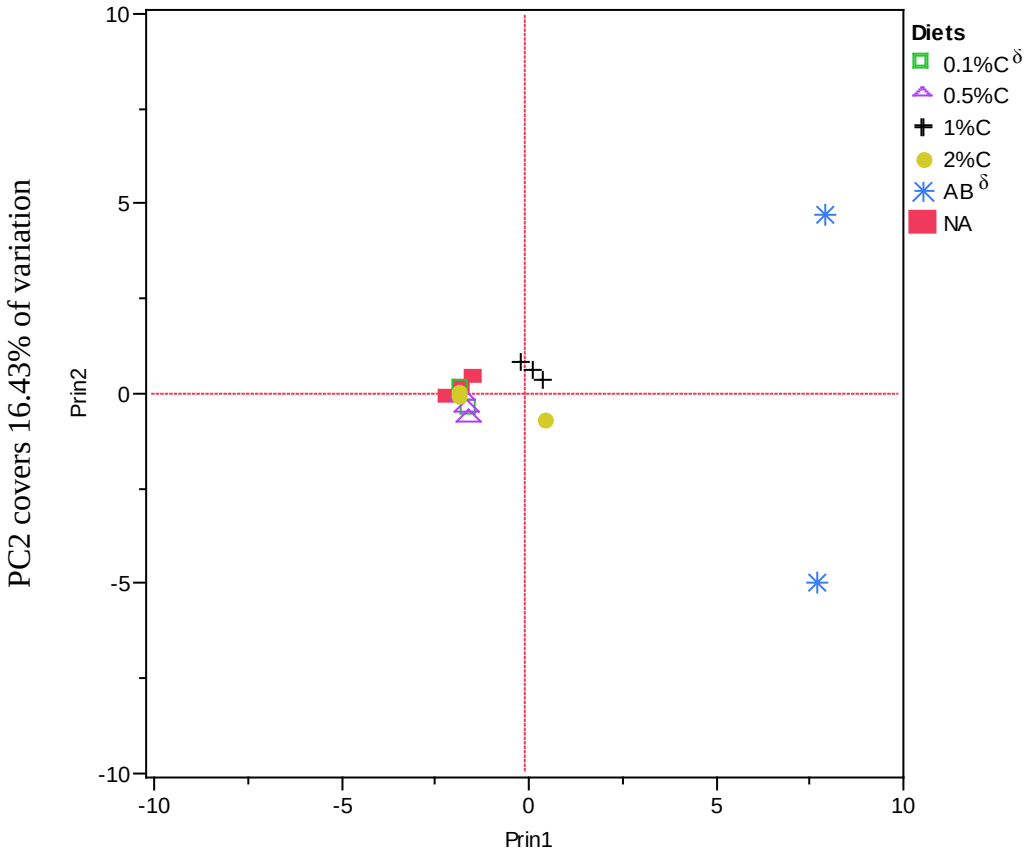

Figure 4. Principal component scatter plot of the ileal digesta at genus level of ETEC K88 infected piglets fed charcoal and non charcoal-based diets. Diets; NA = no additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal. The two principal components cover 67.75% of the variation in the data. $^{\delta}$Two replicate samples were analyzed.

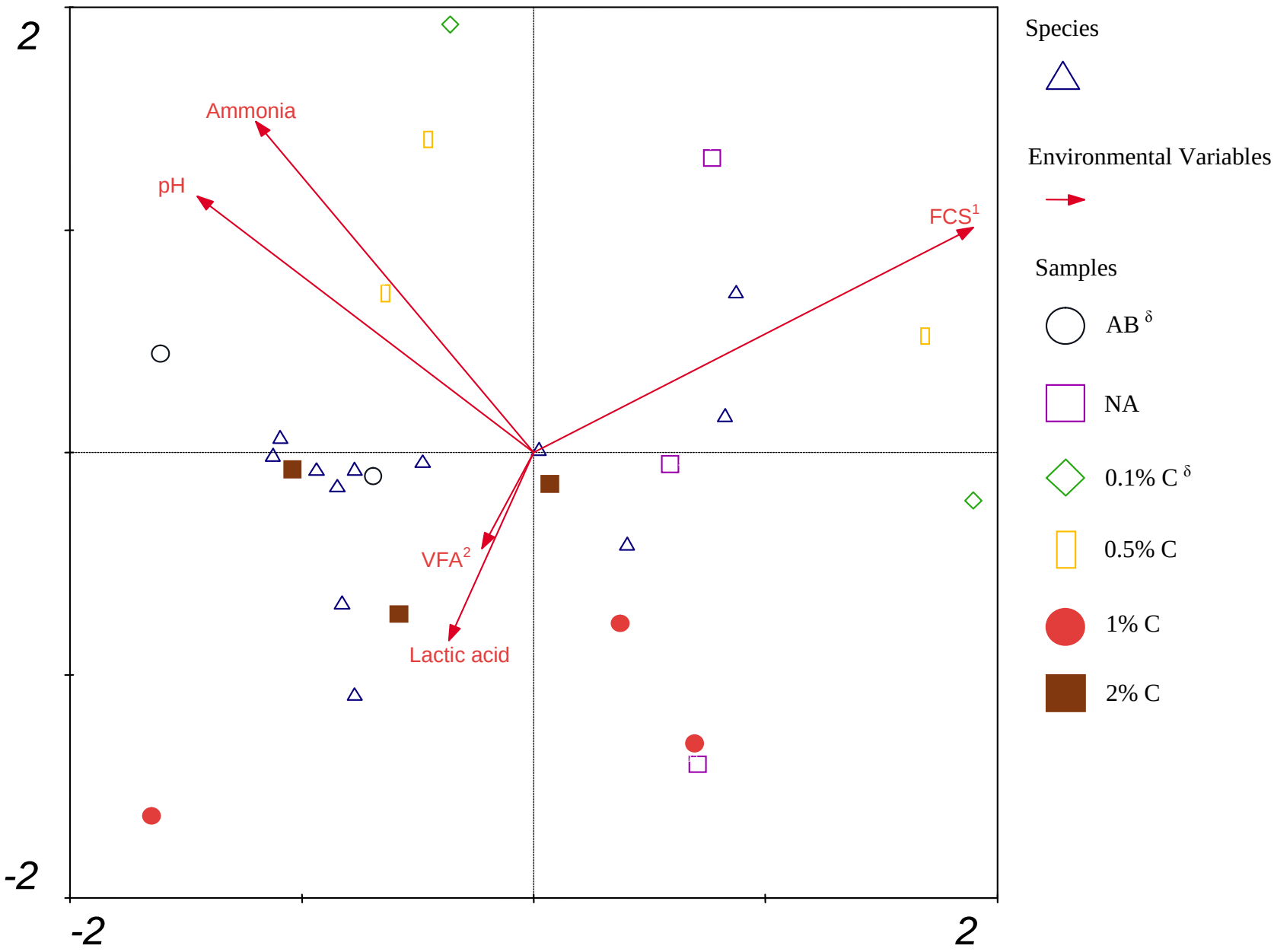
2
-2
-2
2
Ammonia
pH
FCS1
VFA2
Lactic acid
Species
Environmental Variables
Samples
AB δ
NA
0.1% C δ
0.5% C
1% C
2% C

Figure 5. Canonical correspondence analysis (CCA) of the pyrosequenced bacterial 16s RNA genes in the ileal digesta of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets. Diets; NA = no additive, basal diet with no additives; AB = antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal. (Ammonia, $P = 0.28$), (pH, $P = 0.74$), (Lactic acid, $P = 0.72$), (VFA, $P = 0.63$), and (FCS, $P = 0.18$). The distance between the genera shows their unlikeness and their position to the arrowheads shows the effect of the specific environmental variable on them. The length of the arrows shows effects of the environmental variables. The angles between the arrows show the correlations between the parameters; arrows with similar directions demonstrate positive correlation and arrows with opposite direction demonstrate negative correlation.

[1]FCS; Fecal consistency score

[2]VFA; Volatile fatty acids

[δ] Two replicate samples were analyzed.

In the colonic digesta, six phyla were identified with the most predominant phylum being Firmicutes followed by Bacteroides and Proteobacteria (Table 13). The most predominant genus was *Lactobacillus*, followed by uncultured genus of family Ruminococcaceae, and the genera *Blautia, Megasphaera, Fecalibacterium,* Lachnospiraceae Incertae Sedis, and *Roseburia* (Table 14). Dietary treatments had no effect on the colonic microbiota at either the phylum or genus levels ($P > 0.05$), with the exception of a decrease in *Subdoligranulum* ($P < 0.05$) and *Roseburia* ($P < 0.1$) in piglets fed the AB diet. Piglets fed 0.5 to 2 % C diets had a lower percentage of *Prevotella* compared with piglets fed the NA diet, although this was not statistically significant ($P > 0.05$).

Principal component analysis of the genera data from colonic digesta of piglets fed diets with or without charcoal is presented in Figure 8. The first two PC explain 38.69% of the total variation. The results from the PCA demonstrated that there was no dietary treatment effect on the variability of bacterial genera in the colonic digesta of ETEC K88 infected piglets.

Calculation of CCA for colonic digesta microbiota revealed no correlation between dietary treatments and biophysical variables ($P > 0.05$) (Figure 9). However, a trend was observed between a lower FC score and a higher percentage of charcoal in piglets fed 1% C and 2% C diets. Piglets fed the AB diet also had a similar effect. No association was observed between a specific genus and a particular dietary treatment.

Table 13. Phylogenetic composition of bacterial phyla from pyrosequenced 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Phylum	Percentage of sequences in:						SEM[2]	*P*-value[3]
	Diets[1]							
	NA	AB	0.1% C[δ]	0.5% C	1% C	2% C		
Actinobacteria	0.5	0.4	0.2	0.3	0.4	0.2	0.1	NS
Bacteroidetes	5.6	5.1	10.2	3.6	3.5	7.2	2.6	NS
Cyanobacteria	0.1	0.2	0.1	0.2	0.1	0.1	0.1	NS
Firmicutes	92.4	95.3	85.7	94.5	95.2	90.2	3.2	NS
Proteobacteria	1.2	0.5	3.7	1.3	1.7	2.2	1.4	NS
Spirochaetes	0.1	†	†	0.1	0.1	0.1	0.1	NS

[1] Diets: NA = No additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[†]The phyla with lower abundance of 0.10% are omitted.

[δ] Two replicate samples were analyzed.

Table 14. Phylogenetic composition of bacterial genus from pyrosequenced 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Phylum;Family;Genus	Percentage of sequences in: Diets[1]						SEM[2]	*P*-value[3]
	NA	AB	0.1% C[δ]	0.5% C	1% C	2% C		
Actinobacteria								
Coriobacteriaceae; *Collinsella*	0.2	0.3	0.1	0.1	0.2	0.1	0.1	NS
Bacteroidetes								
Prevotellaceae; *Prevotella*	3.9	2.2	5.6	1.0	0.8	1.1	1.1	NS
Prevotellaceae; unclassified	0.4	0.2	0.4	0.5	0.1	0.6	0.2	NS
Prevotellaceae; uncultured	0.7	0.6	1.4	0.3	0.5	1.1	0.5	NS
Rikenellaceae; RC9 gut group	0.4	0.1	0.7	0.3	0.4	0.6	0.2	NS
S247; unclassified	1.0	0.4	1.0	1.1	0.6	0.7	0.3	NS
Firmicutes								
Lactobacillaceae; *Lactobacillus*	43.4	63.9	33.6	55.7	40.5	46.4	7.0	NS
Streptococcaceae; *Streptococcus*	1.2	0.8	0.4	1.7	0.4	0.5	0.6	NS
Clostridiaceae; *Clostridium*	0.5	1.5	0.4	0.3	0.5	0.3	0.4	NS
Clostridiaceae; unclassified	0.2	0.3	0.2	0.6	0.2	0.1	0.3	NS
Clostridiaceae; *Sarcina*	0.1	0.1	0.5	0.2	0.2	0.1	0.3	NS
Family XIII Incertae Sedis; uncultured	0.5	0.1	0.3	0.1	0.1	0.1	0.3	NS
Lachnospiraceae; *Blautia*	6.1	4.0	5.6	4.6	9.9	5.0	1.8	NS
Lachnospiraceae; *Coprococcus*	0.1	†	0.6	0.3	0.2	0.6	0.2	NS
Lachnospiraceae; *Dorea*	0.9	0.3	1.0	0.4	1.9	0.8	0.4	NS
Lachnospiraceae; Incertae Sedis	3.7	1.8	3.2	2.4	5.0	2.9	0.9	NS
Lachnospiraceae; *Lachnospira*	0.1	0.1	0.1	†	0.2	†	<0.1	NS
Lachnospiraceae; *Marvinbryantia*	0.1	0.2	0.2	0.1	0.1	0.2	0.1	NS
Lachnospiraceae; *Oribacterium*	0.4	0.2	0.5	0.2	0.3	0.1	0.1	NS
Lachnospiraceae; *Roseburia*	3.4[ab]	1.1[b]	4.7[a]	2.0[ab]	3.9[ab]	3.3[ab]	0.7	(*)
Lachnospiraceae; unclassified	2.4	1.6	1.9	1.7	2.7	2.8	0.8	NS
Lachnospiraceae; uncultured	2.9	0.9	2.2	1.0	2.5	1.7	0.8	NS
Peptostreptococcaceae; Incertae Sedis	0.3	0.1	0.1	0.1	0.3	0.2	0.2	NS
Ruminococcaceae; *Anaerotruncus*	0.1	†	0.1	0.3	0.1	0.1	0.2	NS
Ruminococcaceae; *Fecalibacterium*	4.7	2.3	6.2	3.2	3.0	3.8	1.1	NS

Table 14 (continued). Phylogenetic composition of bacterial genus from pyrosequenced 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed charcoal and noncharcoal-based diets

Phylum;Family;Genus	Percentage of sequences in: Diets[1]						SEM[2]	*P*-value[3]
	NA	AB	0.1% C[δ]	0.5% C	1% C	2% C		
Ruminococcaceae; Incertae Sedis	0.5	0.3	0.8	0.3	0.6	0.3	0.2	NS
Ruminococcaceae; *Oscillospira*	0.6	0.2	0.3	0.4	0.2	0.4	0.2	NS
Ruminococcaceae; *Ruminococcus*	0.5	0.3	0.6	0.4	0.4	0.9	0.5	NS
Ruminococcaceae; *Subdoligranulum*	1.4^{ab}	0.6^{b}	1.6^{a}	1.1^{ab}	1.5^{a}	1.0^{ab}	0.2	*
Ruminococcaceae; unclassified	0.9	0.3	0.8	0.6	0.7	0.8	0.2	NS
Ruminococcaceae; uncultured	10.8	6.9	7.1	7.0	9.1	8.6	1.5	NS
Veillonellaceae; *Anaerovibrio*	0.2	0.6	0.7	0.1	0.1	0.3	0.3	NS
Veillonellaceae; *Dialister*	0.4	0.2	†	0.2	†	0.4	0.2	NS
Veillonellaceae; *Megasphaera*	3.5	2.8	2.0	2.5	6.9	2.4	2.0	NS
Veillonellaceae; *Mitsuokella*	0.3	0.1	0.1	0.1	0.1	0.3	0.2	NS
Veillonellaceae; *Phascolarctobacterium*	0.3	0.1	0.6	0.1	0.1	†	0.2	NS
Veillonellaceae; *Selenomonas*	0.1	0.1	0.1	†	0.6	0.4	0.4	NS
Veillonellaceae; unclassified	0.2	0.2	0.6	0.1	0.5	0.1	0.2	NS
Veillonellaceae; uncultured	0.7	0.4	1.4	0.4	0.3	0.7	0.3	NS
Erysipelotrichaceae; *Catenibacterium*	0.6	0.3	0.6	0.2	1.0	0.2	0.4	NS
Erysipelotrichaceae; Incertae Sedis	0.5	0.5	0.4	0.5	0.9	0.4	0.2	NS
Erysipelotrichaceae; uncultured	0.3	0.1	0.1	0.1	0.1	0.3	0.3	NS
Proteobacteria								
Neisseriaceae; *Leeia*	0.1	†	0.1	0.1	†	0.3	0.2	NS
Campylobacteraceae; *Campylobacter*	0.2	0.1	0.4	0.5	0.2	0.1	0.2	NS
Succinivibrionaceae; *Succinivibrio*	0.5	0.2	0.2	0.1	1.5	1.3	0.5	NS
Enterobacteriaceae; *Escherichia*	0.2	†	†	0.3	†	0.3	0.2	NS

[1] Diets: NA = No additive, basal diet with no additives; AB = Antibiotic, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[a,b,c] Means with different subscripts within the same row are different.

[†]The genera with lower abundance of 0.10% are omitted.

[δ] Two replicate samples were analyzed.

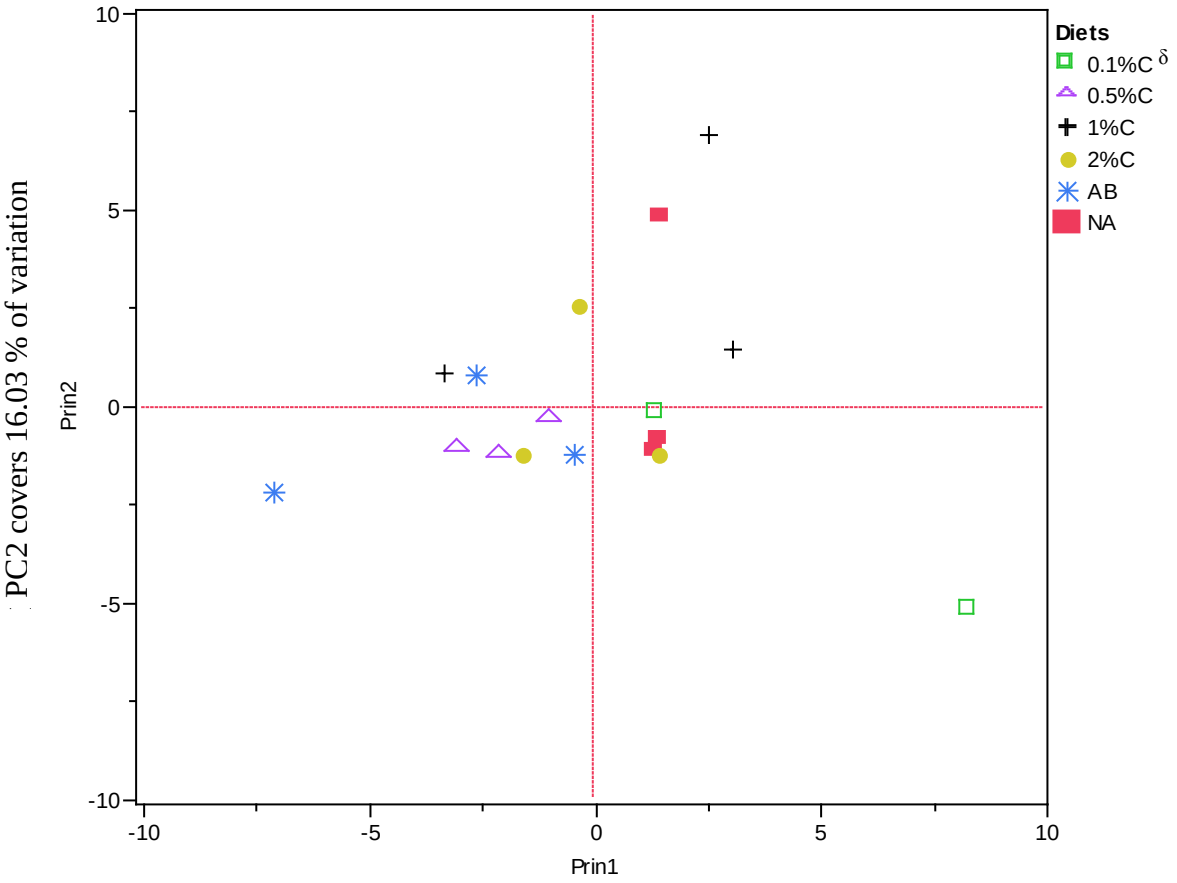

Figure 6. Principal component score plot of the colonic digesta at genus level of ETEC K88 infected piglets fed charcoal and non charcoal-based diets. Diets; NA = no additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal. The two principal components cover 38.69% of the variation in the data.
δ Two replicate samples were analyzed.

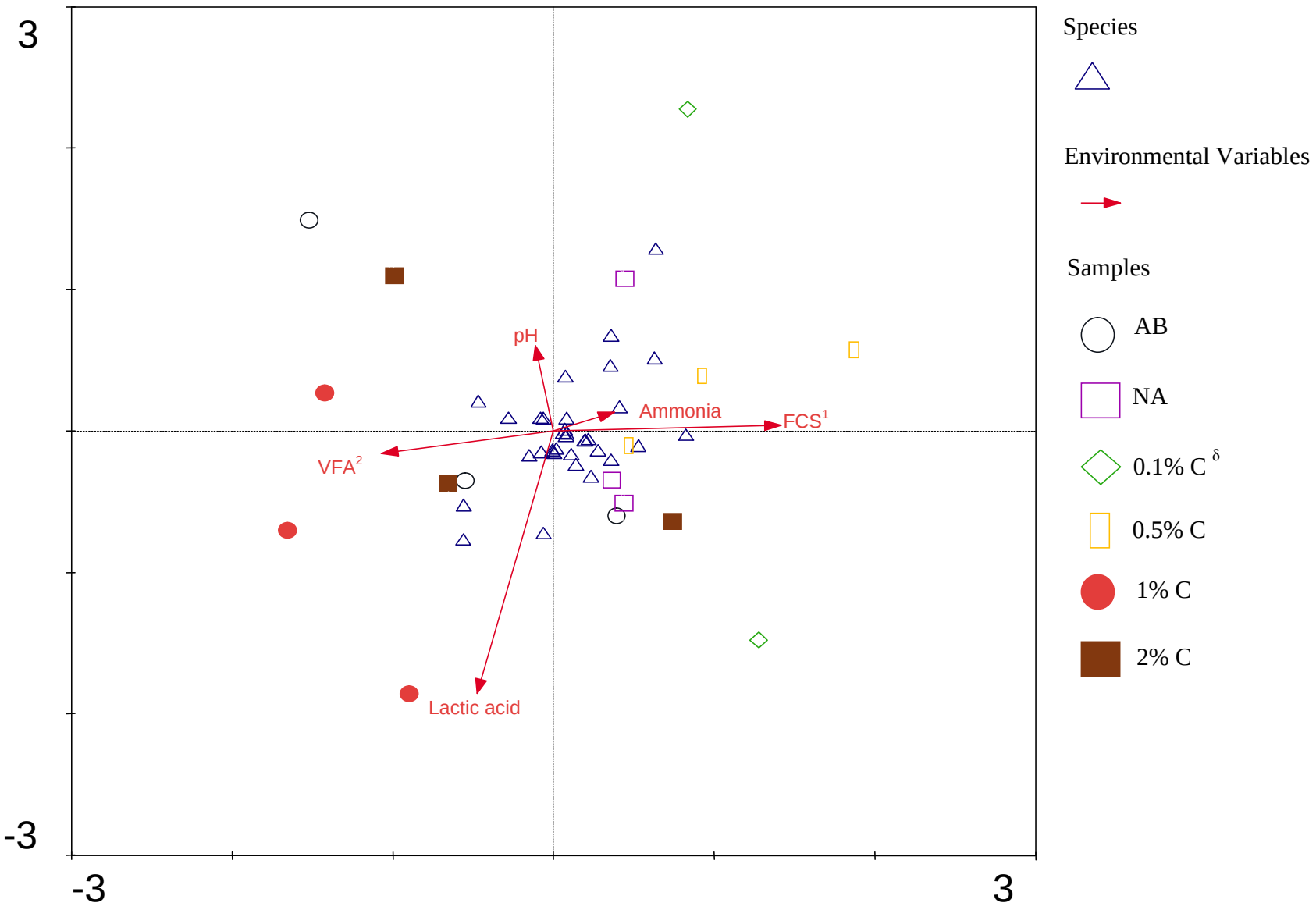
3
-3
-3
3
pH
Ammonia
FCS1
VFA2
Lactic acid
Species
Environmental Variables
Samples
AB
NA
0.1% C δ
0.5% C
1% C
2% C

Figure 7. Canonical correspondence analysis (CCA) of the pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed charcoal and non charcoal-based diets. Diets; NA = no additive, basal diet with no additives; AB = Antibiotics, basal diet + antibiotics; 0.1% C = basal diet + 0.1% charcoal; 0.5% C = basal diet + 0.5% charcoal; 1% C = basal diet + 1% charcoal; 2% C = basal diet + 2% charcoal. (Ammonia, $P = 0.84$), (pH, $P = 0.67$), (Lactic acid, $P = 0.28$), (VFA, $P = 0.54$), and (FCS, $P = 0.44$). The distance between the genera shows their unlikeness and their position to the arrowheads shows the effect of the specific environmental variable on them. The length of the arrows shows effects of the environmental variables. The angles between the arrows show the correlations between the parameters; arrows with similar directions demonstrate positive correlation and arrows with opposite direction demonstrate negative correlation.

[1]FCS; Fecal consistency score

[2]VFA; Volatile fatty acids

[δ]Two replicate samples were analyzed.

2. Crude protein experiment

2.1. pH, ammonia and organic acids

Dietary CP levels had no effect on the ileal and colonic pH ($P > 0.05$) (Table 15). Piglets fed the LPAB diet tended to have a greater pH in the colon compared to piglets fed the HPNA diet ($P < 0.1$) (Table 15). Feeding low-CP diets resulted in a lower ammonia concentration in the ileum ($P < 0.05$) (Table 15). Dietary treatments did not have a significant effect on ammonia concentration in the colon ($P > 0.05$), although there was an increase in the ammonia concentration in the colon of piglets fed HPNA and HPPRO diets (Table 15).

Diets with antibiotics resulted in a lower acetic acid, lactic acid and total overall VFA concentration in the ileum compared to diets with probiotics ($P < 0.05$) (Table 15). Dietary treatments also did not have a significant effect on the OA concentration in the colon ($P > 0.05$) (Table 15).

Table 15. Levels of pH, ammonia, and OA in the ileum and colon of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Items	Diets[1]						SEM[2]	*P*-value[3]		
	High-CP			Low-CP						
	NA	AB	PRO	NA	AB	PRO		A[4]	B[5]	A*B[6]
pH										
Ileum	6.99	7.03	7.28	6.63	6.83	6.95	0.22	NS	NS	NS
Colon	6.28^{b}	6.37^{ab}	6.49^{ab}	6.49^{ab}	6.54^{a}	6.39^{ab}	0.07	NS	NS	(*)
Ammonia, mg/L										
Ileum	23.49	23.73	24.80	20.90	21.29	22.31	1.32	*	NS	NS
Colon	63.29	42.55	61.43	40.33	47.04	40.96	11.16	NS	NS	NS
Ileum OA concentration, mmol/L										
Acetic acid	1.62^{a}	1.08^{b}	2.15^{a}	1.43^{a}	0.70^{b}	1.96^{a}	0.35	NS	*	NS
Propionic acid	0.10	0.10	0.10	0.12	0.05	0.10	0.03	NS	NS	NS
Isobutyric acid	0.03	0.01	0.01	0.00	0.00	0.00	0.01	NS	NS	NS
Butyric acid	0.04	0.00	0.02	0.02	0.00	0.03	0.01	NS	NS	NS
Isovaleric acid	0.00	0.00	0.01	0.00	0.00	0.00	0.01	NS	NS	NS
Valeric acid	0.00	0.01	0.00	0.00	0.00	0.01	0.01	NS	NS	NS
Lactic acid	3.86^{a}	2.03^{b}	5.50^{a}	3.41^{a}	1.27^{b}	5.95^{a}	1.72	NS	*	NS
Total	1.97^{a}	1.20^{b}	2.32^{a}	2.32^{a}	1.00^{b}	2.11^{a}	0.30	NS	*	NS
Colon OA concentration, mmol/L										
Acetic acid	9.29	9.09	11.47	8.91	7.88	10.71	1.56	NS	NS	NS
Propionic acid	3.09	2.57	3.44	3.66	2.47	3.58	0.52	NS	NS	NS
Isobutyric acid	0.32	0.22	0.34	0.19	0.27	0.36	0.05	NS	NS	NS
Butyric acid	1.83	1.54	2.02	1.52	1.62	2.06	0.35	NS	NS	NS
Isovaleric acid	0.17	0.10	0.20	0.11	0.13	0.17	0.04	NS	NS	NS
Valeric acid	0.39	0.18	0.36	0.13	0.20	0.43	0.09	NS	NS	NS
Lactic acid	1.23	1.02	1.08	0.37	0.97	2.05	0.68	NS	NS	NS
Total	15.11	13.70	17.85	14.47	12.57	17.34	2.57	NS	NS	NS

[1]Diets; High-CP = High crude protein, Low-CP = Low crude protein, NA = No additive, AB = Antibiotics, PRO = Probiotics

[2]Pooled standard error of the mean

[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$

[4]Factor A; levels of crude protein

[5]Factor B; additives (no additives/antibiotics/probiotics)

[6]Factor A and B interactions

[a,b,c] Means with different subscripts within the same row are different.

2.2. Gastrointestinal microbial analysis

2.2.1. Culture-independent analysis - Pyrosequencing

After trimming, total individual averages of 12,783 and 12,541 trimmed sequences were obtained from the ileal (Table 16) and colonic (Table 17) digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics, respectively. Sampling coverage of ileal and colonic digesta was ~96.50% and 86.33%, respectively. Rarefraction curves from individual and pooled pyrosequenced 16S rRNA data from the ileal (Figure 10 and 11) and colonic (Figure 12 and 13) digesta of infected piglets fed varying levels of dietary CP supplemented with no additives, antibiotics and probiotics were plotted.

In the ileal digesta, the number of estimated OTUs from individual samples varied from 189 to 403 (Table 16) and in the colonic digesta the OTUs ranged from 560 to 1,209 (Table 17). The number of OTUs from ileal digesta was not affected by dietary treatments ($P > 0.05$) (Table 16). Piglets fed the LPAB diet had the lowest colonic digetsa number of OTUs ($P < 0.05$) (Table 17).

There was no effect of any dietary treatment on richness for the ileal digesta of the piglets ($P > 0.05$) (Table 16). However, diversity and effective species indices were higher in piglets fed the HPAB and LPAB diets compared with piglets fed any other dietary treatment ($P < 0.05$) (Table 16). The richness estimators for the colonic digesta were lower for piglets fed the LPAB diet compared with those fed the HPAB diet ($P < 0.05$) (Table 17). The diversity and effective species indices were lower for piglets fed the LPAB diet compared with those fed HPAB and LPNA diets ($P < 0.05$) (Table 17).

Table 16. Statistical analysis of pyrosequenced bacterial 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Statistics	Diets[1]						SEM[2]	*P*-value[3]		
	High-CP			Low-CP						
	NA[δ]	AB[δ]	PRO	NA[δ]	AB[δ]	PRO[δ]		A[4]	B[5]	A*B[6]
Sequence Matrices										
Trimmed [7]	14,417	13,952	14,467	14,460	6,480	12,924	-	-	-	-
OTUs[8]	189	403	193	244	303	376	85.67	NS	NS	NS
Coverage (%)	98.50	94.50	98.00	97.50	95.00	95.50	0.02	NS	NS	NS
Richness[9]										
Chao1	307.65	740.61	317.35	413.91	492.45	747.12	186.96	NS	NS	NS
ACE	423.76	994.50	399.70	489.26	585.22	791.85	193.53	NS	NS	NS
Diversity[10]										
Shannon	3.17^{b}	4.24^{a}	2.47^{b}	3.17^{b}	4.53^{a}	3.59^{b}	0.58	NS	*	NS
Simpson	0.11^{ab}	0.05^{a}	0.26^{b}	0.12^{ab}	0.03^{a}	0.12^{b}	0.03	NS	*	NS
Effective Species[11]										
Simpson's reciprocal index	9.98^{b}	23.66^{a}	4.02^{b}	8.82^{b}	39.13^{a}	5.26^{b}	3.80	NS	*	NS
Exponential of shannon's index	24.63^{b}	76.70^{a}	12.05^{b}	24.01^{b}	96.40^{a}	49.69^{b}	20.08	NS	*	NS

[1]Diets; High-CP = High crude protein, Low-CP = Low crude protein, NA = No additive, AB = Antibiotics, PRO = Probiotics
[2]Pooled standard error of the mean
[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$
[4]Factor A; levels of crude protein
[5]Factor B; additives (no additives /antibiotics/probiotics)
[6]Factor A and B interactions
[7]Number of trimmed sequences
[8]Number of operational taxonomic units (OTU)
[9]Determined by Chao1 and abundance based coverage estimation (ACE) richness estimators
[10]Determined by Shannon and Simpson diversity indices
[11]Conversion of diversity indices to true diversity (Jost., 2006)
[a,b,c] Means with different subscripts within the same row are different.
[δ] Two sample replicate were analyzed.

Table 17. Statistical analysis of pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Statistics	Diets[1]						SEM[2]	*P*-value[3]		
	High-CP			Low-CP						
	NA	AB	PRO	NA	AB	PRO		A[4]	B[5]	A*B[6]
Sequence Matrices										
Trimmed[7]	12,029	14,206	13,974	12,713	10,640	11,681	-	-	-	-
OTU[8]	972^{ab}	1,209^{a}	1,044^{a}	1,117^{a}	560^{b}	1,034^{a}	97.17	(*)	NS	*
Coverage (%)	86.00	85.00	87.00	85.00	90.00	85.00	0.01	NS	NS	NS
Richness[9]										
Chao1	1,984.63^{ab}	2,459.65^{a}	2,275.84^{ab}	2.220.48^{ab}	1,178.95^{b}	2,024.24^{ab}	251.68	(*)	NS	*
ACE	2,971.89^{ab}	3,714.46^{a}	3,477.67^{ab}	3,188.99^{ab}	1,660.74^{b}	3,055.05^{ab}	396.13	*	NS	*
Diversity[10]										
Shannon	5.87^{ab}	6.10^{a}	5.74^{ab}	6.12^{a}	5.00^{b}	5.98^{ab}	0.23	NS	NS	*
Simpson	0.02	0.01	0.02	0.02	0.03	0.02	0.01	NS	NS	NS
Effective Species[11]										
Simpson's reciprocal index	74.75	85.40	55.28	104.33	45.37	96.94	27.43	NS	NS	NS
Exponential of shannon's index	368.53^{a}	445.22^{a}	326.47^{ab}	478.36^{a}	167.68^{b}	423.16^{a}	71.38	NS	NS	*

[1]Diets; High-CP = High crude protein, Low-CP = Low crude protein, NA = No additive, AB = Antibiotics, PRO = Probiotics
[2]Pooled standard error of the mean
[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$.
[4]Factor A; levels of crude protein
[5]Factor B; additives (no additives /antibiotics/probiotics)
[6]Factor A and B interactions
[7]Number of trimmed sequences.
[8]Number of operational taxonomic units (OTU)
[9]Determined by Chao1 and abundance based coverage estimation (ACE) richness estimators
[10]Determined by Shannon and Simpson diversity indices
[11]Conversion of diversity indices to true diversity (Jost., 2006)
[a,b,c] Means with different subscripts within the same row are different.

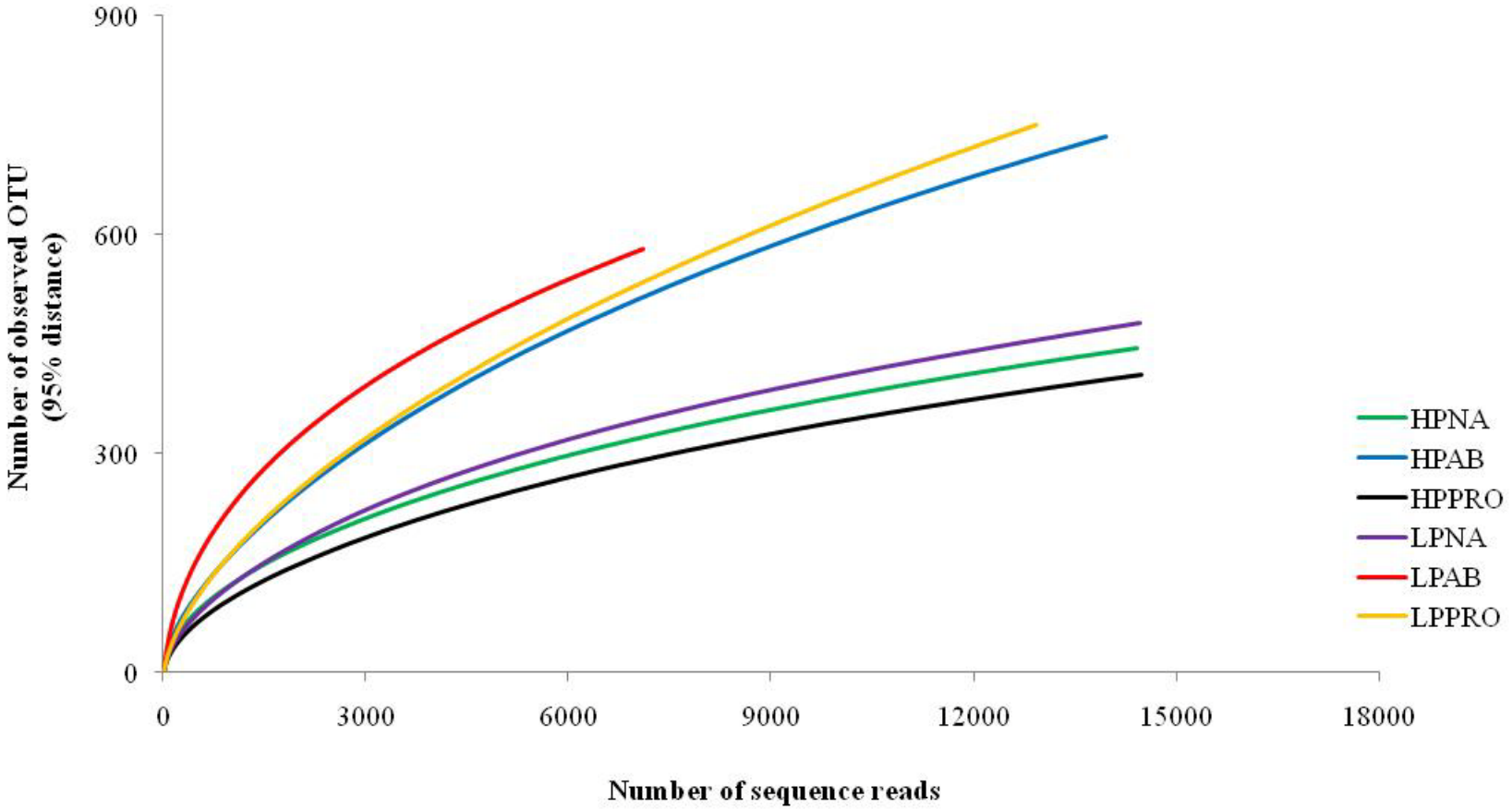

Figure 8. Rarefaction curve analysis of pooled pyrosequenced bacterial 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. Operational toxonomic units (OTU) were defined at 95% similarity. The curves approximate to a plateau state, implying that the majority of the GIT microbiota present in these samples was covered.

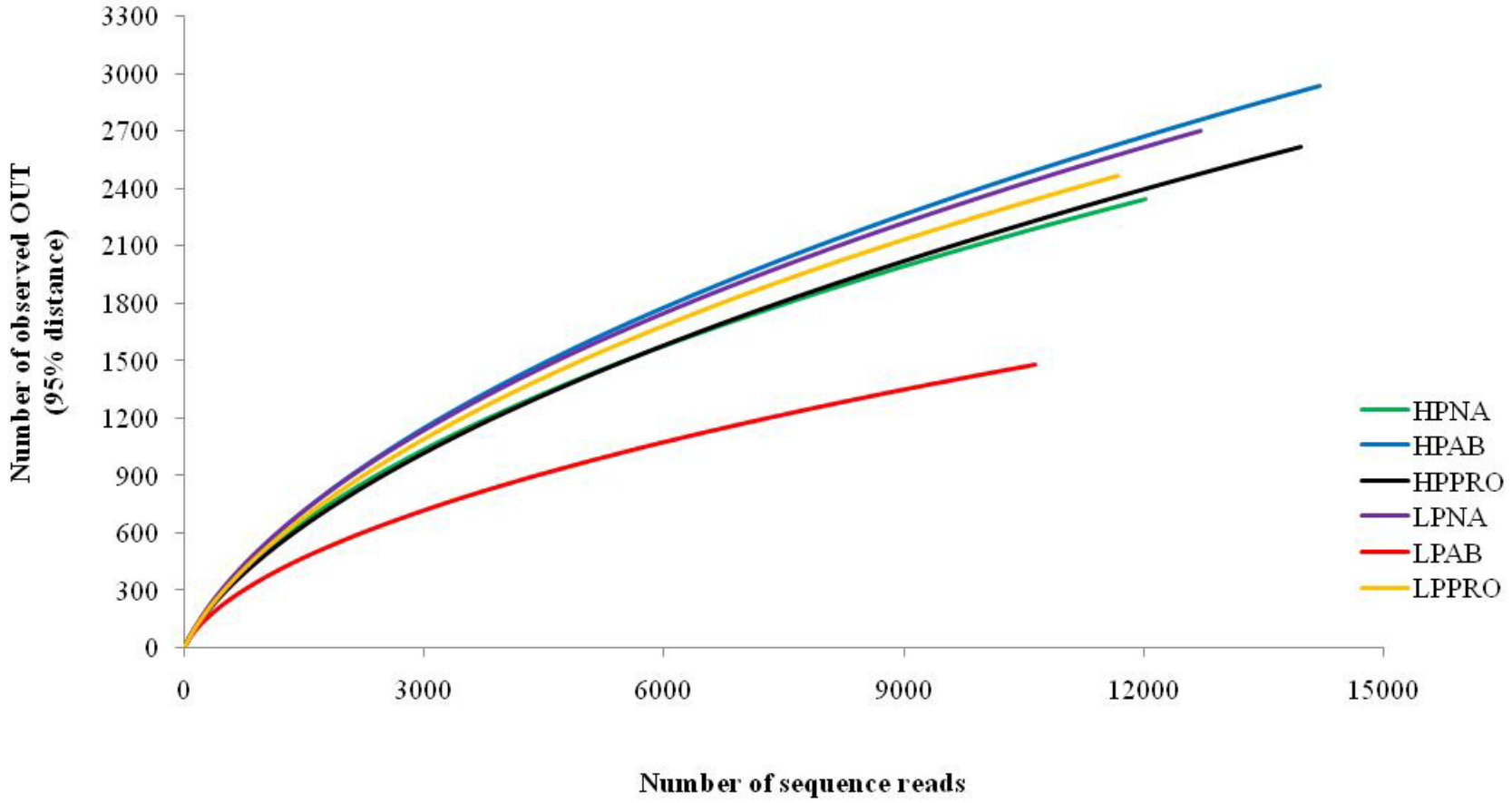

Figure 9. Rarefaction curve analysis of pooled pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. Operational toxonomic units (OTU) were defined at 95% similarity. The curves approximate to a plateau state, implying that the majority of the GIT microbiota present in these samples was covered.

In the ileal digesta five phyla were observed, with the most group predominant being that of Firmicutes followed by Proteobacteria (Table 18). The most predominant genus was *Lactobacillus*, followed by *Clostridium* and uncultured genus of family Peptostreptococcaceae (Table 19). Feeding low-CP diets significantly decreased the Firmicutes ($P > 0.05$) (Table 18). Piglets fed the LPAB diet had the lowest percentage of Firmicutes ($P < 0.05$) (Table 18) and tended to have the highest percentage of Proteobacteria ($P < 0.1$) (Table 18). The ileal digesta of piglets fed a high-CP diet had the highest percentage of the *Lactobacillus* ($P < 0.05$) (Table 19). The ileal digesta of piglets fed the AB diets had the lowest percentage of *Lactobacillus* ($P < 0.05$) (Table 18 and 19). The unclassified genus of family Rs-D42 was found to be the lowest in ileal digesta of piglets fed the LPAB diet ($P < 0.05$) (Table 19). The highest percentage of the genus *Streptococcus* was found in ileal digesta of piglets fed the LPAB diet ($P < 0.05$) (Table 19). The *Clostridium* tended to have the highest percentage in piglets fed the HPAB diet compared with those fed the LPAB diet ($P < 0.1$) (Table 19). The percentage of the uncultured genus of family Peptostreptococcaceae was at its lowest in piglets fed the HPPRO diet compared with piglets fed HPAB, LPAB and LPNA diets ($P < 0.05$) (Table 19). The LPNA diet resulted in the highest percentage of the genus *Turicibacter* ($P < 0.05$) (Table 19).

Principal component analysis of the data for genera in ileal digesta microbiota of piglets fed different levels of dietary CP supplemented with no additives, antibiotics and probiotics are presented in Figure 14. The first two PC explained 64.90% of the total variance. The results from the PCA show different clustering patterns of ileal digesta microbiota at the genus level in piglets fed the LPAB diet.

Table 18. Phylogenetic composition of bacterial phyla from pyrosequenced 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Phylum	Percentage of sequences in:									
	Diets[1]									
	High-CP			Low-CP			SEM[2]	*P*-value[3]		
	NA[δ]	AB[δ]	PRO	NA[δ]	AB[δ]	PRO[δ]		A[4]	B[5]	A*B[6]
Actinobacteria	†	†	0.1	0.1	2.7	0.1	0.2	NS	NS	NS
Bacteroidetes	0.1	1.4	0.2	0.1	3.6	1.9	1.5	NS	NS	NS
Firmicutes	97.2[a]	93.1[a]	98.5[a]	98.1[a]	49.9[b]	95.9[a]	6.4	*	*	*
Proteobacteria	2.6[b]	5.3[b]	1.2[b]	1.7[b]	38.0[a]	1.9[b]	2.6	NS	*	(*)
Unclassified	0.2	0.2	0.1	0.1	2.4	0.2	0.4	NS	NS	NS

[1]Diets; High-CP = High crude protein, Low-CP = Low crude protein, NA = No additive, AB = Antibiotics, PRO = Probiotics
[2]Pooled standard error of the mean
[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$
[4]Factor A; levels of crude protein
[5]Factor B; additives (no additives/antibiotics/probiotics)
[6]Factor A and B interactions
[a,b,c] Means with different subscripts within the same row are different
[†]The phyla with lower abundance of 0.10% are omitted.
[δ]Two replicate samples were analyzed.

Table 19. Phylogenetic composition of bacterial genus from pyrosequenced 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Phylum;Family;Genus	Percentage of sequences in: Diets[1]						SEM[2]	*P*-value[3]		
	High-CP			Low-CP						
	NA[δ]	AB[δ]	PRO	NA[δ]	AB[δ]	PRO[δ]		A[4]	B[5]	A*B[6]
Firmicutes										
Lactobacillacea; *Lactobacillus*	67.1[a]	17.4[b]	85.7[a]	27.1[a]	2.7[b]	41.1[a]	7.3	*	*	NS
Leuconostocaceae; *Weissella*	†	†	†	0.1	0.1	†	0.2	NS	NS	NS
Rs-D42; unclassified	1.1[ab]	4.2[ab]	1.6[ab]	5.3[ab]	0.3[b]	8.4[a]	1.4	(*)	NS	*
Streptococcaceae; *Streptococcus*	0.4[b]	0.1[b]	0.1[b]	1.6[b]	7.4[a]	0.1[b]	0.8	*	*	*
Clostridiaceae; *Clostridium*	19.6[ab]	34.9[a]	13.2[ab]	21.9[ab]	7.1[b]	17.8[ab]	6.1	NS	NS	(*)
Lachnospiracea; Incertae Sedis	†	0.4	†	†	0.9	†	0.3	NS	NS	NS
Lachnospiracea; uncultured	†	0.5	†	†	0.4	0.8	0.3	NS	NS	NS
Lachnospiracea; unclassified	0.1	0.7	0.1	0.2	0.9	0.5	0.5	NS	NS	NS
Peptostreptococcaceae; unclassified	0.4	2.7	0.1	1.8	2.05	1.1	0.8	NS	NS	NS
Peptostreptococcaceae; uncultured	5.6[ab]	14.5[a]	0.3[b]	14.5[a]	13.3[a]	9.6[ab]	2.2	*	*	*
Ruminococcaceae; uncultured	0.1	0.5	0.1	†	0.8	0.8	0.4	NS	NS	NS
Veillonellaceae; *Megasphaera*	†	0.9	0.2	0.4	0.1	0.8	0.4	NS	NS	NS
Erysipelotrichacea; *Turicibacter*	2.0[b]	6.1[b]	1.2[b]	24.2[a]	1.8[b]	6.6[b]	3.9	*	(*)	*
Proteobacteria										
Enterobacteriaceae; *Escherichia*	†	3.8	†	0.1	8.6	0.6	3.0	NS	NS	NS
Pasteurellaceae; *Actinobacillus*	2.0	0.4	0.9	1.3	12.0	†	4.5	NS	NS	NS
Pasteurellaceae; *Haemophilus*	†	†	†	0.2	2.5	†	0.7	NS	NS	NS
Pasteurellaceae; *Pasteurella*	†	0.2	0.1	0.1	4.5	†	1.4	NS	NS	NS
Pasteurellaceae; unclassified	0.1	†	†	0.1	3.7	†	1.2	NS	NS	NS

[1]Diets; High-CP= High protein, Low-CP= Low protein, NA = No additive, AB = Antibiotics, PRO = Probiotics
[2]Pooled standard error of the mean
[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$. [a,b,c] Means with different subscripts within the same row are different.
[4]Factor A; levels of crude protein
[5]Factor B; additives (no additives/antibiotics/probiotics)
[6]Factor A and B interactions
[†]The genera with lower abundance of 0.10% are omitted.
[δ]Two replicate samples were analyzed.

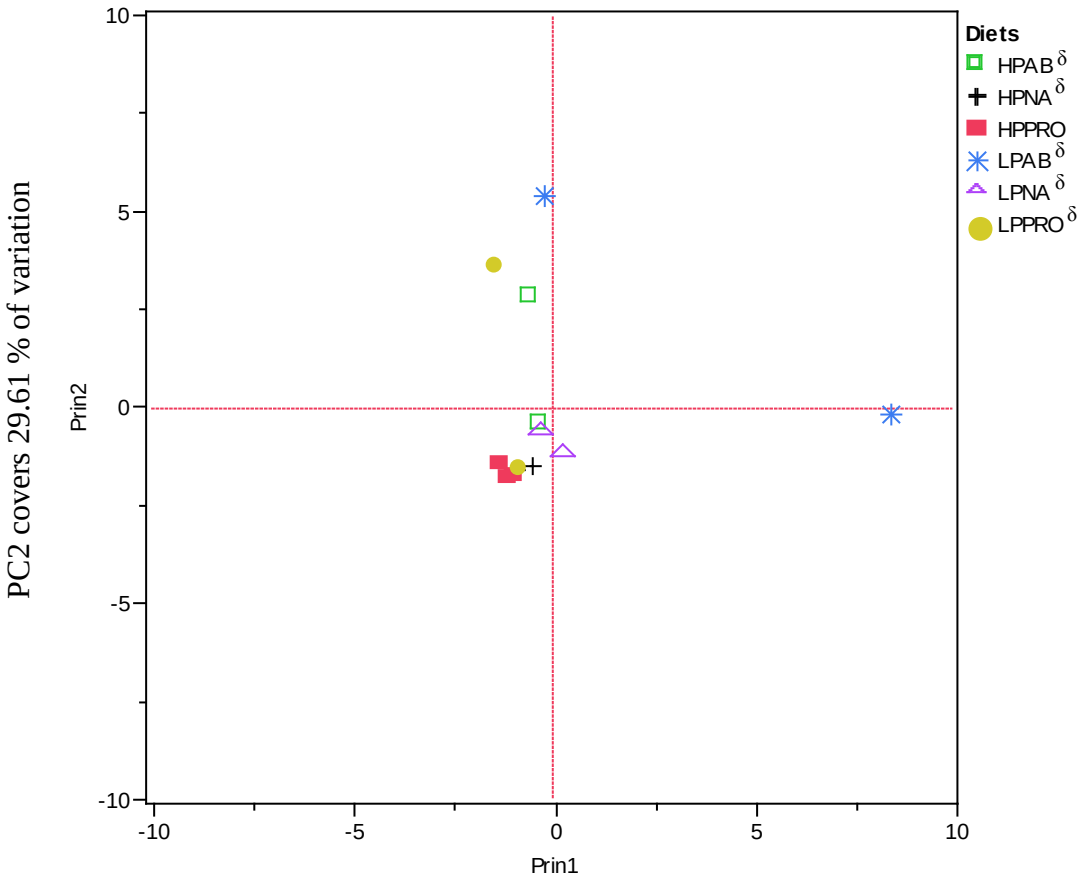

Figure 10. Principal component score plot of the ileal digesta at genus level of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. The two principal components cover 64.90% of the variation in the data.
$^{\delta}$Two replicate samples were analyzed.

Calculation of CCA for the microbiota of the ileal digesta revealed no correlation between dietary treatments and ammonia (P = 0.32), pH (P = 0.21), lactic acid (P = 0.66) and VFA levels (P = 0.73) (Figure 15). However, it appears that there was a positive correlation between the low-CP diets and pH. In addition, a negative trend was observed between the low-CP diets and VFA and lactic acid concentrations. No association was observed for a specific genus and a particular dietary treatment. Nonetheless, it appears that the genus *Lactobacillus* might have an association with high-CP diets. Additionally, the genera *Escherchia* and *Streptococcus* were revealed to have a potential association with the LPAB diet.

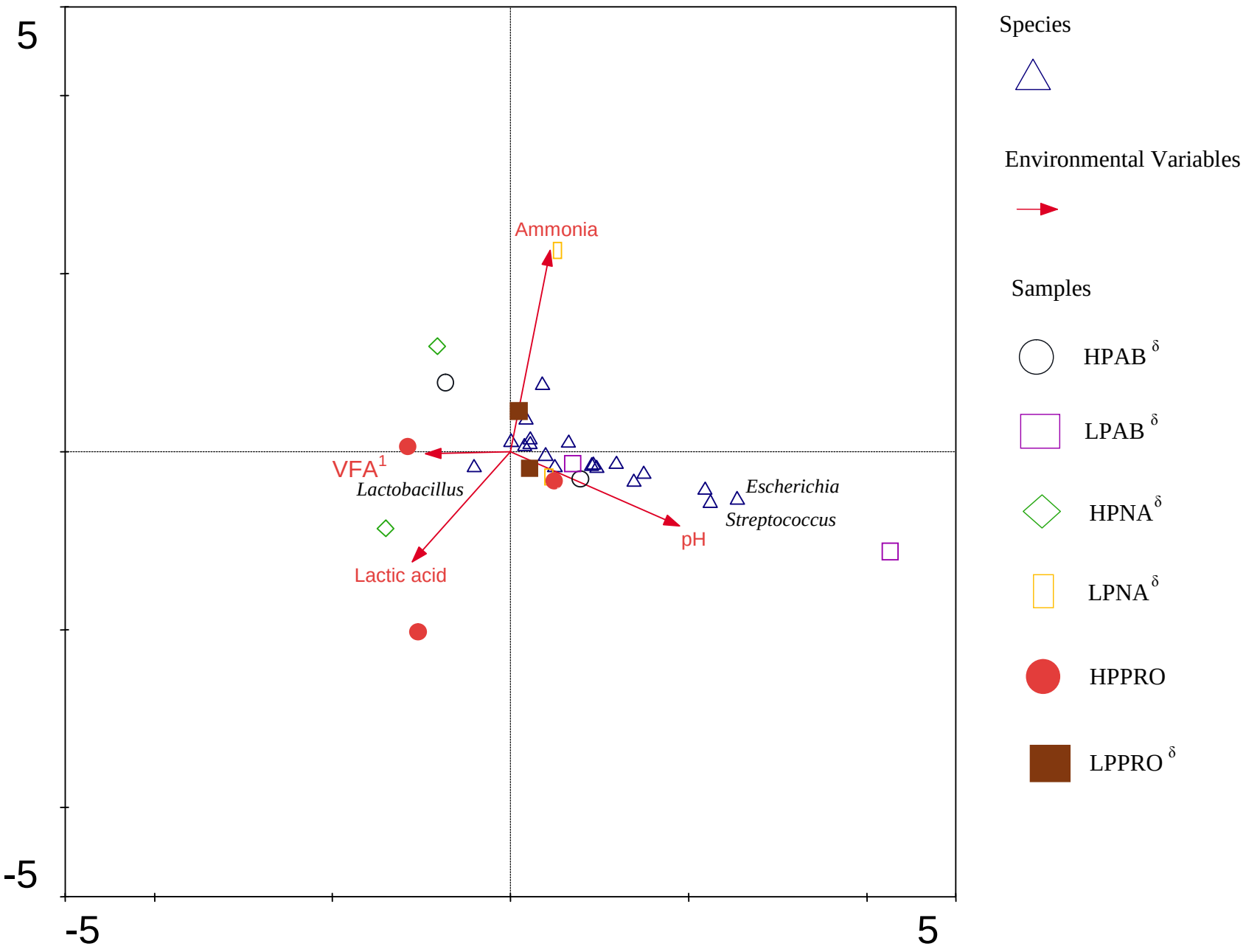
5
-5
-5
5
Ammonia
VFA[1]
Lactobacillus
Lactic acid
pH
Escherichia
Streptococcus
Species
Environmental Variables
Samples
HPAB δ
LPAB δ
HPNA δ
LPNA δ
HPPRO
LPPRO δ

Figure 11. Canonical correspondence analysis (CCA) of the pyrosequenced bacterial 16S rRNA genes in the ileal digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. (Ammonia, P = 0.32), (pH, P = 0.21), (Lactic acid, P = 0.66), (VFA, P = 0.73). The distance between the genera shows their unlikeness and their position to the arrowheads shows the effect of the specific environmental variable on them. The length of the arrows shows effects of the environmental variables. The angles between the arrows show the correlations between the parameters; arrows with similar directions demonstrate positive correlation and arrows with opposite direction demonstrate negative correlation.
[1]VFA; Volatile fatty acids
$^{\delta}$ Two replicate samples were analyzed.

In the colonic digesta 10 phyla were identified, with the most predominant phylum being that of Firmicutes followed by Bacteroides (Table 20). There was no effect of any dietary treatment on the colonic digesta microbiota ($P > 0.05$) (Table 20), except for the unclassified phylum that was higher in HPAB and LPNA diets compared with HPNA and LPAB diets ($P < 0.05$) (Table 20). The most predominant genus in the colonic digesta was *Lactobacillus*, followed by, uncultured genus of family Ruminococcaceae, unclassified genus of family Lachnospiraceae, and uncultured genus of family Lachnospiraceae (Table 21). Feeding high-CP diets resulted in a higher percentage of the genera *Roseburia* and *Megasphaera* in the colonic digesta ($P < 0.05$) (Table 21). The percentage of the unclassified genus of family Rikenellaceae tended to be higher in low-CP diets ($P < 0.1$) (Table 21). Piglets fed the LPPRO diet had a higher percentage of the genus *Xylanibacter* in the colonic digesta ($P < 0.05$) (Table 21). The percentage of the unclassified genus of family Prevotellaceae tended to be lower in the colonic digesta of piglets fed the LPAB diet compared with those fed the LPNA diet ($P < 0.1$) (Table 21). The LPNA diet resulted in a higher percentage of the unclassified genus of family RF16 compared with LPAB, HPNA and HPPRO diets ($P < 0.05$) (Table 21). Piglets fed the LPPRO diet had the highest percentage of the unclassified genus of family RS-D42 in their colonic digesta ($P < 0.05$) (Table 21). The percentage of *Clostridium* was lower in colonic digesta of piglets fed the LPAB diet compared with those fed the LPPRO diet ($P < 0.05$) (Table 21). The uncultured genus of family Peptostreptococcaceae was found to be higher in the LPPRO diet compared with LPNA and HPPRO diets ($P < 0.05$) (Table 21). The percentage of the genus *Anaerovibrio* tended to be higher in colonic digesta of piglets fed the LPNA diet compared with those fed HPAB, HPPRO, LPAB and LPPRO diets ($P < 0.1$) (Table 21). Piglets fed the LPPRO diet had a higher percentage of *Turicibacter* in the colonic digesta compared with those fed the LPAB diet ($P < 0.05$) (Table 21).

Table 20. Phylogenetic composition of bacterial phyla from pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Phylum	Percentage of sequences in:									
	Diets[1]						SEM[2]	*P*-value[3]		
	High-CP			Low-CP						
	NA[δ]	AB[δ]	PRO	NC[δ]	AB[δ]	PRO[δ]		A[3]	B[4]	A*B[5]
Actinobacteria	0.2	0.1	0.2	0.2	0.6	0.1	0.3	NS	NS	NS
Bacteroidetes	23.0	26.6	13.8	31.0	21.7	23.7	4.9	NS	NS	NS
Cyanobacteria	0.8	0.1	0.1	0.5	0.2	0.2	0.3	NS	NS	NS
Fibrobacteres	†	†	†	1.3	†	†	0.2	NS	NS	NS
Firmicutes	74.4	68.9	83.8	70.0	69.8	73.2	4.6	NS	NS	NS
Planctomycetes	0.3	0.2	0.7	0.1	0.2	0.2	0.3	NS	NS	NS
Proteobacteria	0.4	0.8	0.5	1.3	1.8	0.4	0.4	NS	NS	NS
Spirochaetes	1.2	2.3	0.3	1.5	0.8	0.5	0.7	NS	NS	NS
Synergistetes	†	0.2	†	0.3	1.3	†	0.6	NS	NS	NS
----------Unclassified----------										
Unclassified	0.3[b]	0.7[a]	0.6[ab]	0.7[a]	0.3[b]	0.6[ab]	0.1	NS	NS	*

[1]Diets; High-CP = High crude protein, Low-CP = Low crude protein, NA = No additive, AB = Antibiotics, PRO = Probiotics
[2]Pooled standard error of the mean
[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$
[3]Factor A; levels of crude protein.
[4]Factor B; additives (no additives/antibiotics/probiotics)
[5]Factor A and B interactions
[a,b,c] Means with different subscripts within the same row are different.
[†]The phyla with lower abundance of 0.10% are omitted.
[δ] Two replicate samples were analyzed.

Table 21. Phylogenetic composition of bacterial genus from pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Phylum;Family;Genus	Percentage of sequences in: Diets[1]						SEM[2]	*P*-value[3]		
	High-CP			Low-CP						
	NA	AB	PRO	NA	AB	PRO		A[4]	B[5]	A*B[6]
Bacteroidetes										
Bacteroides	0.2	0.3	0.4	0.3	0.3	0.1	0.1	NS	NS	NS
Porphyromonadaceae; *Parabacteroides*	0.8	1.2	0.2	1.3	1.1	1.8	0.6	NS	NS	NS
Prevotellaceae; *Prevotella*	3.5	3.4	2.2	5.9	5.1	2.8	1.8	NS	NS	NS
Prevotellaceae; *Xylanibacter*	1.5^{b}	1.6^{b}	0.3^{b}	1.1^{b}	0.6^{b}	4.3^{a}	0.5	(*)	(*)	*
Prevotellaceae; unclassified	2.6^{ab}	2.3^{ab}	2.2^{ab}	4.5^{a}	0.3^{b}	1.7^{ab}	0.8	NS	(*)	(*)
Prevotellaceae; uncultured	3.3	4.6	3.6	5.4	3.0	3.5	1.0	NS	NS	NS
RF16; unclassified	†^{b}	0.3^{ab}	†^{b}	0.6^{a}	†^{b}	0.2^{ab}	0.2	NS	NS	*
Rikenellaceae; RC9 gut group	5.7	3.2	2.8	4.2	5.0	4.0	1.4	NS	NS	NS
Rikenellaceae; unclassified	0.1	†	0.1	0.2	0.2	0.2	0.1	(*)	NS	NS
S247; unclassified	3.4	2.8	2.1	3.0	3.9	2.6	0.9	NS	NS	NS
p-2534-18B5 gut group; unclassified	0.1	0.2	0.1	†	†	0.3	0.2	NS	NS	NS
Fibrobacteres										
Fibrobacteraceae; *Fibrobacteres*	0.1	0.1	0.1	1.1	0.1	0.1	0.3	NS	NS	NS
Firmicutes										
Lactobacillaceae; *Lactobacillus*	20.1	9.7	20.5	9.2	17.5	12.8	6.6	NS	NS	NS
Rs-D42; unclassified	0.6^{b}	0.6^{b}	0.2^{b}	0.3^{b}	0.4^{b}	2.3^{a}	0.3	(*)	(*)	*
Clostridiaceae; *Clostridium*	4.2^{ab}	4.1^{ab}	1.9^{ab}	1.9^{ab}	0.8^{b}	7.6^{a}	1.4	NS	*	*
Family_XIII Incertae Sedis; *Mogibacterium*	0.2	0.2	0.1	0.2	0.4	0.1	0.1	NS	NS	NS
Family XIII Incertae Sedis; unclassified	0.3	0.4	0.6	0.4	0.2	0.4	0.2	NS	NS	NS
Family XIII Incertae Sedis; uncultured	0.7	0.5	0.8	0.5	0.5	0.5	0.2	NS	NS	NS
Lachnospiraceae; *Blautia*	1.7	1.1	2.0	1.2	2.8	0.9	0.6	NS	NS	NS
Lachnospiraceae; *Dorea*	0.3	0.2	0.5	0.2	0.4	0.3	0.2	NS	NS	NS
Lachnospiraceae; Incertae Sedis	3.4	3.7	4.1	4.4	5.1	2.1	1.2	NS	NS	NS
Lachnospiraceae; *Oribacterium*	0.2	0.1	†	0.2	0.2	0.1	0.1	NS	NS	NS
Lachnospiraceae; *Roseburia*	3.6	7.0	4.3	0.7	2.5	1.8	1.4	*	NS	NS

Table 21 (continued). Phylogenetic composition of bacterial genus from pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Phylum;Family;Genus	Percentage of sequences in: Diets[1]						SEM[2]	*P*-value[3]		
	High-CP			Low-CP						
	NA	AB	PRO	NA	AB	PRO		A[4]	B[5]	A*B[6]
Lachnospiraceae; unclassified	5.8	10.4	10.7	10.6	5.5	7.6	2.2	NS	NS	NS
Lachnospiraceae; uncultured	5.5	8.3	11.5	9.4	6.2	5.6	2.2	NS	NS	NS
Peptostreptococcaceae; Incertae Sedis	0.7	0.7	0.2	1.1	0.5	0.6	0.2	NS	NS	NS
Peptostreptococcaceae; unclassified	0.8	1.3	0.2	0.2	0.9	1.3	0.5	NS	NS	NS
Peptostreptococcaceae; uncultured	0.6^{ab}	1.2^{ab}	0.1^{b}	0.1^{b}	0.4^{ab}	1.7^{a}	0.3	NS	NS	*
Ruminococcaceae; *Anaerotruncus*	0.3	0.2	0.9	0.3	1.0	0.3	0.4	NS	NS	NS
Ruminococcaceae; *Fecalibacterium*	0.7	1.5	0.5	0.6	0.3	0.6	0.3	NS	NS	NS
Ruminococcaceae; Incertae Sedis	0.4	0.5	1.0	0.4	1.3	0.6	0.5	NS	NS	NS
Ruminococcaceae; *Oscillospira*	0.9	1.0	0.7	0.7	0.4	0.8	0.2	NS	NS	NS
Ruminococcaceae; *Ruminococcus*	1.5	1.9	1.3	1.1	1.0	2.2	0.5	NS	NS	NS
Ruminococcaceae; *Subdoligranulum*	0.3	0.5	0.3	0.6	0.5	0.3	0.1	NS	NS	NS
Ruminococcaceae; unclassified	1.7	2.6	3.0	2.8	1.2	2.8	0.7	NS	NS	NS
Ruminococcaceae; uncultured	10.2	5.6	15.0	7.5	11.9	12.3	2.8	NS	NS	NS
Veillonellaceae; *Anaerovibrio*	3.5^{ac}	0.8^{bc}	†[bc]	6.5^{a}	0.1^{bc}	1.4^{bc}	1.1	NS	NS	(*)
Veillonellaceae; *Megasphaera*	1.7	0.9	1.2	0.1	0.6	0.5	0.6	(*)	NS	NS
Veillonellaceae; *Phascolarctobacterium*	0.3	0.6	0.2	0.6	0.4	0.4	0.2	NS	NS	NS
Erysipelotrichaceae; Incertae Sedis	0.2	0.2	0.3	0.1	0.1	0.2	0.2	NS	NS	NS
Erysipelotrichaceae; *Turicibacter*	1.9^{ab}	1.5^{ab}	0.2^{ab}	0.3^{ab}	†[b]	2.4^{a}	0.6	NS	NS	*
Erysipelotrichaceae; uncultured	0.4	0.3	1.8	0.2	1.2	0.2	0.5	NS	NS	NS
Planctomycetes										
Planctomycetaceae; p-1088-a5 gut group	0.4	0.2	0.7	0.1	0.2	0.2	0.3	NS	NS	NS
Proteobacteria										
Campylobacteracea; *Campylobacter*	†	0.1	†	0.2	0.4	†	0.1	NS	NS	NS
Succinivibrionaceae; *Succinivibrio*	†	0.1	0.1	0.8	0.2	0.9	0.3	NS	NS	NS
Enterobacteriaceae; *Escherichia*	†	0.2	†	†	0.1	†	0.1	NS	NS	NS

Table 21 (continued). Phylogenetic composition of bacterial genus from pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Phylum;Family;Genus	Percentage of sequences in:									
	Diets[1]						SEM[2]	*P*-value[3]		
	High-CP			Low-CP						
	NA	AB	PRO	NA	AB	PRO		A[4]	B[5]	A*B[6]
Spirochaetes										
Spirochaetaceae; *Treponema*	1.2	2.4	0.3	1.4	0.8	0.5	0.7	NS	NS	NS

[1]Diets; High-CP = High crude protein, Low-CP = Low crude protein, NA = No additive, AB = Antibiotics, PRO = Probiotics
[2]Pooled standard error of the mean
[3]Significance: NS, $P > 0.1$; (*), $P < 0.1$; *, $P < 0.05$
[3]Factor A; levels of crude protein
[4]Factor B; additives (no additives/antibiotics/probiotics)
[5]Factor A and B interactions
[a,b,c] Means with different subscripts within the same row are different.
[†]The genera with lower abundance of 0.10% are omitted.

Principal component analysis of the data for genera in colonic digesta microbiota of piglets fed different levels of dietary CP supplemented with no additives, antibiotics and probiotics is presented in Figure 16. The first two PC explained 34.98% of the total variance. The results from the PCA show no effect of dietary treatments on the variability of bacterial genera in the colonic digesta of piglets.

Calculation of CCA for the colonic digesta microbiota revealed no correlation between dietary treatments and ammonia (P = 0.52), pH (P = 0.91), lactic acid (P = 0.72 and VFA levels (P = 0.75) (Figure 17). However, a negative trend was observed between the level of ammonia and the low-CP diets. No association was observed for a specific genus and a particular dietary treatment.

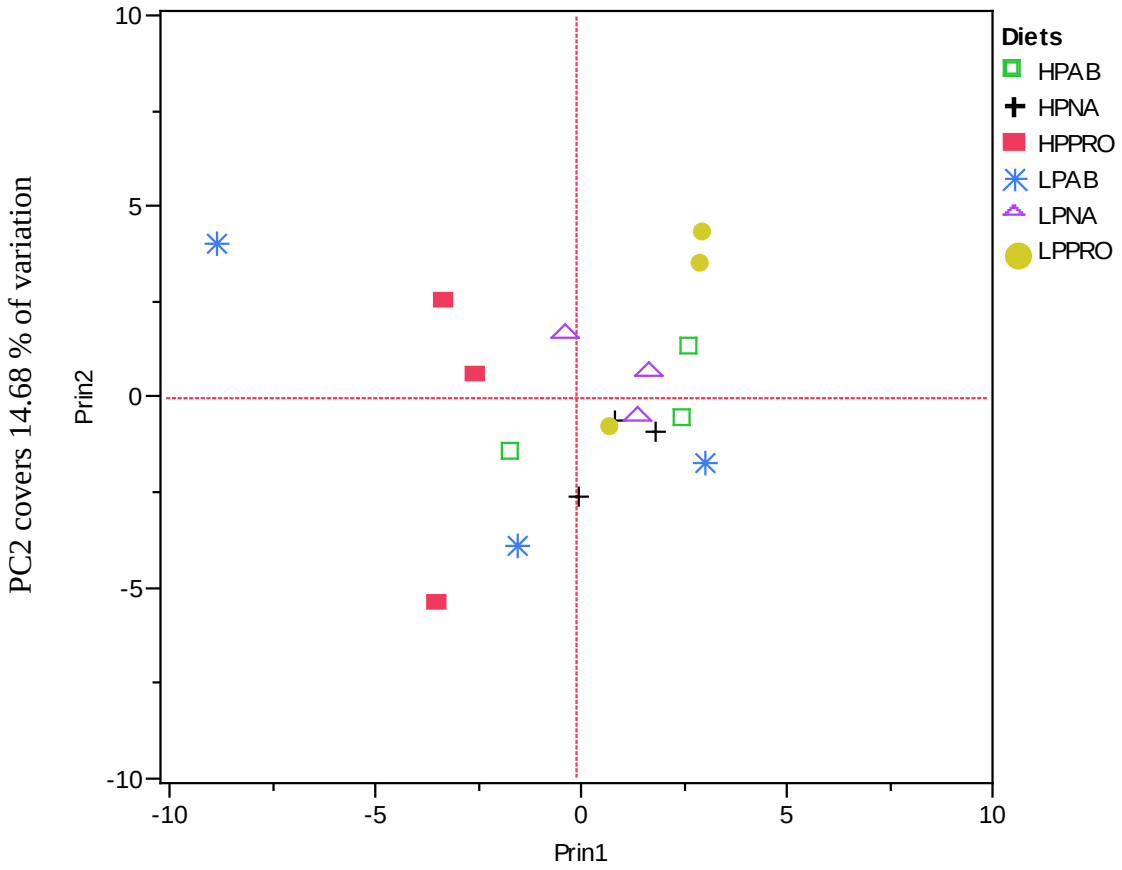

Figure 12. Principal component score plot of the colonic digesta at genus level of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. The two principal components cover 34.98% of the variation in the data.

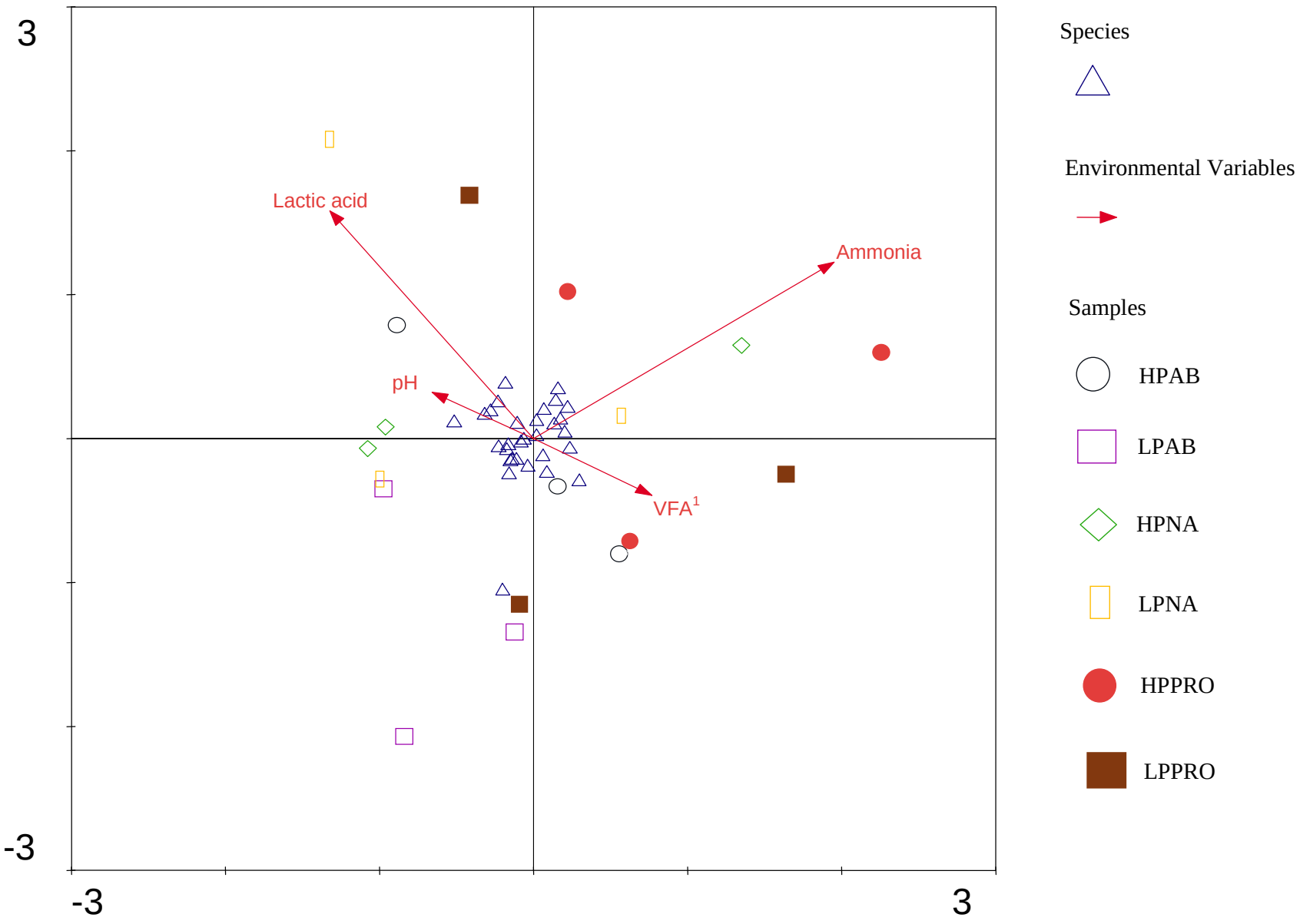
3
-3
-3
3
Lactic acid
Ammonia
pH
VFA1
Species
Environmental Variables
Samples
HPAB
LPAB
HPNA
LPNA
HPPRO
LPPRO

Figure 13. Canonical correspondence analysis (CCA) of the pyrosequenced bacterial 16S rRNA genes in the colonic digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. (Ammonia, P = 0.52), (pH, P = 0.91), (Lactic acid, P = 0.72), (VFA, P = 0.75). The distance between the genera shows their unlikeness and their position to the arrowheads shows the effect of the specific environmental variable on them. The length of the arrows shows effects of the environmental variables. The angles between the arrows show the correlations between the parameters; arrows with similar directions demonstrate positive correlation and arrows with opposite direction demonstrate negative correlation.
[1]VFA; Volatile fatty acids

2.2.2. Culture-independent analysis – Q-PCR

Relative quantification of specific bacterial groups in the ileal and colonic digesta of ETEC K88 infected piglets fed different levels of dietary CP supplemented with no additives, antibiotics and probiotics were determined (Figures 18 and 19).

The populations of *Lactobacillus* spp. and *E. coli* in the ileal digesta significantly increased in response to feeding high-CP diets ($P < 0.05$). Diets supplemented with AB resulted in a significant decrease in the populations of Clostridial Cluster IV, Cluster IV *Ruminococcus*, *Roseburia spp.*, *E. rectale*, *Bacteroides spp.*, *Lactobacillus* spp. and *E. coli* in the ileal digesta ($P < 0.05$). The population of *B. adolescentis* also decreased, although not significantly ($P > 0.05$). Probiotics had no effect on quantified bacterial populations in the ileal digesta ($P > 0.05$).

The population of *E. coli* in the colon tended to be higher in response to feeding high-CP diets ($P < 0.1$). Diets supplemented with AB resulted in decreased populations of Clostridial Cluster IV ($P < 0.1$), *Bacteroides spp.* ($P < 0.1$), *B. adolescentis* ($P < 0.1$), *Lactobacillus* spp. ($P < 0.05$) and *E. coli* ($P < 0.05$) in the colonic digesta. The population of Cluster IV *Ruminococcus spp.*, *Roseburia spp.* and *E. rectale* also decreased but not significantly ($P > 0.05$). Probiotics also had no effect on quantified bacterial populations in the colonic digesta ($P > 0.05$).

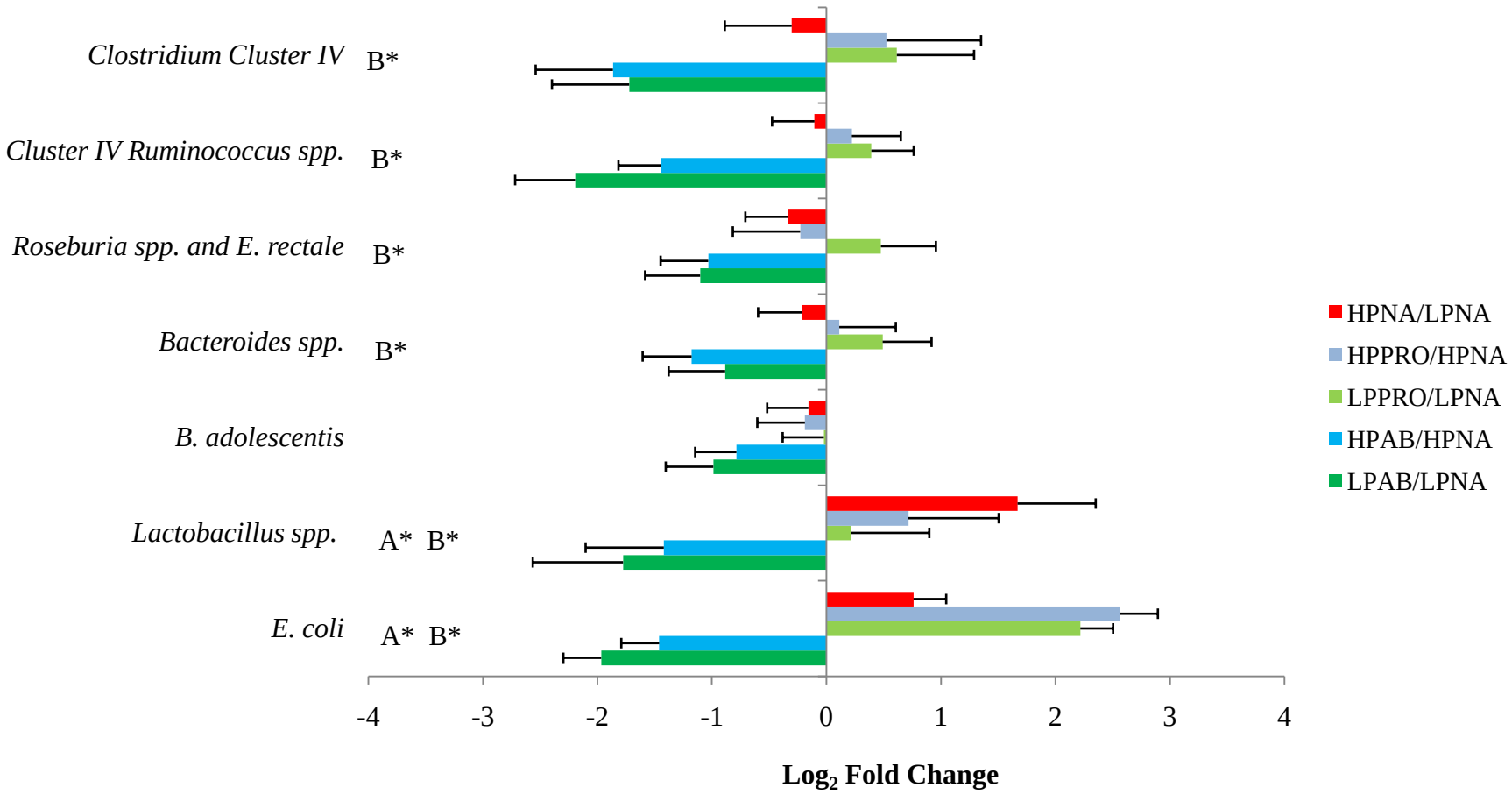

Figure 14. Log_2 fold changes of select bacterial groups in the ileal digesta of ETEC K88 infected piglets fed different experimental diets based on quantitative real-time PCR. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. Factor A; levels of crude protein, Factor B; additives (no additives/antibiotics/probiotics). Significant differences and tendencies were noted as $P < 0.05$, *, and $P < 0.1$, (*), respectively.

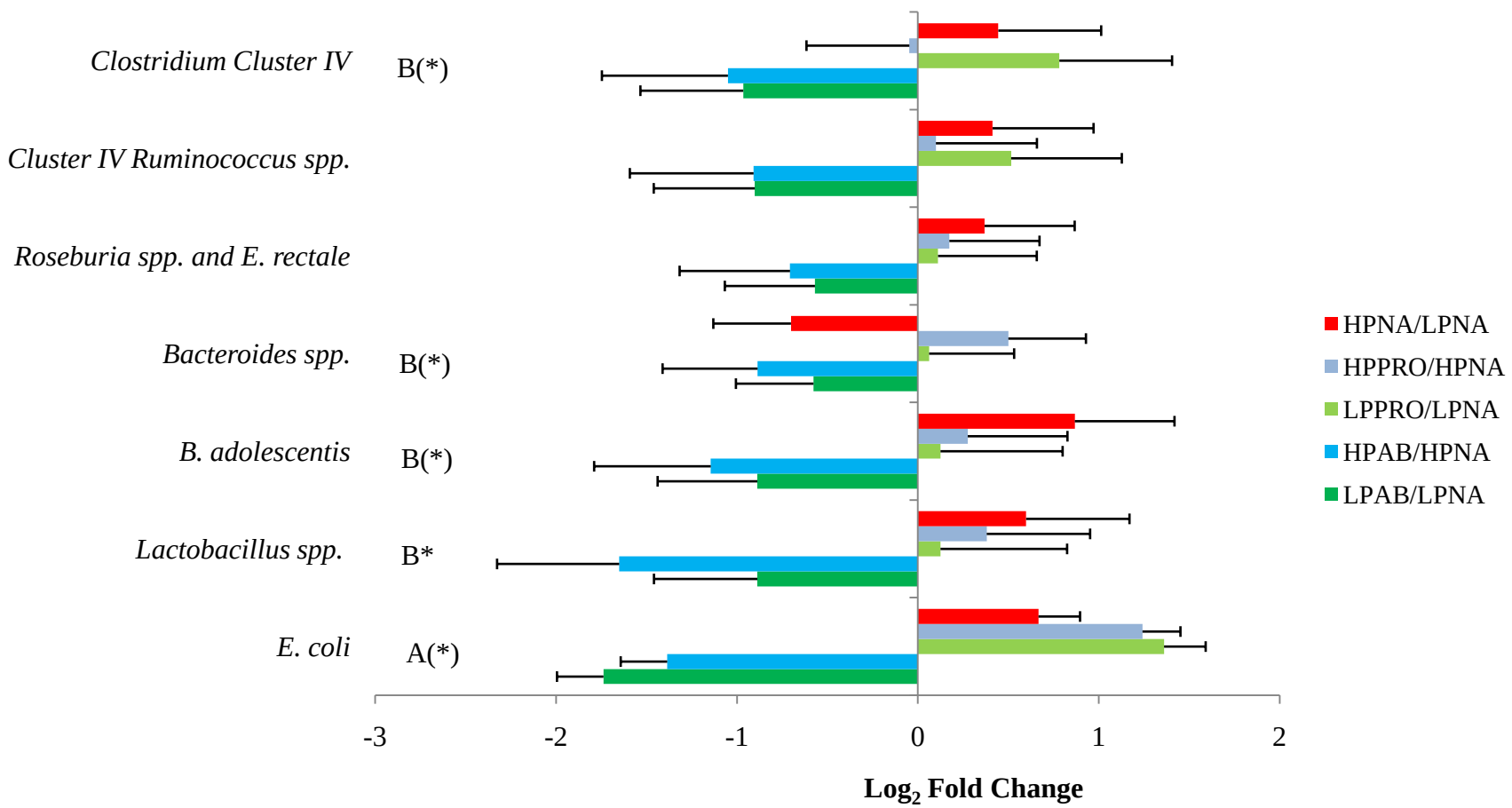

Figure 15. Log_2 fold changes of select bacterial groups in the colonic digesta of ETEC K88 infected piglets fed different experimental diets based on quantitative real-time PCR. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. Factor A; levels of crude protein, Factor B; additives (no additives/antibiotics/probiotics). Significant differences and tendencies were noted as $P < 0.05$, *, and $P < 0.1$, (*), respectively.

2.3. Inflammatory cytokines and receptors - PCR array

Effect of different dietary treatments on the inflammatory cytokines and receptors of 84 genes in the ileal tissue of ETEC K88 infected piglets were also determined. Amongs the 84 selected genes, BCL-6, C-3, CCL-13, CCL-24, CCL-25, CCL-3, CCL-8, CCR-1, CCR-4, CCR-7, CEBPB, CX3CR-1, CXCL-12, CXCL-5, IL-1 α, IL-1 ß, IL-22, IL-5, IL-8, IL-9, TNF, TOLLIP, XCR1 were actually detected (Figure 20). The mRNA expression of IL-1ß was significantly increased in ETEC K88 infected piglets fed the high-CP diets ($P < 0.05$). The effect of diet, supplemented with PRO, was similar to those supplemented with AB.

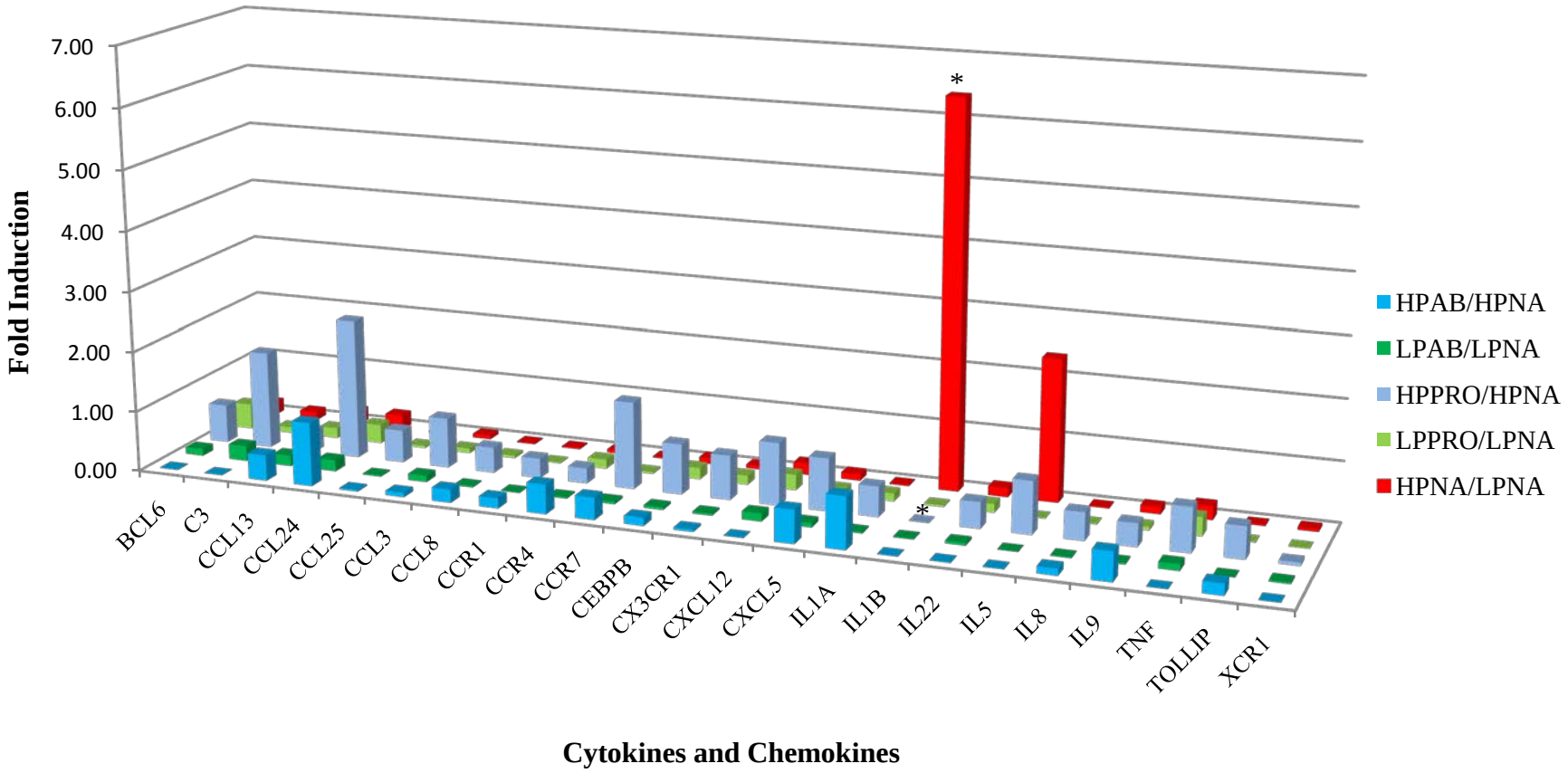

Figure 16. Fold induction of inflammatory cytokines and receptors expression in the ileal tissue of ETEC K88 infected piglets fed different experimental diets. Diets; HPNA = High crude protein with no additives, HPAB = High crude protein + antibiotics, HPPRO = High crude protein + probiotics, LPNA= Low crude protein with no additives, LPAB = Low crude protein + antibiotics, LPPRO = Low crude protein + probiotics. Significant differences and tendencies were noted as $P < 0.05$, *, and $P < 0.1$, (*), respectively.

DISCUSSION

1. Charcoal experiment

Post-weaning diarrhea caused by ETEC K88 is a major challenge to the swine industry often resulting in huge economical losses from decreased performance and high morbidity (Cutler *et al*., 2007, Fairbrother *et al*., 2005, Krause *et al*., 2010). Overuse of antibiotics as a traditional strategy to treat or prevent PWD has given rise to an increase in antibiotic resistant ETEC K88 and other normal GIT flora (Krause *et al*., 2010, McEwen & Fedorka-Cray., 2002). Following the ban of antibiotics in the European Union in 2006, efforts have been increased to find alternatives to antibiotics. Charcoal has demonstrated superior adsorbent qualities for bacteria, bacterial toxins, and harmful gasses such as ammonia (Naka *et al*., 2001, Pegues *et al*., 1979, Richardson., 1917). With numorus micropores, charcoal has a non-selective adsorptive capacity to bind to a variety of molecules (Chandy & Sharma., 1998, Naka *et al*., 2001). To date, little is known about the potential effects of charcoal in pig diets, with limited peer reviewed literature regarding the addition of charcoal to the diet. In this study, we investigated the efficacy of different doses of charcoal in early-weaned piglets experimentally infected with ETEC K88 and measured the incidence of PWD, performance and gut microbial population. Our findings indicate that inclusion of charcoal up to 2% of the diet had no adverse effects on BW gain, ADFI, ADG and G:F ratio. However, there was a numerical decrease in the ADG of piglets fed the 2% C diet compared with those fed the NA diet. It is speculatated this occurred due to a decrease in the availability of some nutrients by adding more charcoal (Rotter *et al*., 1989). In

addition, the black appearance of the diet with 2% charcoal might have visually affected the perception of the feed to the piglets, thus resulting in a reduction in ADFI. Willman *et al.*, (1947) conducted seven trials and reported that charcoal had no significant effect on ADFI, ADG and G:F ratio in pigs fed charcoal diets (Willman & Morrison., 1947). However, Evvard *et al.* (1927) observed that adding charcoal to the diet of pigs slightly improved feed intake. Frolich *et al.* (1934) also found that pigs fed a charcoal diet had higher weight gains compared to those fed noncharcoal-based diets. Parallel studies on chickens demonstrated that inclusion of charcoal increases ADFI and BW gain in chickens (Dalvi & Ademoyero., 1984, Jindal *et al*., 1994). Another study on chicks and poults demonstrated that charcoal had no influence on an animal's performance (Edrington *et al*., 1997, Kubena *et al*., 1990). These discrepancies in results might be due to the properties of charcoal, feed-intake duration, composition of the basal diet or other parameters (Edrington *et al*., 1997).

In this experiment, four pigs remained clinically healthy without showing any signs of diarrhea post-infection. This could be due to either the absence of ETEC K88 receptors on intestinal brush border cells or suppression of ETEC K88 cells by host's innate immune system (Li *et al*., 2009).

Our data indicates that inclusion of charcoal at 1% and 2% of the diet had a significant reduction on scours incidence of infected piglets. In addition, we observed a decrease in ileal and colonic digesta counts of ETEC K88, although this was not statistically significant. A study conducted more than 50 years ago (Lentz., 1932) showed that charcoal is an effective substance in reducing scours in pigs, but in *vivo* studies using charcoal are lacking. Watarai *et al.* (2008) demonstrated that oral administration of charcoal containing wood vinegar liquid (Nekka-Rich) had an increased adsorption efficacy for *Cryptosporidium parvum.* They reported that Nekka-

Rich was useful in reducing diarrhea caused by *C. parvum*in calves. In general, charcoal contains numerous micropores that makes it a powerful adsorbent in removing bacteria and their associated toxins (Knutson *et al*., 2006, Naka *et al*., 2001, van der Mei *et al*., 2008, Watarai., 2008). The adsorptive capability of charcoal relies on its pore size as removal of larger molecules requires larger pores and removal of smaller molecules needs smaller pores (Chandy & Sharma., 1998). Although, there was a decrease in the number of ETEC K88 in ileal and colonic digesta, we are not certain that this charcoal has pores of sufficient diameter required to remove this pathogen. Indeed, charcoal included up to 2% of the diet was highly effective in reducing ileal-mucosal attached ETEC K88 in piglets. Recently, it has been shown that LT knocked-out isolates resulted in decreased diarrhea and intestinal bacterial colonization in gnotobiotic pigs (Berberov *et al*., 2004). Although the efficacy of charcoal in removing ETEC K88 entrotoxins was not measured, we speculate that charcoal may have had an effect in reducing LT and subsequent lower ileal-mucosal attached ETEC K88 and the severity of diarrhea. However, further studies are needed to assess the effects of charcoal on ETEC K88 entrotoxins.

In addition, we observed that charcoal at a higher concentration (1-2%) had a greater therapeutic effect against diarrhea caused by ETEC K88. Hence, results of such effects might underline the fact that charcoal at a high concentration does provide sufficiently more pores with an appropriate diameter to remove ETEC K88 and/or its entrotoxins from the GIT of infected piglets.

Ammonia, a toxic compound found in trace quantities in the ileum and colon of animals, is produced from microbial deamination of amino acids and urea hydrolysis (Gaskins., 2001). High concentrations of ammonia can negatively influence gut health by increasing bacterial and viral infections (Lin & Visek., 1991, Visek., 1978). Charcoal, as an indigestible substance, can

be effective in removing harmful gases such as ammonia (Richardson., 1917). Charcoal has traditionally been used in animal housing to reduce the fecal odor by adsorbing ammonia (Samanya & Yamauchi., 2002). However we failed to observe the significant effect of charcoal in reducing the ileal and colonic ammonia concentration. We also observed no significant effect of charcoal on ileal and colonic pH.

In this study, we investigated the effect of dietary charcoal on the bacterial composition and diversity from ileal and colonic digesta in piglets infected with ETEC K88. The analysis was accomplished by pyrosequencing of the highly conserved 16S rRNA gene of bacteria. The majority of publications, primarily focused on the effect charcoal had regarding only a few specific microorganisms such as *E. coli*, *S. tryphimurium*, *S. enteric*a serovar *entritidis* and *S. aerus* (Knutson *et al*., 2006, Naka *et al*., 2001, Shi *et al*., 2007, Watarai., 2008, Watarai & Tana., 2005). Other studies simply focused on the effect charcoal had on toxins produced from certain microorganisms such as *Vibrio cholera* entrotoxin (Stoll *et al*., 1980), *Staphylococcal* entrotoxin B (Hoffman *et al*., 2007) and ochtoxin A produced by certain *Aspergillus* and *Penicillium* species of fungi (Edrington *et al*., 1997). An in *vitro* study conducted by Naka *et al.* (2001) illustrated that charcoal had a lower binding affinity to the GIT bacteria *E. faecium*, *B. thermofilum* and *L. acidophilus* compared to *E. coli* O157:H7. Watarai *et al.* (2005) reported similar observations in chickens fed charcoal. They demonstrated that charcoal might be used in clinical applications due to its effective binding capability to *S. enteritidis* than to the normal GIT flora, such as *E. faecium.* Our results indicate that charcoal had no effect on the composition and diversity of ileal and colonic microbiota. This was also further confirmed with PCA and CCA analyses. The gut as an ecosystem can be reviewed from a biodiversity point of view taking into account the structure of the entire microbial community. It has been demonstrated that a

reduction in microbial richness and diversity can negatively affect GIT health (Krause *et al*., 2010). Therefore, we could conclude that charcoal was beneficial in reducing the incidence and severity of PWD without affecting biodiversity and function of the piglet gut ecosystem infected with ETEC K88. To the best of our knowledge, this is the first study to report the effect of charcoal on the GIT microbiome of piglets infected with ETEC K88, thus we could not compare our results to other published values.

Further results from the pyroseqencing data found that *Lactobacillus* is the main population in the ileal and colonic digesta of piglets. Lactic acid bacteria such as lactobacilli have an inhibitory role on the adhesion of ETEC to the ileal mucosa (Hillman *et al*., 1995, Pluske *et al*., 2002). However, no major change was observed in the *Lactobacillus* population in the ileal and colonic digesta of piglets fed charcoal. This was also further confirmed by measuring the OA concentration in the ileal and colonic digesta. It is possible that no alteration in OA concentration resulted from non-affinity of charcoal to bind to the GIT normal flora bacteria. This also may account for no observed changes in the ileal and colonic pH.

Our findings suggested that including 1-2% charcoal into the diet could be effective in reducing the incidence and severity of PWD in ETEC K88 infected piglets without affecting the GIT bacteria and their fermentation end products. Charcoal did not appear to have any negative effects on ADG, ADFI and G/F ratio in ETEC K88 infected piglets. We can conclude that charcoal can be used prophylactically against the ETEC K88 infection.

2. Crude protein experiment

Low performance and high morbidity as a result of PWD caused by ETEC K88 in early-weaned pigs can have tremendous economic consequences to the swine industry (Cutler *et al*., 2007, Fairbrother *et al*., 2005, Krause *et al*., 2010). Sub-therapeutic use of dietary antibiotics in animal diets as a growth promoter has increased concerns as the number of antibiotic resistant bacteria is increasing. Consequently, several strategies have been looked at as alternatives to antibiotics (Krause *et al*., 2010, McEwen & Fedorka-Cray., 2002). Manipulation of animal diets is considered an effective strategy to control the incidence and severity of PWD in early-weaned pigs without the use of dietary antibiotics. It has been demonstrated that a decrease in CP reduces protein availability required for pathogenic bacteria proliferation in the GIT (Prohászka & Baron., 1980). In addition, probiotics have also been effective against ETEC through production of natural microbial compounds such as bacteriocins (Setia *et al*., 2009, Stahl *et al*., 2004). In this study, we evaluated the effect of different levels of CP supplemented with antibiotics and several probiotic *E. coli* strains on the GIT microbiome of ETEC K88 infected piglets. We hypothesized that feeding a low-CP diet supplemented with probiotic would have a beneficial effect on the GIT bacteria and overall immune response of ETEC K88 infected piglets. This combination could be an effective alternative to dietary antibiotics in reducing PWD.

Feeding a low-CP diet to early-weaned pigs has demonstrated a beneficial effect on the GIT microbiome and its immune response (Lynch *et al*., 2007, Opapeju *et al*., 2010, Wellock *et al*., 2006). Other studies used two different levels of dietary CP (17 and 23%), which were also used in this study (Opapeju *et al*., 2009, Stein & Kil., 2006). We observed that ileal ammonia concentration was significantly lower in low-CP fed piglets. In addition, there was a trend of

lower colonic ammonia concentration in low-CP fed piglets. These observations indicate lowered protein fermentation in low-CP fed piglets which are similar to results of previous studies (Bikker *et al*., 2006, Nyachoti *et al*., 2006).

Higher buffering capacity of high-CP diets (Partanen & Mroz., 1999) results in higher gastric pH, leading to increased proliferation of bacteria (Htoo *et al*., 2007). To lower the gastric pH, more gastric HCl needs to be produced (Schutte., 2000). Inability to produce enough gastric HCl in early-weaned piglets is one of the main causes of PWD (Cranwell., 1995). Nyachoti *et al*., (2006) reported a decrease in ileal digesta pH in low-CP (17% CP) fed piglets. A low pH, along with a lower concentration of ammonia and higher VFA concentration of digesta, are typical indicators of a healthy ecosystem. Such associations with a lower ammonia concentration were not found in this study, although a trend was observed for a lower ileal pH in low-CP diets based on CCA analysis. Similar inconsistencies have also been reported previously where low-CP diet did not coincide with an effect on VFA (Bikker *et al*., 2006) or pH level (Htoo *et al*., 2007).

In this study, changes to the composition and diversity of ileal and colonic microbiota of ETEC K88 infected piglets were sought using a culture-independent technique, called pyrosequencing. In response to different levels of CP supplemented with antibiotics and probiotic *E. coli* strains, statistical analysis of the pyrosequenced 16S rRNA gene revealed that within ileal digesta at the phylum level Firmicutes decreased while Proteobacteia increased. This is in agreement with a previous study that reported the same pattern in the mucosal associated bacteria in mice treated with antibiotics (Hill *et al*., 2010). At the genus level, the *Lactobacillus* population of ileal digesta from high-CP diets significantly increased. Based on the CCA analysis, a similar trend of increasing *Lactobacillus*, lactic acid and VFA concentration was

observed in the ileal digesta from high-CP diets. The increased *Lactobacillus* population from the high-CP diet could be due to a greater amount of available protein (casein), which stimulates bacterial proliferation (Wellock *et al*., 2008). It has been shown that lactic acid bacteria such as *Lactococcus lactis* and *Lactobacillus helveticus* are able to degrade the casein in order to generate bioactive peptides (Kunji et al., 1998, Minervini et al., 2003, Pritchard & Coolbear., 1993). Therefore, the increase in the lactobacilli population may arise from higher amounts of casein in high-CP diet. This result was further confirmed with Q-PCR on select microorganisms from the ileal digesta. It was revealed that *E. coli* and *Lactobacillus* populations significantly increased in the ileal digesta of high-CP diets. This supports the idea that a high-CP diet provides a more suitable environment for colonization and proliferation of pathogens, such as ETEC, consequently increasing the risk of PWD (Prohászka & Baron., 1980, Wellock *et al*., 2006).

Feeding a low-CP diet indicates then that a lower protein: carbohydrate ratio will be available to the GIT bacteria. Such an environment is conducive to increase non-proteolytic bacteria; therefore we would expect to see an increase in glycolytic bacteria. Contrary to what we expected, the low-CP diet did not affect the bacteria we selected for analysis in the ileal digesta, which included *Bacteroides spp., Roseburia spp. and E. rectale,* Clostridial cluster IV, Cluster IV *Ruminococcus spp.,* and *B. adolescentis.* The LPPRO diet resulted in a lower prevalence of the genus *Turicibacter* in the piglets' ileal digesta. *Turicibacter* is a relatively unknown genus and its presence in pigs has been previously reported (Gagnon *et al*., 2007, Kishimoto *et al*., 2006). The bacterium *Turicibacter* is a putative pathogen and may cause sub-clinical infection (Rettedal *et al*., 2009). Further isolation of any *Turicibacter* bacteria is necessary to determine its role in the GIT and the potential effects of probiotic *E. coli* strains on these bacteria.

Antibiotic supplemented diets have a significant effect on the diversity and bacterial population of ileal digesta. The genus *Streptococcus* was found to be higher in the LPAB fed piglets. This is an interesting result as antibiotic supplementation was anticipated to decrease the pathogenic bacteria within the host including the *Streptococcus* population. These changes in microbial composition might be due to the replacement of antibiotic susceptible strains with resistant bacteria (Baquero *et al*., 1998, Onishi *et al*., 1974). Another challenge to using dietary antibiotics, based on Q-PCR analysis, is a decrease in the number of specific beneficial bacterial groups including *Bacteroides spp., Roseburia spp.* and *E. rectale,* Clostridial cluster IV, Cluster IV *Ruminococcus spp.,* and *B. adolescentis*. Pyrosequencing of the 16S rRNA gene of the bacterial populations of ileal digesta revealed that the genus *Lactobacillus* was significantly lower in piglets fed diets supplemented with antibiotics. A similar study with antibiotic treated mice reported a reduction in the genus *Lactobacillus* (Hill *et al*., 2010). It can be concluded that the use of dietary antibiotics adversely affects the bacterial population of the GIT.

Statistical analysis of the pyrosequencing data from colonic digesta in ETEC K88 infected piglets revealed a decreased diversity in LPAB diets compared with the control. This indicates an adverse effect of the dietary antibiotics on the GIT bacteria. There was no significant effect of dietary CP levels on the bacterial population, ammonia and OA concentration in the colon. This lack of significance was also further confirmed by PCA and CCA analyses. This is also in agreement with a previous study (Bikker *et al*., 2006) that concluded there was no effect of dietary CP levels on colonic bacteria of piglets. Our findings suggest that changing dietary CP per se does not affect the colonic bacteria, indicating involvement of other environmental factors (Bikker *et al*., 2006, Nyachoti *et al*., 2006). The genus *Roseburia* significantly increased in high-CP diets. This observed increase is in contrast with a previous study where its population

increased in low-CP diets (Opapeju *et al*., 2009). *Roseburia* is a common butyrate-producing bacterium in the colon (Ramirez-Farias *et al*., 2009) that grows and proliferates in a low pH environment (Walker *et al*., 2005). However, this was not true in our study, which can be explained by the excessive protein available within the gut in high-CP diets. The results from the Q-PCR analysis of the colonic digesta indicated a tendency for a higher *E. coli* population in high-CP diets and a lower bacterial population of groups including *Bacteroides spp.,* Clostridial cluster IV, and *B. adolescentis, Lactobacillus* and *E. coli* in piglets fed diets containing dietary antibiotics. While a higher *E. coli* population might be due to higher levels of casein found in a high-CP diet, lower total microbial bacteria counts might be due to the bacteriocidal effect from the antibiotics. We also found a significant increase in the population of the genus *Xylanibacter* in digesta samples from the LPPRO fed piglets. The *Xylanibacter* are known for SCFA production, however, the role the probiotic *E. coli* strains played to increase these numbers is unknown.

Epithelial cells are a primary line of defense and play a major role in the passage of microorganisms to underlining tissues (Schierack *et al*., 2006). In response to a bacterial infection, recruitment of immunological cells such as neutrophils and macrophages, mediated by pro-inflammatory cytokines and chemokines, will occur (Burger & Dayer., 2002, Dube *et al*., 2001). Interleukin-1ß is considered an important mediator of a pro-inflammatory response (Burger & Dayer., 2002, Dube *et al*., 2001). We hypothesized that by lowering the dietary CP level and supplementing the diet with a colicin-producing *E. coli* probiotic effective against ETEC K88 (Setia *et al*., 2009) could favorably reduce the colonization and proliferation of ETEC K88 in the ileum. The analysis of inflammatory cytokines and receptors in the ileal tissue of ETEC K88 infected piglets demonstrated a significant decrease in IL-1ß in low-CP diets.

Bhandari *et al.*, (2010), previously reported a decrease in ileal mucosal counts of ETEC K88 in response to a low-CP diet. Hence, the decreased mRNA expression of IL-1ß can be explained by the lower number of ileal mucosal ETEC K88. This is in agreement with a previous report (Opapeju *et al*., 2010) where the measurement of IL-1ß was measured from blood serum samples. Immunomodulatory effects of the low-CP diet in ETEC K88 infected piglets might be due to the lower protein availability for ETEC proliferation (Prohászka & Baron., 1980, Wellock *et al*., 2008). In addition, a low-CP diet has demonstrated a decrease in the alteration of the gut architecture resulting in inhibition of translocation of bacteria and their toxins across the gut wall (Opapeju *et al*., 2008, Opapeju *et al*., 2009).

Probiotic supplementation reduced the mRNA expression of IL-1ß in the ileal tissue of piglets fed a high-CP diet. It was previously demonstrated that the efficacy of probiotic *E. coli* strains (UM2 and UM7) can out-compete ETEC K88 by means of colicin N, S4, B, and D (Krause *et al*., 2010, Setia *et al*., 2009). Here we confirmed the effectiveness of probiotic *E. coli* strains against ETEC K88 in reducing an increased IL-1ß response to an ETEC K88 infection. To our knowledge, this is the first study to investigate the effect of different levels of dietary CP on mRNA expression of cytokines in the ileal tissue of ETEC K88 infected piglets. Therefore, we could not compare our results to other studies. Together with previous findings by Bhandari *et al.*, (2010), we concluded that feeding a low-CP diet supplemented with probiotic *E. coli* strains reduces inflammatory responses by down-regulating IL-1ß.

Our study found that administration of a low-CP diet supplemented with probiotic *E. coli* strains (UM2 and UM7) resulted in a lower proteolytic fermentation in the ileum, a healthier GIT microbiota and a reduced pro-inflammatory immune response. We concluded that dietary

CP supplemented with probiotic *E. coli* strains is an effective combinationin in reducing the incidence and severity of PWD.

CONCLUSION

Based on our findings we can conclude that:

1. Diets supplemented with dietary antibiotics adversely affect the GIT microbiota by reducing the number of beneficial bacteria such as lactobacilli, decreasing the diversity of the ecosystem and increasing the antibiotic-resistance strains. This indicates a necessity to find new alternatives for antibiotics.
2. Differing doses of charcoal supplemented in the diet had no significant effect on ADG, ADFI, and G:F of piglets infected with ETEC K88.
3. Charcoal at different dietary levels caused no shifts in the GIT microbiota of ETEC K88 infected piglets. This indicates no binding affinity of charcoal to the normal GIT flora.
4. Charcoal fed at 1 and 2% was effective in reducing scours in ETEC K88 infected piglets. In addition, feeding charcoal reduced the ileal-mucosal attached ETEC K88. These demonstrate the benefit of feeding charcoal in reducing the incidence and severity of PWD.
5. Different dietary CP levels caused no major shifts in the ileal and colonic microbiota other than increasing *Lactobacillus* and *E. coli* populations in ileal digesta of ETEC K88 infected piglets. This implies the involvement of other factors in controlling of the GIT microbiota.
6. Feeding a low-CP diet resulted in a lower ileal ammonia concentration. This suggests a low-CP diet may improve the health of the GIT.

7. Probiotic *E. coli* strains caused no major shifts in the GIT microbiota of ETEC K88 infected piglets.
8. Feeding a low-CP diet significantly decreased the pro-inflammatory response (IL-1ß) in ETEC K88 infected piglets. This is in support of the idea that a low-CP diet reduces a pro-inflammatory response resulting from the proliferation and colonization of pathogenic bacteria such as ETEC K88.
9. Supplementing diets (high/low-CP) with probiotic *E. coli* strains was found to be effective at reducing the pro-inflammatory response (IL-1ß) in ETEC K88 infected piglets. This emphasizes the efficacy of probiotic *E. coli* strains against ETEC K88 and their subsequent suppression of the pro-inflammatory response (IL-1ß) caused by ETEC K88.

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APPENDIX

Table 1. Phylogenetic composition of bacterial genera with lower abundance of 0.10 % from pyrosequenced 16S rRNA data in ileal digesta of ETEC K88 infected piglets fed charcoal and non-charcoal based diets

Phylum; Family; Genus
Acidobacteria
Acidobacteriaceae; uncultured
Actinobacteria
Actinomycetaceae; *Actinomyces*
Corynebacteriaceae; *Corynebacterium*
Dietziaceae; *Dietzia*
Mycobacteriaceae; *Mycobacterium*
Geodermatophilaceae; *Blastococcus*
Microbacteriaceae; *Microbacterium*
Micrococcaceae; *Rothia*
Coriobacteriaceae; *Atopobium*
Coriobacteriaceae*; Collinsella*
Coriobacteriaceae; Enterorhabdus
Coriobacteriaceae*; Slackia*
Coriobacteriaceae; unclassified
Coriobacteriaceae; uncultured
Bacteroidetes
Porphyromonadaceae; *Parabacteroides*
Prevotellaceae; *Prevotella*
Prevotellaceae; unclassified
Prevotellaceae; uncultured

Rikenellaceae; RC9 gut group

Rikenellaceae; unclassified

S24-7; unclassified

p-2534-18B5 gut group; unclassified

Chitinophagaceae; *Flavisolibacter*

Deinococcus-Thermus

Thermaceae; *Meiothermus*

Firmicutes

Alicyclobacillaceae; *Alicyclobacillus*

Paenibacillaceae; *Paenibacillus*

Planococcaceae; *Lysinibacillus*

Planococcaceae; uncultured

Staphylococcaceae; *Macrococcus*

Staphylococcaceae; *Staphylococcus*

Aerococcaceae; *Aerococcus*

Carnobacteriaceae; *Carnobacterium*

Enterococcaceae; *Vagococcus*

Lactobacillaceae; unclassified

Leuconostocaceae; *Leuconostoc*

Leuconostocaceae; *Weissella*

Streptococcaceae; *Lactococcus*

Clostridiaceae; *CandidatusArthromitus*

Clostridiaceae; unclassified

Family XIII Incertae Sedis; *Mogibacterium*

Family XIII Incertae Sedis; unclassified

Family XIII Incertae Sedis; uncultured

Lachnospiraceae; *Butyrivibrio-Pseudobutyrivibrio*

Lachnospiraceae; *Coprococcus*

Lachnospiraceae; *Dorea*

Lachnospiraceae; *Epulopiscium*

Lachnospiraceae; *Howardella*

Lachnospiraceae; *Lachnospira*

Lachnospiraceae; *Marvinbryantia*

Lachnospiraceae; *Oribacterium*

Lachnospiraceae; *Roseburia*

Lachnospiraceae; *Syntrophococcus*

Peptococcaceae; *Peptococcus*

Peptostreptococcaceae; Incertae Sedis

Ruminococcaceae*; Anaerofilum*

Ruminococcaceae*; Anaerotruncus*

Ruminococcaceae*;* Incertae Sedis

Ruminococcaceae*; Oscillospira*

Ruminococcaceae*; Ruminococcus*

Ruminococcaceae*; Subdoligranulum*

Ruminococcaceae*;* unclassified

Veillonellaceae*; Acidaminococcus*

Veillonellaceae*; Allisonella*

Veillonellaceae*; Anaerovibrio*

Veillonellaceae*; Dialister*

Veillonellaceae*; Mitsuokella*

Veillonellaceae*; Phascolarctobacterium*

Veillonellaceae*; Selenomonas*

Veillonellaceae*; Succiniclasticum*

Veillonellaceae*; Veillonella*

Veillonellaceae; unclassified

Veillonellaceae; uncultured

Erysipelotrichaceae*; Catenibacterium*

Erysipelotrichaceae; Incertae Sedis

Erysipelotrichaceae*; Sharpea*

Erysipelotrichaceae*; Solobacterium*

Erysipelotrichaceae; unclassified

Erysipelotrichaceae; uncultured

Proteobacteria

Methylobacteriaceae*; Methylobacterium*

Rhizobiaceae; *Rhizobium*

Rhodobacteraceae*; Paracoccus*

Mitochondria*;* unclassified

Alcaligenaceae*; Bordetella*

Comamonadaceae*; Acidovorax*

Comamonadaceae*; Delftia*

Comamonadaceae*; Pelomonas*

Comamonadaceae*; Ramlibacter*

Comamonadaceae; unclassified

Oxalobacteraceae*; Duganella*

Oxalobacteraceae*; Oxalobacter*

Oxalobacteraceae*; unclassified*

Neisseriaceae*; Leeia*

Neisseriaceae; unclassified

Neisseriaceae*;* uncultured

Desulfovibrionaceae*; Desulfovibrio*

Campylobacteraceae*; Campylobacter*

Helicobacteraceae*; Helicobacter*

Succinivibrionaceae*; Succinivibrio*

Shewanellaceae*; Shewanella*

Enterobacteriaceae*; Klebsiella*

Enterobacteriaceae*; Moellerella*

Pasteurellaceae*; Haemophilus*

Pasteurellaceae*; Pasteurella*

Pasteurellaceae*; Phocoenobacter*

Pasteurellaceae; unclassified

Moraxellaceae*; Acinetobacter*

Moraxellaceae*; Moraxella*

Pseudomonadaceae*; Pseudomonas*

Xanthomonadaceae*; Lysobacter*

Xanthomonadacea; *Stenotrophomonas*

Spirochaetes

Spirochaetaceae*; Treponema*

Table 2. Phylogenetic composition of bacterial genera with lower abundance of 0.10 % from pyrosequenced 16S rRNA data in colonic digesta of ETEC K88 infected piglets fed charcoal and non-charcoal based diets

Phylum; Family; Genus
Acidobacteria
Acidobacteriaceae; uncultured
Actinobacteria
Corynebacteriaceae; *Corynebacterium*
Coriobacteriaceae; *Atopobium*
Coriobacteriaceae; *Enterorhabdus*
Coriobacteriaceae; *Olsenella*
Coriobacteriaceae; *Slackia*
Coriobacteriaceae; unclassified
Coriobacteriaceae; uncultured
Bacteroidetes
BS11 gut group; unclassified
Bacteroidaceae
Bacteroidaceae; *Bacteroides*
Porphyromonadaceae; *Butyricimonas*
Porphyromonadaceae; *Parabacteroides*
Prevotellaceae*; Xylanibacter*
RF16; unclassified
Rikenellaceae*; Alistipes*
Rikenellaceae*;* unclassified
p-2534-18B5 gut group; unclassified

Deferribacteraceae

Deferribacteraceae; *Mucispirillum*

Fibrobacteraceae

Fibrobacteraceae; *Fibrobacter*

Firmicutes

Alicyclobacillaceae; *Alicyclobacillus*

Bacillaceae; *Bacillus*

Staphylococcaceae; *Staphylococcus*

16d63.751; unclassified

Enterococcaceae; *Enterococcus*

Lactobacillaceae; *Pediococcus*

Lactobacillaceae; unclassified

Leuconostocaceae; *Leuconostoc*

Leuconostocaceae; *Weissella*

Rs-D42; unclassified

Clostridiaceae; *Candidatus Arthromitus*

Clostridiaceae; unclassified

Eubacteriaceae; *Anaerofustis*

Family XIII Incertae Sedis*; Eubacterium*

Family XIII Incertae Sedis*; Mogibacterium*

Family XIII Incertae Sedis*;* unclassified

Lachnospiraceae; *Acetitomaculum*

Lachnospiraceae; *Butyrivibrio*

Lachnospiraceae; *Butyrivibrio-Pseudobutyrivibrio*

Lachnospiraceae; *Catabacter*

Lachnospiraceae; *Hespellia*

Lachnospiraceae; *Howardella*

Lachnospiraceae; *Syntrophococcus*

Peptococcaceae; *Peptococcus*

Peptococcaceae; uncultured

Peptostreptococcaceae; unclassified

Peptostreptococcaceae; uncultured

Ruminococcaceae; *Anaerofilum*

Ruminococcaceae; *Oscillibacter*

Veillonellaceae; *Acidaminococcus*

Veillonellaceae; *Allisonella*

Veillonellaceae; *Schwartzia*

Veillonellaceae; *Succiniclasticum*

Veillonellaceae; *Veillonella*

Erysipelotrichaceae; *Sharpea*

Erysipelotrichaceae; *Solobacterium*

Erysipelotrichaceae; *Turicibacter*

Erysipelotrichaceae; *Turicibacter*

Erysipelotrichaceae; unclassified

Planctomycetaceae; p-1088-a5 gut group

Proteobacteria

Candidatus Liberibacter; unclassified

Acetobacteraceae; *Acetobacter*

Rhodospirillaceae; *Thalassospira*

Rhodospirillaceae; uncultured

Anaplasmataceae; *Wolbachia*

Mitochondria; unclassified

Alcaligenaceae; *Sutterella*

Burkholderiaceae; *Burkholderia*

Burkholderiaceae; *Ralstonia*

Comamonadaceae; *Delftia*

Comamonadaceae; *Tepidimonas*

Comamonadaceae; unclassified

Oxalobacteraceae; *Oxalobacter*

Oxalobacteraceae; unclassified

Desulfovibrionaceae; *Desulfovibrio*

GR-WP33-58; unclassified

Helicobacteraceae; Helicobacter

Aeromonadaceae; Aeromonas

Succinivibrionaceae; unclassified

Enterobacteriaceae; *Buchnera*

Pasteurellaceae; *Actinobacillus*

Pasteurellaceae; *Pasteurella*

Moraxellaceae; *Acinetobacter*

Moraxellaceae; *Psychrobacter*

Pseudomonadaceae; *Pseudomonas*

Spirochaetes

Spirochaetaceae; *Spirochaeta*

Spirochaetaceae; *Treponema*

Spirochaetaceae; unclassified

Synergistetes

Synergistaceae; *Cloacibacillus*

Synergistaceae; *Pyramidobacter*

Verrucomicrobia

Verrucomicrobiaceae; *Akkermansia*

Table 3. Phylogenetic composition of bacterial genera with lower abundance of 0.10 % from pyrosequenced 16S rRNA data in ileal digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Phylum; Family; Genus
Actinobacteria
Actinomycetaceae; *Actinomyces*
Actinomycetaceae; *Arcanobacterium*
Corynebacteriaceae; *Corynebacterium*
Intrasporangiaceae; *Janibacter*
Micrococcaceae; *Rothia*
Nocardioidaceae; *Nocardioides*
Propionibacteriaceae; *Propionibacterium*
Propionibacteriaceae; *Tessaracoccus*
Coriobacteriaceae; *Atopobium*
Coriobacteriaceae; *Collinsella*
Coriobacteriaceae; *Enterorhabdus*
Coriobacteriaceae; *Olsenella*
Coriobacteriaceae; marine group
Coriobacteriaceae; unclassified
Coriobacteriaceae; uncultured
Bacteroidetes
Bacteroidaceae; *Bacteroides*
Porphyromonadacea; *Butyricimonas*
Porphyromonadacea; *Dysgonomonas*
Porphyromonadacea; *Paludibacter*
Porphyromonadacea; *Parabacteroides*
Porphyromonadacea; *Porphyromonas*

Porphyromonadacea; unclassified

Prevotellaceae; *Prevotella*

Prevotellaceae; *Xylanibacter*

Prevotellaceae; unclassified

Prevotellaceae; uncultured

Rikenellaceae; *Alistipes*

Rikenellaceae; RC9 gut group

Rikenellaceae; unclassified

p-2534-18B5 gut group; unclassified

Flavobacteriaceae; *Bergeyella*

Chlorobi

BSV26; unclassified

Fibrobacteres

Fibrobacteraceae; *Fibrobacter*

Firmicutes

Family XI Incertae Sedis; *Gemella*

Staphylococcaceae; *Macrococcus*

Staphylococcaceae; *Staphylococcus*

Aerococcaceae; *Flacklamia*

Aerococcaceae; unclassified

Carnobacteriaceae; *Carnobacterium*

Carnobacteriaceae; *Granulicatella*

Enterococcaceae; *Enterococcus*

Enterococcaceae; *Vagococcus*

Lactobacillaceae; *Pediococcus*

Lactobacillaceae; unclassified

Streptococcaceae; *Lactococcus*

Clostridiaceae; *Sarcina*

Clostridiaceae; unclassified

Family XIII Incertae Sedis; *Eubacterium*

Family XIII Incertae Sedis; *Mogibacterium*

Family XIII Incertae Sedis; unclassified

Family XIII Incertae Sedis; uncultured

Family XI Incertae Sedis; *Anaerococcus*

Lachnospiraceae; *Acetitomaculum*

Lachnospiraceae; *Blautia*

Lachnospiraceae; *Butyrivibrio-Pseudobutyrivibrio*

Lachnospiraceae; *Catabacter*

Lachnospiraceae; *Catonella*

Lachnospiraceae; *Coprococcus*

Lachnospiraceae; *Dorea*

Lachnospiraceae; *Epulopiscium*

Lachnospiraceae; *Howardella*

Lachnospiraceae; Incertae Sedis

Lachnospiraceae; *Lachnospira*

Lachnospiraceae; *Marvinbryantia*

Lachnospiraceae; *Moryella*

Lachnospiraceae; *Oribacterium*

Lachnospiraceae; *Roseburia*

Lachnospiraceae; *Syntrophococcus*

Peptococcaceae; *Peptococcus*

Peptostreptococcaceae; *Filifactor*

Peptostreptococcaceae; *Peptostreptococcus*

Ruminococcaceae; *Anaerofilum*

Ruminococcaceae; *Anaerotruncus*

Ruminococcaceae; *Fecalibacterium*

Ruminococcaceae; Incertae Sedis

Ruminococcaceae; *Oscillibacter*

Ruminococcaceae; *Oscillospira*

Ruminococcaceae; *Ruminococcus*

Ruminococcaceae; *Subdoligranulum*

Ruminococcaceae; unclassified

Ruminococcaceae; uncultured

Veillonellaceae; *Acidaminococcus*

Veillonellaceae; *Allisonella*

Veillonellaceae; *Anaerovibrio*

Veillonellaceae; *Mitsuokella*

Veillonellaceae; *Phascolarctobacterium*

Veillonellaceae; *Quinella*

Veillonellaceae; *Selenomonas*

Veillonellaceae; *Succiniclasticum*

Veillonellaceae; *Veillonella*

Veillonellaceae; uncultured

Erysipelotrichaceae; *Erysipelothrix*

Erysipelotrichaceae; Incertae Sedis

Erysipelotrichaceae; *Sharpea*

Erysipelotrichaceae; *Solobacterium*

Erysipelotrichaceae; unclassified

Erysipelotrichaceae; uncultured

Erysipelotrichaceae; *Mollicutes*

Anaeroplasmataceae; *Anaeroplasma*

Fusobacteria

Family XIII; unclassified

CFT112H7; unclassified

Fusobacteriaceae; *Fusobacterium*

Leptotrichiaceae; *Leptotrichia*

Leptotrichiaceae; *Streptobacillus*

Leptotrichiaceae; unclassified

Proteobacteria

Planctomycetaceae; p-1088-a5 gut group

Bradyrhizobiaceae; *Blastobacter*

Bradyrhizobiaceae; *Rhodopseudomonas*

Bradyrhizobiaceae; unclassified

Brucellaceae; uncultured

Mitochondria; unclassified

Sphingomonadaceae; *Sphingopyxis*

Alcaligenaceae; *Pelistega*

Alcaligenaceae; *Sutterella*

Alcaligenaceae; unclassified

Comamonadaceae; unclassified

Neisseriaceae; *Alysiella*

Neisseriaceae; *Conchiformibius*

Neisseriaceae; *Neisseria*

Neisseriaceae; unclassified

BVA18; unclassified

GR-WP33-58; unclassified

Campylobacteraceae; *Campylobacter*

Helicobacteraceae; *Helicobacter*

Helicobacteraceae; *Sulfuricurvum*

Succinivibrionaceae; *Anaerobiospirillum*

Succinivibrionaceae; *Succinivibrio*

Enterobacteriaceae; *Cedecea*

Enterobacteriaceae; *Citrobacter*

Enterobacteriaceae; *Leclercia*

Enterobacteriaceae; *Raoultella*

Enterobacteriaceae; unclassified

Enterobacteriaceae; *Morganella*

Enterobacteriaceae; unclassified

Moraxellaceae; *Acinetobacter*

Moraxellaceae; *Moraxella*

Thiotrichaceae; *Leucothrix*

Xanthomonadaceae; unclassified

Spirochaetes

Leptospiraceae; *Leptospira*

Spirochaetaceae; *Spirochaeta*

Spirochaetaceae; *Treponema*

Spirochaetaceae; uncultured

Synergistetes

Synergistaceae; *Cloacibacillus*

Synergistaceae; *Pyramidobacter*

Verrucomicrobia

Verrucomicrobiaceae; *Akkermansia*

Table 4. Phylogenetic composition of bacterial genera with lower abundance of 0.10 % from pyrosequenced 16S rRNA data in colonic digesta of ETEC K88 infected piglets fed different levels of dietary crude protein supplemented with no additives, antibiotics and probiotics

Phylum; Family; Genus
Actinobacteria
Corynebacteriaceae; *Corynebacterium*
Dermatophilaceae; *Dermatophilus*
Nocardioidaceae; *Aeromicrobium*
Coriobacteriaceae; *Atopobium*
Coriobacteriaceae; *Collinsella*
Coriobacteriaceae; *Enterorhabdus*
Coriobacteriaceae; unclassified
Coriobacteriaceae; uncultured
Elev-16S-1332; unclassified
Elev-16S-1332; unclassified
Bacteroidetes
BS11 gut group; unclassified
Porphyromonadaceae; *Barnesiella*
Porphyromonadaceae; *Butyricimonas*
Porphyromonadaceae; *Paludibacter*
Porphyromonadaceae; unclassified
Rikenellaceae; *Alistipes*
Flavobacteriaceae; unclassified
Flammeovirgaceae; *Persicobacter*
Chloroflexi

Anaerolineaceae; uncultured

Deferribacteres

Deferribacteraceae; *Mucispirillum*

Firmicutes

Bacillaceae; *Anoxybacillus*

Staphylococcaceae; *Staphylococcus*

Carnobacteriaceae; *Carnobacterium*

Enterococcaceae; *Enterococcus*

Enterococcaceae; *Vagococcus*

Lactobacillaceae; *Pediococcus*

Lactobacillaceae; unclassified

Leuconostocaceae; *Weissella*

Streptococcaceae; *Lactococcus*

Streptococcaceae; *Streptococcus*

Clostridiaceae; *Candidatus Arthromitus*

Clostridiaceae; *Sarcina*

Clostridiaceae; unclassified

Eubacteriaceae; *Eubacterium*

Family XIII Incertae Sedis; *Anaerovorax*

Family XIII Incertae Sedis; *Eubacterium*

Family XI Incertae Sedis; *Finegoldia*

Family XI Incertae Sedis; *Helcococcus*

Lachnospiraceae; *Acetitomaculum*

Lachnospiraceae; *Anaerostipes*

Lachnospiraceae; *Butyrivibrio-Pseudobutyrivibrio*

Lachnospiraceae; *Catabacter*

Lachnospiraceae; *Coprococcus*

Lachnospiraceae; *Epulopiscium*

Lachnospiraceae; *Howardella*

Lachnospiraceae; *Lachnospira*

Lachnospiraceae; *Marvinbryantia*

Lachnospiraceae; *Parasporobacterium-Sporobacterium*

Lachnospiraceae; *Robinsoniella*

Lachnospiracea; *Shuttleworthia*

Peptococcaceae*; Peptococcus*

Peptococcaceae; unclassified

Peptococcaceae; uncultured

Ruminococcaceae*; Anaerofilum*

Ruminococcaceae*; Hydrogenoanaerobacterium*

Ruminococcaceae*; Oscillibacter*

Veillonellaceae*; Acidaminococcus*

Veillonellaceae*; Allisonella*

Veillonellaceae; *Dialister*

Veillonellaceae; *Mitsuokella*

Veillonellaceae; *Quinella*

Veillonellaceae; *Selenomonas*

Veillonellaceae; *Succiniclasticum*

Veillonellaceae; *Veillonella*

Veillonellaceae; unclassified

Erysipelotrichaceae*; Asteroleplasma*

Erysipelotrichaceae*; Catenibacterium*

Erysipelotrichaceae; *Erysipelothrix*

Erysipelotrichaceae*; Sharpea*

Erysipelotrichaceae*; Solobacterium*

Erysipelotrichaceae*;* unclassified

Anaeroplasmataceae; *Anaeroplasma*

Fusobacteria

CFT112H7; unclassified

Fusobacteriaceae; *Fusobacterium*

Lentisphaerae

Victivallaceae; uncultured

Planctomycetes

Planctomycetes; *SM1A02*

Urania-1B-19 marine sediment group; unclassified

Urania-1B-19 marine sediment group; uncultured

Proteobacteria

Phyllobacteriaceae; *Ahrensia*

Rhodobacteraceae; *Roseobacter_clade*

Rhodobacteraceae; NAC11-7 lineage

Rhodobacteraceae; uncultured

Rhodospirillaceae; *Defluviicoccus*

Rhodospirillaceae; *Pelagibius*

Rhodospirillaceae; Thalassospira

Rhodospirillaceae; uncultured

Mitochondria; unclassified

Deep_1; unclassified

Erythrobacteraceae; *Erythrobacter*

Erythrobacteraceae; unclassified

Sphingomonadaceae; *Sphingopyxis*

Alcaligenaceae; *Sutterella*

Comamonadaceae; *Comamonas*

Comamonadaceae; unclassified

Oxalobacteraceae; *Oxalobacter*

Bdellovibrionaceae; *Bdellovibrio*

Nitrospina; unclassified

Desulfovibrionaceae; *Desulfovibrio*

GR-WP33-58; unclassified

Sva1033; unclassified

Helicobacteraceae; *Helicobacter*

Helicobacteraceae*; Sulfurovum*

Succinivibrionaceae; *Ruminobacter*

Chromatiaceae; *Nitrosococcus*

Chromatiaceae; *Thiorhodovibrio*

Enterobacteriaceae ; *Citrobacte*

Enterobacteriaceae*; Providencia*

Pasteurellaceae; *Actinobacillus*

Pasteurellaceae; Pasteurella

Pasteurellaceae; unclassified

Pseudomonadaceae; *Pseudomonas*

Thiotrichaceae; *Leucothrix*

Thiotrichaceae; *Thiothrix*

Sinobacteraceae; marineBenthic_group

Spirochaetes

Spirochaetaceae; *Spirochaeta*

Spirochaetaceae; unclassified

Synergistetes

Synergistaceae; *Cloacibacillus*

Synergistaceae; *Pyramidobacter*

Synergistaceae; unclassified

Verrucomicrob

Verrucomicrobiaceae; *Akkermansia*
